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CONTENTS

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OMICS-TECHNOLOGIES TO ANALYZE INDIVIDUAL BOVINE OOCYTES FOR IDENTIFICATION OF DEVELOPMENTAL OOCYTE COMPETENCE BIOMARKERS

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Abstract

Oocyte quality is a capacity to be fertilized and to develop into viable embryo and this is crucial for reproductive biotechnologies in farm animals. Technical progress and possible miniaturization of «omics» technologies made possible application of transcriptomic, proteomic and lipidomic methodologies to single oocyte, and thus to search molecular factors representing possible markers of oocyte quality. Oocyte quality is determined by its follicular environment and affects transcript, protein and lipid composition of an oocyte, which has to progress through maturation — a final step before fertilization, crucial for the acquisition of oocyte developmental competence. Here we describe the examples of «omics» analysis performed on single bovine oocytes and their neighboring cumulus cells through the comparison of the oocytes with different competence to maturate and to develop blastocyst in vitro. In particular, we focus to original technologies of proteomics and lipidomics based on mass spectrometry phenotyping of intact cells and identification of molecular biomarkers.

Keywords: «omics» technologies, single oocyte, bovine

The assessment of gamete quality is important for reproductive biotechnologies, since high quality is essential in conceiving embryos with full development ability. In general, 20-50 % of high-producing lactating dairy cows have already experienced pregnancy loss during the first week of gestation [1]. Although many different factors — like genetic background (race, quantitative trait loci etc), physiological stage (age, parity, lactation...) or environmental impacts (nutrition, temperature stress etc) — can affect the reproductive efficiency in dairy cows, 10-20 % of artificial insemination failure and pregnancy losses could be explained by fertilization failure and early embryo mortality. In modern dairy cow, like Holstein, fertilization rate is typically above 80%, thus early embryonic losses may be linked to compromised oocyte quality due to a poor follicular microenvironment, suboptimal reproductive tract environment for the embryo, and/or inadequate maternal − embryonic communication [2]. Indeed, oocyte maternal factors are at the first line to assure embryo development, before the activation of its proper genome, at the stage of 8-16 cells in cattle.

The understanding of the determinants of oocyte competence to be fertilized and to assure embryo viability during the first cleavagesis in progress through large-scale analysis of oocyte molecular factors. In the recent decade numerous "omics" technologies, which encompass genomics, transcriptomics, proteomics, metabolomics (including lipidomics) and epigenomics disciplines,

allowed the significant advances in the understanding of oocyte biology [3].

Oocyte acquires its developmental potential inside of the follicle, and the most important stage before an oocyte becomes ready for fertilization, is oocyte maturation. It is now possible to initiate early embryonic development by co-incubating oocytes with treated spermatozoa following a protocol known as in vitro fertilization (IVF), which is routinely used in reproductive biotechnologies in cattle. The original process includes the administration of high levels of gonadotropins to stimulate oocyte maturation and ovulation *in vivo*; whereas *in vitro* oocyte can resume meiosis without gonadotropins. Oocyte maturation is a transition from immature germinal vesicle transcriptionally silenced oocyte to metaphase-II stage which is accompanied by polar body extrusion, ooplasm reorganization, and molecular modifications including changes in protein abundance and post-translational modifications [4]. Oocyte maturation plays an important role in the acquisition of oocyte developmental competence, referred as oocyte quality, which at the same time is crucial for embryo quality. Maturation can be accomplished in vitro starting from the oocytes recovered from small antral follicles at the stage prior to selection and dominance, by using specific culture media, following a protocol known as in vitro maturation (IVM). IVM is promising technique in animal reproduction biotechnology, especially in genetic merit dairy cows, where the success rate after artificial insemination may be low. In cattle, immature oocytes can be obtained from the ovaries either by ovum pick up or after slaughter, then subjected to IVM, fertilized and developed to transferable embryos. Comparing immature and mature oocytes demonstrating contrasted developmental capacity, by using different "omics" technologies helps to reveal molecular factors involved in oocyte quality.

Unlike spermatozoa, the main problem with implementing of "omics" approaches like transcriptomics, proteomics and metabolomics to the female gametes is low biological material content in single oocyte. In bovine full-grown oocyte, total quantities of RNA, protein or lipids are estimated to approximatively 2-3 ng, 80-130 ng and 200-250 ng per oocyte, respectively. Nevertheless, significant progress in the adapting of "omics" tools to single oocyte was observed during the last years.

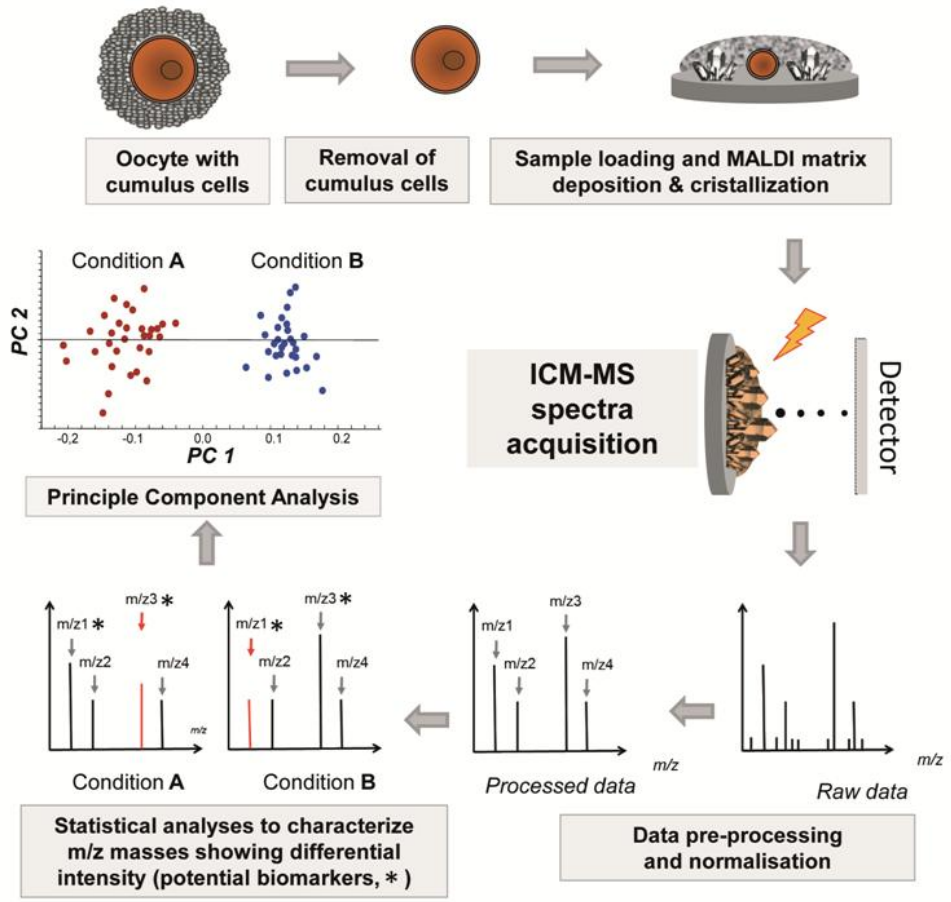
Oocyte transcriptomics. Global analysis of bovine oocyte transcriptome since 2003 [5] is now became a routine approach because of development of RNA amplification methods, new generation RNA sequencing and global annotation of bovine genome. Few or even single oocytes are sufficient to amplify RNA and perform transcriptomic analysis, so gene expression of more or less competent oocytes could be compared. For example, genes *SLC25A16, PPP1R14C, ROBO1, AMDHD1* and *MEAF6* were shown to be differentially expressed in the oocytes, obtained from the Monbeliarde animals with high or low oocyte potential to produce viable embryos after *in vitro* maturation (IVM) [6]. The comparison of more competent oocytes from the large follicles (> 8mm) with the oocytes with lower competence from the small follicles (< 3 mm) allowed the identification of several genes involved in crucial functions such as transcriptional regulation (*TAF2*), chromatin remodeling (*PPP1CB*), energy production (*SLC25A31*), as well as transport of key molecules within the cell (*NAGPA, CYHR1*, and *SLC3A12*) [7]. Oocyte which were denuded from their cumulus cells before IVM were less competent than those matured within oocyte-cumulus complex, and some differently abundant transcripts were detected by microarray hybridization, although the most differences between these groups of oocytes were found in their lipid composition [8].

However, the relevance of oocyte transcriptomics has limitations because full-grown oocytes are transcriptionally quiescent, and therefore the difference in

transcript abundance between immature and mature oocytes is mainly related to either RNA degradation or degree of polyadenylation at 3'UTR [9, 10]. Consequently, in the oocytes, there is mainly no correlation between transcript abundance and level of the corresponding proteins: we have confirmed this for the main actors of oocyte meiotic maturation, like Aurora kinases, cyclin B1, CDK1, c-MOS, and CPEB1 in bovine oocyte during IVM [11].

Oocyte proteomics. In bovine oocyte, maturation process is accompanied with protein neosynthesis and different post-translational modifications (PTM), as protein phosphorylation or acetylation. Proteomic changes that occur in the oocyte during maturation and that define the quality of *in vitro* matured oocytes are not enough known, and so there is not an accurate way of evaluating/monitoring how different IVM protocols can affect the process, at single oocyte level. Proteomics has been employed with this objective. Classical proteomic approaches using gel separation and tandem mass spectrometry (MS) after liquid chromatography (LC-MS/MS) require a high amount of cells – from several hundreds to thousands oocytes per condition. The reason for this is that despite the oocyte being the largest cell in the organism (about 120 μm in diameter in cattle), it contains relatively low intra-cellular protein content. In average, bovine oocyte contains about 100 ng total proteins of which approximately 15% is attributed to zona pellucida – a thick extracellular coat which consists mainly of glycoproteins. Several studies have reported functional proteomic approaches performed on pools of immature and mature bovine oocytes [12, 13]. Numerous proteins including putative markers of oocyte developmental competence including proteins needed for fertilization, reprogramming, embryo genome activation and first cleavages during early embryo development were identified. Very recently, new efficient sample preparation using paramagnetic bead technology allowed the identification of more than 400 proteins in single human oocyte [14]. However, this procedure is very complex, and is not convenient for high-throughput quantitative analysis on individual oocytes.

In our laboratory, we have adapted original approach, Intact Cells Matrix-Assisted Laser Desorption/Ionization time-of-flight Mass Spectrometry (ICM-MS), to analysis of single bovine oocytes (Fig.). Being initially developed for bacteria phenotyping [15], ICM-MS has the ability to detect the most intense native molecular ions present directly in the biological sample over a relatively wide mass range (typically 100- 25,000 Da), with a high sensitivity (from the picomolar to the femtomolar range) and a high tolerance for contaminants such as salts. In addition, ICM-MS allows detection of very small proteins and peptides which are often lack when using classic LC-MS/MS methods. The method included only a few and simple preparatory including stripping oocytes from cumulus cells and washing them to eliminate any somatic cells and salt excess. The plating procedure is simple: oocyte was loaded onto a MALDI plate, overlayed with an adequate matrix and allowed to dry. Analysis was here performed using UltrafleXtreme MALDI-TOF/TOF instrument (Bruker) but other MALDI-TOF spectrometer may be also used. Using adequate pre-processing and bioinformatic analysis tools, ICM-MS profiles showed characteristic spectral features according to their real maturation stage or quality, and thus differential analysis is possible between different conditions. Consequently, ICM-MS constitutes a powerful tool for the direct analysis of complex peptide/protein mixtures within biological specimens such as crude extracts and intact cells and identify differential molecules, as it was shown for analysis on boar spermatozoa isolated from four different epididymal regions (immature to mature stage) [16]. As ICM-MS is based on the comparison of peptide and small proteins profiles rather than the comparison of single biomarkers, it is theoretically more prone to



Typical ICM-MS workflow. Individual oocytes are washed, then loaded to MALDI plate and recovered with the appropriate matrix. Lipid or peptide/protein spectra are obtained by MALDI-TOF mass spectrometer. Spectra are processed and aligned, and then total spectra intensity and peak heights are measured. Potential markers are discovered by statistical analysis of the spectra between different conditions. Principal component analysis is performed to discriminate the oocytes in different conditions.

identify phenotypic differences associated with a certain physiological, pathological or experimental condition, notably with a subfertility versus high fertility as shown for chicken semen [17]. ICM-MS is in fact, the possibility of using whole, untreated cells (i.e. no extraction or pre-fractionation), high throughput, ease of operation, simple sample preparation and the existence of established data-processing platforms and software for processing and quantification of spectral peaks (normalized peak height values) makes this technique particularly promising for applicationsin reproductive biotechnologies for analysis of different cells of reproductive organs.

To discover the possible factors of oocyte quality, we compared individual oocytes with different developmental competence using ICM-MS technology. Reproducible proteins/peptides fingerprints gathering a more than two hundreds of peaks were obtained for single oocytes, both immature and after IVM[18]. Immature and mature oocytes were easily discriminated by Principle Component Analysis using a number of peaks which varied significantly between the correspondent groups. Moreover, peptide/protein fingerprints obtained by ICM-MS allowed discrimination of the oocytes at the same maturation stage but with different developmental potential after IVF; for example, in vivo versus in vitro mature oocytes or oocytes after IVM, matured either with or without cu-

mulus cells, or oocytes from prepubertal calves versus adult cows.

ICM-MS analyses should be completed with the identification of the peaks present in the spectra according to their specific m/z (mass to charge ratio) values. Peak masses correspond to native, endogenous molecules present in the sample. Of all the MS-based identification approaches currently available, top-down (TD) proteomics by high resolution nano-LC-MS/MS constitute the best approach to identify endogenous peptidoforms and proteoforms, as it involves measurement of an intact molecular species and direct fragmentation, thus providing with a complete description of the primary structure of the protein and of its modifications [19]. Pool of oocyte-cumulus complexes and follicular cells was used to extract total proteins which were then employed for TD identification of the peaks which were observed in oocyte ICM-MS spectra. TD approach allowed identification of more than 350 of unique proteins which were represented by either intact small size proteins (environ 15%) or N-terminal, C-terminal or intermediate fragments of the proteins higher molecular weights. These fragments were shown to be products of proteolytic activities of different endopeptidases targeting specific sites orsubstrates. Numerous PTMs were also evidenced on these proteoforms. Among the identified markers of oocyte maturation, we found several known proteins involved in cytoskeleton organization (alfa- and beta-thymosin), chromosome organization (histones) or protein degradation (ubiquitin) [20].

Oocyte lipidomics. Intracellular lipids have several roles including structural functions while they are components of membranes, energy storage and molecular signaling. The importance of oocyte lipid metabolism during oocyte maturation for it developmental competence is now formerly recognized [21]. Active lipolysis, fatty acid synthesis and oxidation permanently occur in bovine oocyte and surrounding cumulus cells and increased during IVM [22]. Bovine oocyte is rich in lipid droplets inclusion, and during IVM the total lipid quantity decreased [8]. Different methodologies of lipid analysis were applied to the oocytes in different species and allowed characterize different lipid classes of oocyte content. Analysis of complex lipid species in single bovine oocyte has been reported using MALDI MS, and some species of sphingomyelins, phosphatidylcholines and triacylglycerols were detected [23].

We adapted ICM-MS to establish lipid fingerprints from single bovine oocytes. The analytic procedure was similar to peptide/protein profiling except specific matrix and parameters of spectra acquisition. Lipids were easily detectable by ICM-MS, presented as numerous peaks of different intensity. By measurement of normalized peak height of all detected ions we compared lipid fingerprints of immature oocytes and oocytes after IVM and demonstrated significant increase in abundance of several peaks, identified as phosphatidylcholines and sphingomyelins, whereas abundance of two lower weight species (possibly free fatty acids) was significantly decreased [24].

Limitations of “omics” analysis of single oocytes. Technical hurdles of “omics” analysis of single oocyte caused by the minimal amount of biological material are evident and require a miniaturization of several methodologies for extracting and measurement of biological material. It seems being of less importance for transcriptomics because of the possibility of RNA amplification; however this step introduces the biases to real representability of the specific RNAs due to differential enrichment rate depending on either the difference in expression level or to transcript length. Also, the question of data normalization is recurrent in the analyses of the oocytes at different maturation stages.

Correct preservation of the samples before analysis is crucial. Whether oocyte RNA is enough protected from the ribonucleases by zona pellucida and,

in addition, RNA protectors are also available, oocyte lipids and proteins require reinforced more protection even stored frozen. Indeed, lipidomics must be performed as soon as possible after collection because of massive oxidation and degrading of lipids under atmospheric oxygen, and lipid profiles significantly change during storage at –80 °C. Proteins seem to be more stable however ICM-MS performed with fresh oocytes generates much more informative and reproducible spectra than with frozen ones.

Whether an oocyte is used for molecular analysis, its capacity to develop to embryo could not be directly assessed. Oocyte quality therefore could be evaluated by the measurement of developmental competence of the oocytes matured in similar conditions or from the same animal as those which were analyzed. However, the variability between the oocytes recovered from the same animal and /or from the follicle of the similar size is very important. In fact, the quality of the oocyte at the start of the maturation process is thought as the key factor determining the proportion of oocytes developing to the blastocyst after IVM [25]. Therefore, this variability of individual oocytes must be considered before analysis by providing for sufficient number of biological replicates.

Use of cumulus cells to search non-invasive biomarkers of oocyte quality. Cumulus cells are physically and metabolically coupled with the enclosed oocyte, and due to permanent exchanges of small molecules (ions, metabolites, amino and fatty acids, AMPc etc), cumulus may reflect oocyte physiological status. Therefore, whether a part of cumulus cells are taken for analysis, the oocyte may be kept for fertilization and embryo development. A system capable of supporting the IVM, IVF and development of immature bovine oocytes to the blastocyst stage in an individually identifiable manner was reported [26]. More abundant in biological material, cumulus cells from individual oocytes could be used for transcriptomic analysis and gene expression data were correlated with embryo development of correspondent oocytes and thus allowed identification of the markers associated with oocyte quality [27]. Among these genes, 1-acylglycerol-3-phosphate O-acyltransferase 9, *AGPAT9*, involved in lipid metabolism, was over-expressed in cumulus cells of the oocytes stopped at 2-8 cells.

In our laboratory, we adapted ICM-MS profiling for analysis of both lipids and proteins in cumulus cells, surrounding individual oocyte by using either all detached cells or cumulus biopsies. ICM-MS of lipids in cumulus cells allowed clear discrimination of the profiles according to maturation stage or metabolic status of the correspondent oocytes [22]; ICM-MS protein/peptides signatures of cumulus cells were also specific and grouped in accordance with oocyte conditions.

By using the TD approach earlier described, numerous peaks from cumulus cell protein and lipid profiles were identified, and they constitute now the database for further research.

Thus, different “omics” technologies became available tools for analysis of individual oocytes and somatic follicular cells. Oocyte transcriptomics, proteomics and lipidomics on both oocyte and surrounding cumulus cells generate data in order to establish the accurate, fast and affordable tests that can help in the assessment of oocyte quality in assisted reproduction biotechnologies of farm animals. The combination of original mass spectrometry approaches, ICM-MS and TD, proved to be a suitable strategy to identify markers of oocyte quality in bovine using limited biological samples.

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NEW METHODS OF SEMEN ANALYSIS BY CASA

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Abstract

Semen analysis constitutes the base for the establishment of fertility of a male. In addition, the number of insemination doses to produce from an ejaculate depends on the defined sperm characteristics. As soon as image analysis techniques were applied to biological problems the andrology was one of the first fields benefited both in the evaluation of genital track and sperm function. The improvement of image analysis expands possibilities of using developed systems in medical practice and animal husbandry. In ISAS®PBos («PROISER — Projectes i Serveis R+D S.L.», Spain) the semen analysis done includes concentration, motility and morphology. The systems with optimal rate of frame capture and transmission at images processing are developed for a two-dimensional analysis of spermatozoa motility in different species with regard to the spermatozoa sizes. ISAS®PBos calculates the percentage of morpho-abnormalities analysing the presence of cytoplasmic droplets and coiled tails base on the images used for motility analysis, and allows calculation of the optimal number of doses to produce from a particular ejaculate. Breed-dependent morphological diversity of spermatozoa found in different species, and a disclosure of structured subpolations of spermatozoa in the ejaculate, lead reproductive biology to the next level and open new prospects for the practice of animal breeding. In the study of spermatozoa at the subpopulation level the multivariate statistics which is based on an analysis of the principal components is applicable. In statistical estimation and mathematical modeling, it is proposed to use the Bayesian approach, on the basis of which a mathematical toolkit for estimating sperm quality will be developed in the near future. Essentially, the shortcomings of the early methods used for semen analysis are due to modifications of real motility of germ cells in counting chambers and the real shape and size of the spermatozoa under dehydration, fixation, staining and mounting. ISAS® 3DTrack and Trumorph® for estimation of sperm motility and morphology, respectively, avoid the limitations. ISAS® 3DTrack device, a lensless laser microscope, allows the analysis in a depth around 100 μm. Moreover, the analysis of the correspondent hologram allows the analysis of track in three dimensions what is also a big novelty. Trumorph® technique offers the maximum projection of the cells making it possible to obtain images of high resolution and definition of cells components in a wide range of species, including bull. ISAS® 3Fun with ISAS®3Fun kit and the correspondent software for automatic analysis is a new method enables a clear distinction of spermatozoa with intact plasmalemma and acrosome which are essential for sperm function.

Keywords: spermatozoa, motility, morphology, fertility, subpopulations, computer-assisted sperm analysis, statistical analysis

Semen analysis constitutes the base for the establishment of fertility ability of a male. In addition, the number of insemination doses to produce from an ejaculate depends on the defined sperm characteristics.

Traditionally, the semen evaluation was done by an experienced technician, analysing each sample under the microscope, basically for concentration and motility. When possible, concentration was estimated by using a good counting chamber (Neubauer, Bürker etc), when not it is just estimated

in the same time motility is done. For this the most common procedure is to make an approximation to 5 % value, not counting cell by cell but like a subjective approach. Regarding morphology it is not possible to do in a routine daily work and it is applied basically for control quality programs [1] but not for insemination seminal doses production.

In a traditional way, subjective motility and concentration were the most used parameters [2], while morphology assessment had a secondary place because it takes much more time to be performed, the definition of a universal pattern of classification is more difficult [3, 4], and all the process lacks convenient precision (5, 6 Nevertheless, Morphological characteristics of spermatozoa are genetically determined [7], relative to the spermatogenesis and epididymal maturation processes (8), and so being more informative than the motility that is much more affected by environmental factor [9].

As soon as image analysis techniques were applied to biological problems one of the first fields benefited was the Andrology, both into the evaluation of genital track and sperm function [10-13]. The entrance in the personal computerization era implies a fast appearance of computerized image analysis processes to evaluate concentration [14], motility [15, 16], and morphology [17, 18] including electron microscopy images [19]. Successful results of the predictive value of automated CASA [Computerized Assisted Semen Analysis] systems parameters [20, 21] derivates in the appearance of multiple commercial brands. The basic components of a CASa system included from the beginning, the microscope [with good phase contrast, if possible negative], the video-camera, the computer and the software [Fig. 1].

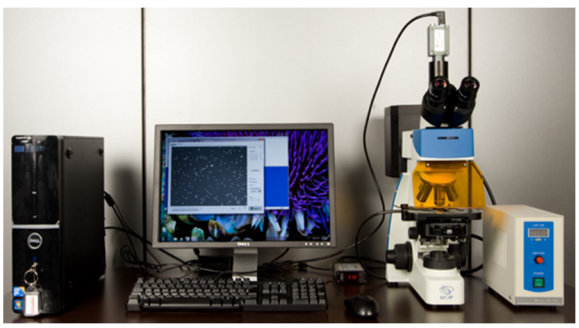

Fig. 1. Components of a CASA system.

The first generation of CASA systems were based on poor informatics tools, requiring a lot of computation time and resulting too much expensive. These facts limited their use to basic research and led to the widespread idea that its use is not recommended to routine human clinical or livestock production. One of the aims of PROISER (Projectes i Serveis R+D S.L., Spain) was the overcoming of this situation with the design of the ISAS®P series, originally devoted on boar seminal doses production but amplified to other species, including bull.

Centering our attention on ISAS®PBos, the semen analysis done includes concentration, motility and morphology. For concentration analysis the use of well-defined counting chambers is needed and, for this, we have developed specific chambers, both reusable and disposable [Fig. 2]. In the market it is possible to dispose of different designs but not all fit a good distribution of cells. In disposable chambers, this is due to rheologic and capillary forces and de design following parallel and thin way results in the best distribution [22-24]. When reusable chambers are used, to optimize the design and glass composition is also needed. Superficial tension of the glass due to surface ions implies a bad distribution related with the time of cover glass deposition [25].

These questions also affect the motility analysis, taking into account that

the cells are forced to move in a plane. For a cell having about 70 μm in length, movement in a space of only 10 μm of width produces a clear motility artifact. Depending on the species, the introduction of chambers of 20 μm reduces this effect, even it doesn't disappear (see later about for the analysis on high depth chambers.).

Fig. 2. Examples of reusable and disposable counting chambers.

Another significant aspect related with the quality of motility analysis refers to frame rate of sequence capture. Motility is defined by several kinematic parameters, some of which are very sensitive to the lapse between images capture. Particularly the VCL [curvilinear velocity] increases following an exponential curve [A. Valverde et al., unpublished results]. So only when the frame rate is close to the asymptotic value the kinematic parameters are really representative, being calculated for bull on 160 fps [26].

Now we are working in the establishment, species by species of the best conditions for the bidimensional motility analysis, it means the best chamber depth and characteristics, the optimal frame rate, the time of charge and analysis etc.

Regarding morphology analysis, the classical approach implied the use of different staining techniques (27). This process includes steps of dehydration, fixation and staining, all of them modifying the real structure and dimensions of the cell and this producing different level of artifacts when observing the cells (28). ISAS®PBos calculates the percentage of morpho-abnormalities analysing the presence of cytoplasmic droplets and coiled tails base on the images used for motility analysis, it means cells on suspension and using negative phase contrast. At his magnification (10×) it is not possible to obtain more detailed information about sperm morphology/morphometry. Fluorescence techniques could be also applied but, in this using this technique, only some head components could be measured (29). In the case of ISAS®PBos the offered report allows to the calculation of the optimal number of doses to produce from a particular ejaculate based, alternatively, on the quality of the analysed sample or on the historical results of the last five post-thawed analysis of samples from the considered animal.

Semen cryopreservation limits. The most important limit to the use of semen doses for AI (artificial insemination) is the time for the maintenance of the doses. In the beginning of the AI both males and females were placed in the same farm, in fact this is even now the common practice in some species like fox. But the evolution of the use of AI derivates to specific farms for each purpose, it means males for seminal doses production, females for calving and other for the growth of the animals. In this case, the possibility to preserve the doses results fundamental. The refrigeration of the samples was the first approach, and this continues being the most used in species like boar or rabbit. But the distance at which the samples can be transported and the time for its use is short, limiting the possible interchange of genetics in long distances. The introduction of freezing techniques allowed to solve this problem producing samples that, theoretically, could be transported around the world and for no limited time. This combination of techniques of freezing/thawing process is the most common now in human and bull species and a lot of different protocols were

developed in the last decades.

But this technique causes different alterations in the semen quality, like increase of oxidative stress conducing to changes in motility, DNA fragmentation, acrosome reaction etc [30]. The evaluation of these changes is very difficult if not impossible following manual evaluation of semen quality and thus, the use of CASA technology is particularly interesting for this determinations [31, 32].

Effect of genistein on bull sperm after freezing and thawing

Group	Viability	Sperm DNA fragmentation
Control	61.1±1.3[a]	6.8±0.7[a]
Genistein 30 min	51.6±1.3[b]	5.3±0.6[ab]
Genestein 60 min	50.2±1.4[b]	4.7±0.6[b]

In an attempt to improve the problem of oxidative stress on spermatozoa during cryopreservation the effect of addition of genistein was evaluated on bull semen [33]. We observed that the percentage of cells with high level of DNA fragmentation was lower after treatment with the antioxidant genistein (Table), but this reduction was in parallel to a decrease in motility and vitality, this treatment only seems to be indicated to samples with high DNA fragmentation levels or for ICSI procedure.

In other paper we asked recently, what we speak when we refer to dog semen (C. Soler et al., in press). In the case of dog we observed significant differences between the morphometric characteristics of spermatozoa from different breeds. It seems to be obvious that this differences must exist when we consider breeds so much different like British bulldog, chihuahua and German shepherd dod but until now must of the scientific literature has mixed different breed in the same work just like a dog. The same question could be also applicable to bull, where a lot of well-defined breeds, even inside *Bos taurus* were defined, but more if we included also the *Bos indicus*. In fact some studies have compared both species and morphometrical differences have been observed [34]. In the present we are performing one experiment comparing four different breeds trying to advance on this interesting topic that has two kinds of implications, just from the biological point of view and to define new approaches to how manage the ejaculates defining specific protocols for each breed

Looking to the future. Seminal subpopulations structure. In spite of the advances achieved in the last decades, the predictive capacity of the *in vitro* analysis on potential fertility of semen remains still limited [35, 36]. This limitation of the "classical" seminal parameters although may be improved using combined statistical analyses of various sperm quality parameters [37, 38]. As consequence, the complexity of the semen analysis has progressively been increased with the hope of improving fertility predictions [39, 40]. However, some of these quality parameters are highly correlated, with merely increasing the number of analytical tests not always improve the predictive ability of the spermiogram [28, 38, 41].

The application of high mathematics analysis tools to the study of semen characteristics has revealed the existence of sperm subpopulations in the ejaculate. This has been observed in the last decade in a great number of species and conditions, even its significance is not yet clear (42-47). The application of high mathematics analysis tools to the study of semen characteristics has revealed the existence of sperm subpopulations in the ejaculate. This has been observed in the last decade in a great number of species and conditions, even its significance is not yet clear (28). How this could happen is the most exiting research we can approach in the next decades until to achieve a good comprehension of the phenomena.

In a recent paper we have compared the "classical and advanced" mathematical approaches to the problem to evaluate the seminal characteristics. It was put on evidence that the Classical approaches, based on ANOVA or even

MANOVA, are no good enough to define the sperm population, being necessary the introduction of the use of multivariate statistics based on principal component analysis to define the subpopulation structure as a better definition of the real semen cell composition. In addition, the presence of differences between different ejaculates of the same animal indicates that the subpopulation structure, even having a genetic basis, could be influenced by environmental (external and internal) factors (48). Looking to this problem, the recent introduction of Bayesian approaches to the evaluation of semen quality is offering a new tool that will be developed in the next time (49-51).

New tools for new approaches. In any case, the analysis done until now, both for motility and morphology present an additional limitation, referred to the fact they must be considered like artifacts. The motility analysis had been requiring the observation using optical microscopy and this means that the depth of view is very low, and this implies that counting chambers depth cannot be higher than 20 μm. Taking into account that the length of sperm, depending on the species, could be considered higher than 50 μm it implies that the movement of the spermatozoa inside the chambers is non "natural" being constricted and so modified in its real pathway. In reference to morphology, all the techniques used until now implied the modification of the real shape and size of the spermatozoa, including processes of dehydration, fixation, staining and mounting (52).

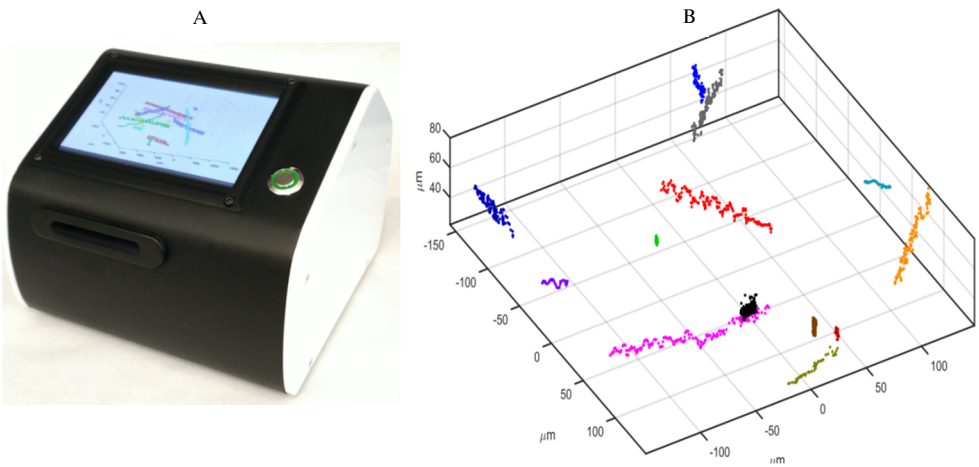

Fig. 3. ISAS®3DTrack device (A) **Tridimensional representation of boar sperm motility by using ISAS®3DTrack** (B).

To overcome this artifactual production, our team has developed two new facilities: the ISAS®3DTrack (Fig. 3, A, B) and Trumorph® (Fig. 4, A, B) for both motility and morphology analysis, respectively.

As we pointed out before, it is needed to significantly increment the depth in which cells must move during the analysis and for this we have developed the ISAS3DTrack, a lensless laser microscope that allows the analysis in a depth around 100 μm. Moreover, the analysis of the correspondent hologram allows the analysis of track in three dimensions what is also a big novelty. In essence, the proposed technology becomes in the basis architecture of a reduced cost, portable and compact system design of lensless holographic microscope with an illumination/detection scheme based on wavelength multiplexing, working with single hologram acquisition and using a fast convergence algorithm for image processing. Altogether, the proposed imaging platform allows high-resolution (μm range) phase-retrieved (twin image elimination) quantitative phase imaging of dynamic events (video rate recording speed) (M. Sanz et al.,

2015, 2017).

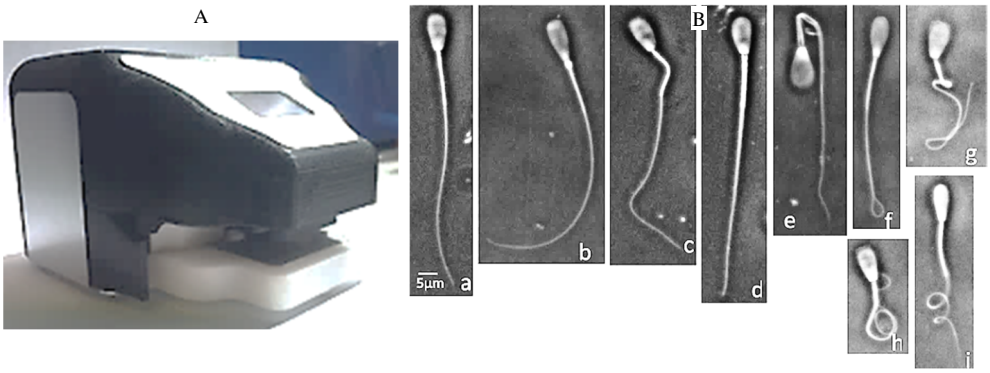

Fig. 4. Trumorph® device (A) **and different morphologies of bull sperm observed using Trumorph® technique** (B; a, b, c, d, e, f, g, h, i)**.** Image (a) represents what is considered a normal cell.

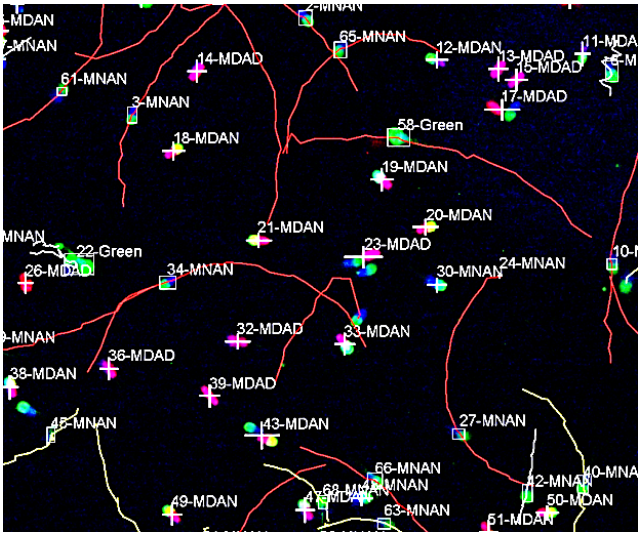

Fig. 5. Staining patterns of boar spermatozoa using the ISAS® 3Fun kit. Scale bar = 5 µm (left) **and computer-assisted sperm analysis of the sperm trajectories after staining with the ISAS® 3Fun kit (right).**

On the other hand, the Trumorph® technique (Fig. 5, A) is based on application of heating to immobilise the cells and a little pressure to spread the seminal plasma offering the maximum projection of the cells. With the use of objectives of 40×, negative phase contrast, it is possible to obtain images of high resolution and definition of cells components in a wide range of species, including bull (Fig. 5, B) (46, 53, 54).

Finally, fluorescent markers and flow cytometry allow the assessment of numerous structural and functional characteristics of spermatozoa in large populations (55, 56), but this technique is complex and too much expensive to be applied in a routine work for doses calculation. With the aim to avoid these limitations we have developed the ISAS®3Fun kit and the correspondent software for automatic analysis. This new method enables a clear distinction of spermatozoa with intact plasmalemma and acrosome, which are essential for sperm function (57), no altering the motility and so offering the possibility to analyse these three parameters simultaneously and cell by cell (see Fig. 5).

So, our proposal for the close future must to combine, using advanced mathematical analysis, as much as possible seminal parameters, including both the “classical” and the new ones to define a new mathematical pathway for predicting fertility. This effort must to start by the definition of the optimal conditions for the use of any of the tools considered. Some work on this was done but it must be completed. The analysis of the best possible data will rend a new way

to approach both the best comprehension of the subpopulations structure meaning from the evolutive and physiological points of view and the improvement in the seminal evaluation and seminal doses production for assisted reproduction techniques.

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ADDITIVES USED IN EXTENDERS TO IMPROVE THE FREEZABILITY OF RAM SEMEN IN RECENT YEARS: a mini review

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Abstract

One of the most important reasons why artificial insemination does not spread as much in cattle as sheep is that ram sperm is highly fragile against cryodamage and consequently the optimum fertility results can not be obtained from cervical inseminations compared to laparoscopic insemination. To establish an ideal ram sperm freezing model, many studies have been carried out for more than 60 years and various methods have been tested by making great efforts. One of the topics where these tests have been done intensively in recent years are additives used in freezing extenders in order to increase the tolerance of sperm against oxidative stress in frozen-thawed ram semen. In this review, covering the studies carried out between the years 2000-2016, we have mostly compiled the additives used in ram semen freezing media which have given better results compared to others supplements. It is understood that, usually, seminal plasma and proteins, thiol compounds, enzymatic antioxidants, sugars, fatty acids and vitamins have been studied as additive agents in recent years. As a result, this review suggests us that further studies are needed to explore the novel techniques and new additives, and their combinations should be tested in different doses in order to install an ideal cryopreservation template for ram semen. Furthermore, even though addition of various compounds to freezing media have improved freezability of ram semen in experimental conditions, anyway these results must be confirmed in field studies. Therefore, the value of sperm which potential fertility is predicted from laboratory survey must be compared with conception or lambing rates.

Keywords: ram semen, additives, freezability, extender

While the basic techniques related to sperm freezing have done positive effects and important advances mostly on dairy cattle husbandry, any remarkable success has not been provided in sheep. Substantially, these matter is caused mainly by from two reasons following as: i] intolerance of ram sperm against cryopreservation, ii] the physiological and anatomic factors in sperm transport. It is well known subject for a long time that freezing and thawing of ram sperm have caused significant membran and genetic damage [1]. As cervical insemination has given poor fertility results compared to laparascopic inseminations, artificial insemination with frozen semen has not become widespread in sheep husbandry [2]. On the other hand, the expenses of ram semen freezing are almost 20 times more than those of bull semen freezing cost [3]. B Recently, investigators have focused mostly on especially composition, determination of *in vivo* properties and effects of different cryoprotectants, antioxidants and specific supplements in sperm freezing [4].

We understood from literature documented here, no comprehensive review of the researches on frozen ram sperm has been found from that date into the past 16 years. During 2000s, the attentions of researchers have mostly concentrated on additives for improving membrane repair and increasing progressive motility in ram semen cryopreservation. It seems that while base extenders used in ram semen freezing has not been much changed, many various supplements has been tested from the beginning of 2000's to now.

However, in this review, only additives that often have improving effects on frozen-thawed ram semen are mentioned. The additives studied between 2000-2016 years are reviewed here and considerations has presented below.

Seminal plasma and its protein components (SPPs). The role of seminal plasma in reproductive technologies such as sperm freezing and sex determination is already proven and it is also agreed that seminal plasma addition to ram semen freezing medium had a improving effects on post-thaw motlitity and morphological damage [5, 6]. Furthermore, although seminal plasma proteins show protective effect in freezing and thawing processes of ram semen, interestingly the same effect has not been in bull semen [7].

When seminal plasma proteins are used alone or combined with other compounds such as oleic/linoleic acids and vitamin E etc., the protective effect is not only observed in fresh sperm but also after freezing and thawing, so that SPPs provide regular regenerative SOD distribution by using combined with oleic / linoleic acids and vitamin E [6]. However, still, more investigations are necessary to solving of biological complicacies, and then identify and synthesize favorable proteins within seminal plasma [8].

Antioxidants. As sperm membrane contains higher of unsaturated fatty acids than those of somatic cells, it is inclinable to oxidative stress, thus plasma and mitochondrial membrane is highly susceptible against cold shock. Moreover, sperm has not is any restoration mechanism in prevent cell injuries caused by ROS. The antioxidants which are mainly present in seminal plasma in semen have limited protective effect [9, 10]. Moreover, antioxidants are used not only for protecting of membrane integrity of sperm cell, but also, the membrane damage of embryo and oocytes by depressing of lipid peroxidation and ROS production [11].

Thiol compounds. It has been known that thiol compounds have an scavenger effect for elimination of cytoxins and ROS which give cellular defects caused by ROS [12]. In recent years, thiol compunds such as l-erhothioneine [LE],Cystein and N-aceytyl cysteine have been frequently studied. It has been observed that thiol compounds have given the improving effect of ram semen freezability [13-18].

Cystein. Cystein is also one of thiol compunds which protects against oxidative stress damage of ram sperm cell during cryopreservation processes. The addition of cysteine to ram semen freezing medium has provided high motility rates and catalase level [14].

N-acetyl cystein [NAC]. NAC which is a precursor of intracellular GSH biosynthesis, has been rarely studied by researchers in ram semen freezing [17, 18].

Enzymatic antioxidants. Enzymatic antioxidants protect cells prevent from cellular membrane damage with neutralizing ROS produced by cellular components. Enzymatic antioxidants contain superoxide dismutase [SOD], catalase, glutathione peroxidase [GPx], and glutathione reductase [GR] [19].

Some researchers have claimed that the supplementations of catalase [20], SH-glutathione [GSH], SOD [21, 22], Gpx and Tempo or Tempol [as SOD mimics] [23] have improved in semen quality in frozen-thawed samples.

Non-enzymatic antioxidants and sugars (trehalose and sucrose). Synthetic antioxidants or food supplements such as vitamins, and minerals, *BSA* [bovine serum albumin, trehalose, vitamin C, vitamin E, zinc,cysteine, taurine, hypotaurine, and lutathione etc. has been accepted as non-enzymatic antioxidants [19 According to the results of some studies, BSA, cysteine, lycopene [24], trehalose, taurine, cysteamine, hyaluronan [24] and combination of sucrose and trehalose combination [25] in freezing diluents have given satisfacto-

ry percentages of motility, viability and acrosomal membrane integrity.

Lecithin. Despite mechanism of action of lecithin on sperm plasma stabilization during freezing and thawing is not clearly know, it has been reported that lecithin and other lipidic supplements protect and stabilize the sperm membrane by substation of phospholipids, thereby increasing resistance to freezing. Some studies demonstrate that use, alone or combined with some compounds, of 1.5 % soya bean lecithin has given better spermatological values motility, viability and intact membrane compared to hyaluronic acid (HA) [26] and egg yolk [27] additions, and also could be alternative a cryoprotectant aid instead of egg yolk [28]. Although egg yolk has been found as superior to lecithin in a study [29], when compared to in another study, it has been reported that lecithin could be used instead of egg yolk. In Bakhtiari rams, spermatological results obtained from the diluents which included 1 % lecithin, 20 % egg yolk and 7 % glycerol were superior in the freezing process and it was concluded that lecithin could be used instead of egg yolk [30].

Fatty acids (*FA*). Long-chain polyunsaturated fatty acids (LCP-UFA) of the n-3 series provide suitable stabilization by maintaining of liquidity and elasticity of cell membran, thus, supplementation of (polyunsaturated fatty acids) (PUFA) increase the sperm cell number, motility and sperm fertilizing potential by strengthening against lipid peroxidation in mamals [31)].

Fatty acids, such as oleic acid, linoelic acids, n-3 (ω-3) FA have been used by investigators in order to support the sperm against oxidative stress damage and obtain an optimum cryosurvival in frozen-thawed semen. It has been alleged that the combination of oleic/linoleic acids with vitamin E and SPPs (6), some other agents, e.g. fatty acid amide hydrolase (FAAH) [32], and ω-3 FA provided protection in frozen-thawed ram sperm.

Vitamins. Currently, it has been understood that the vitamin E [31, 33], vitamin C [34-38] and vitamin B_{12} [39, 40] additions to semen freezing diluents have enhanced the spermatological properties by eliminating of ROS damage to sperm cells.

Hormones. It has reported that there is a relationship between hormones and sperm damage [41, 42]. From articles scanned here, no study was not seen which has been searched the directly addition of hormones to ram semen freezing extenders except for one study which established the effect of melatonin hormone. In this study, 1 mM melatonin additions were found to be quite successful in terms of total and progressive motility, intracellular ATP concentrations and DNA integrity, especially in viability rates. In addition, in vitro embryonic development was highest in the group with 1 mM melatonin [43].

Other substances. Some alternative substances have been tested, as alone or combined by, with/without various glycerol concentrations in extenders for improving freezability of ram semen [33, 44, 45].

One of surfactant detergents, Equex, is known that stabilizes the cell membrane, protects from cellular toxication and membrane damage after freezing and thawing semen. With respect to results of a research the addition of 0.75 % Equex STM® yielded better spermatological percentages (motility, viability, membrane integrity) and DNA fragmentation compared to control samples. Moreover, in two trials comparing methyl formamide (MF) and ethylene glycol (EG) with glycerol (G) it has been observed that glycerol gave more cryosurvival quality [44] than MF and EG additions [33], however, the nearest results to glycerol (5 %) were provided in the sample concentrated with EG 3 % (Table). On the other hand, the addition of 3 % glycerol ve 1.5 mg CLC/120×10^6 spermatozoa combination to freezing diluent was more amendatory compared to other groups, despite glycerol decreased the viability [45].

Various additives used for cryopreservation of ram semen and reported frozen—thawed spermatological quality

Research	Base extender	Additives	Quantity	Motility		Percentage			
				1	2	3	4	5	6
Aisen et al., 2002	TCFY10+G3	Trehalose	100 mM	65				50	
Baran et al., 2004	TCGz	SP	7.5 %	30			56 AA		56
Uysal et al., 2007	TCFY10	GSSG	5 mM	60		78	3 AA	65	10
	TCFY10	BSA	20 mg/ml	51		78	4 AA	55	12
	TCFY10	Cystein	10 mM	59		73.5	3 AA	41	19.2
	TCFY10	Likopen	800 μg	57.2		70.5	7 AA	49	16.3
Bucak et al., 2007	TCFY10	Taurine	25 mM	63.0		73.0	6 AA	44,0	23.7
Bucak et al., 2008	TCFY10+G5	Cystein	5 mM	61.0		27	10 AA	48	30
Marti et al., 2008	SMY+G7+Galactose 112 мМ	+SPP+ol-lin+vit E	4 mg+25 mM + 2 mM	48.8		36.3		27,0	
Anghel et al., 2009	TCGzY20+G5	Cystein	5 mM	60		60		60	12
	TCGzY20+G5	Cystein	10 mM	72		70		68	17
Uysal et al., 2009	TCGzY15+G5	Trehalose	100 mM	72.0		74.5		66,1	28.7
Forouzanfar et al., 2010	TCFY20+G7	Lechitin	1 %	51.9		48.1			
Maia et al., 2010	TGzY	Catalase	50 μg	69-75	27-30				
Silva et al., 2011	TCFY20+G5	SOD	100 U/ml	58.4	9.4		33.5		
	TCFY20+G5	GSH	2 mM	49.45	9.19		32		
Succu et al., 2011	TCFY20+G4	Melatonin	1 mM	45.9	31	68.7			
Silva et al., 2012	TCFY20	Glycerol	5 %		49,2		33.3		
	TCFY20	EG	3 %		41,7		39.8		
Silva et al., 2012	TCFY20+G5	Vit E	120 μM	80	14		55	45	
Ari et al., 2012	SMEGsY10+G5	LE	10 mM		23	27.1	30	37.1	
Towhidi et al., 2013	Andromed	Vit E+ω-3-FA	0.1 mM+ 1 ng/mL	37	33	35			3
Das Graças et al., 2013	TCGsY20	MF	3 %	38					77 N
	TCGsY20	Glycerol	5.3 %	50					84 N
Motamedi-Mojdehi et al., 2014	TCFY20+G3	CLC	1.5 mg	45				32	
Santiani et al., 2014	SMFY5+G7	Tempo	1 mM	52			41		
Šterbenc et al., 2014	TCFY20+G14	Equex	0.75 %	78	26	88		60	
Najafi et al., 2014	TCFY20+G7	SL	1.5 %	53	56			45	
Emamverdi et al., 2014	TY20	SL	1.5 %	56	26	39		51	
Mata et al., 2015	TES-TCF+G4	SL	3.5 %	52			28 AA		
Talebiyan et al., 2015	TCGsY25+G7	FAAH	0.025 IU/ml	66	27 Fast				
Yıldız et al., 2015	SMEGsY10G5	LE	10 mM	26		27	49 AA	29	52
Panyaboriban et al., 2015	TCFY15G5+ +0,5 % Equex	Trehalose+ +Sucrose	30 mM	79		84	82		
Nalley et al., 2016	TCF	Y-Omega 3	20 %				60		
Câmara et al., 2016	TY10G6	Catalase							
Arı et al., 2016.	TY20G7	GPx	5 IU/ml	40	23	36	37	50	72

N o t e. 1 и 2 — total motility and progressive motility, %, 3 — viability, 4 — acrosome membrane integrity, 5 — hypo osmotic test, 6 — total anomaly. T — Tris, C — citric acid, F — fructose, Gs — glucose, Y — egg yolk, G — glycerol, SM — skim milk, GSSG — oxidized glutathione: BSA — bovine serum albumin, SP — seminal plasma, SPP + ol-lin — seminal plasma proteins + oleic/linoleic acid, SOD — superoxide dismutase, GSH — glutathione, EG — ethylene glycol, MF — methyl formamide, LE — L-ergothioneine, ω-3-FA — ω-3 fatty acids, CLC — cholesterol-loaded cyclodextrin, SL — soybean lecithin, FAAH — fatty acid amide hydrolase, NAC — N-acetyl cystetin, AA — acrosomal anomaly, N — normal. Underlined numbers show addition rates of glycerol and egg yolk to base extender.

The influence of different additives on frozen-thawed sperm parameters is presented in the table. It is seen that in most of studies Tris-based exdender with egg yolk and glycerol has been used. Considering the table, it is noticed that while some of studies' results are very high, some of them are very poor. It should be not ignored that these differences may depend on the specific properties and doses of additives and kind of base extender used [46] as well as freezing method [47], type of cryoprotectant [33], glycerolisation method, glycerol ratio [48], ram individuality and season [49, 50].

Thus, in this review, we only endeavored to divulge additives that provide a good improvement in post-thaw ram semen quality because ram spermatozoa is susceptible against freezing process. In today's conditions, we believe that thorough technological advances in semen freezing techniques and analyzing systems such as assessment of kinematic parameters, determination of genet-

ic, mitochondrial and membrane defects in sperm, the effects of additives or various supplementations on ram semen freezability will be better explored. On the other hand, as each antioxidant and supplement have different influences on post-thaw semen properties, the choice of extender additives mustn't be done randomly. Before installation of experimental design, more detailed preliminary research on the many aspects of supplement agent must be done because of the preventing detrimental effects of ram semen freezing procedure and antioxidant mechanism of additive compounds is highly complicated. Cryopreservation of ram semen is a subtle matter and needed to study deeply and multifactorial experiment designs in future. Although many investigators have made valuable efforts and obtained good laboratory results in ram semen freezing a cryopreservation method giving satisfactory fertility rates with cervical insemination has not been established yet. As a result, further studies must be carried out to explore the unknown aspects of various supplements and tested with combination of different compounds and in different doses in order to install an ideal cryopreservation template in ram semen. Furthermore, even though addition of various antioxidants, hormones and the some other substances to freezing mediums have improved freezability of ram semen in experimental conditions, these results must be confirmed in field studies. Thereby, the sperm potential fertility predicted from laboratory survey must be compared with conception or lambing rates.

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BIODIVERSITY ASSESSMENT IN INTERSPECIES HYBRIDS OF THE GENUS *Ovis* USING STR AND SNP MARKERS

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Abstract

Introgression of wild and domestic species is regarded as a promising way to improve genetic diversity in populations of farm animals. The aim of our study was to investigate the influence of introgression of the wild species (argali) on genetic diversity of interspecific hybrids with domestic sheep using STR and SNP markers. Samples included original parental forms: Romanov sheep (ROM, $n = 35$), representing the «domestic» form (*Ovis aries*), and argali (OAM, $n = 10$), characterizing the «wild» form (*O. ammon polii*), male hybrid F1, obtained by surgical insemination of Romanov ewe by argali sperm (F_1, $n = 1$), and back crosses obtained by crossing Romanov ewes with hybrid F_1 ram (BC_1, $n = 38$) and hybrid BC_1 rams (BC_2, $n = 14$). The analysis of 11 STR loci (BLT001B, CSRD247, FCB20, CSAP36, MAF65, McM147, OarCP49, D5S2, HSC, BMS2213 and INRA23) was carried out on the ABI PRISM 3130xl genetic analyzer. For SNP genotyping we used Ovine SNP50K BeadChip. After quality control, 9 STR loci and 8591 SNPs were left for the analysis. Statistical calculations were performed in GenAIEx 6.5, PLINK v1.07, HP-Rare 1.1, GENETIX 4.05 and STRUCTURE 2.3.4. Regardless the type of DNA marker, ROM, compared with OAM, was characterized by a higher level of genetic diversity, assessed by observed heterozygosity (H_o) and allelic richness (Ar). Hybridization resulted in an increase in this parameter in the F_1 hybrid. In groups BC_1 and BC_2, the H_o values, calculated per STR and SNP, were higher than similar in the parental forms. In BC_1 and BC_2 groups the Ar values, estimated by SNP-markers, were reducing in comparison with F_1 and were intermediate in comparison with the same in the parental forms. The changes in the Ar values, based on STR data, had the character of a trend in groups BC_1 and BC_2. Principal component analysis (PCA), performed by using SNP-markers, showed a more objective distribution pattern of the studied animals according to their origin in the coordinates space. In case of SNP data, PC1 was sufficient for clearly differentiation of groups OAM, ROM, F_1 and $BC_1 + BC_2$. In summary, the first two components (PC1 and PC2) were responsible for 25.87 % of the SNP variability and for only 12.46 % of the STR variability. Principal component 3 (PC3), which was responsible for 6.16 % of SNP variability, made it possible to differentiate BC_1 and BC_2 groups, whereas at the application of STR these groups were localized as a common cluster. The results of STRUCTURE analysis showed that association of the investigated individuals into clusters, based on STR-profiles, did not match their origin, while the formation of clusters by SNP-markers was corresponded to the actual origin of animals. We found that both types of tested DNA markers were suitable for detecting changes in genetic diversity through hybrids generation. Nevertheless, a significant advantage of using multiple SNP-markers for the differentiation of hybrids from the parental forms was shown.

Keywords: interspecific hybrids, introgression, genetic diversity, SNP, STR, genus *Ovis*

Major evolutionary levers, such as recombinations and natural selection, act slowly and with a few exceptions lead to a definite effect after a long time, but through introgressive hybridization these events can be significantly accelerated [1-5]. Introgressive hybridization is the process of including alleles of one species in the gene pool of another on the basis of a repeated backcrosses of an interspecific hybrid with one of the parental forms [6-9]. In nature, introgression, as a rule, occurs between closely related species [10, 11], and is also characteristic of hybrid zones, or zones of contact of two species [1]. Nevertheless, the greatest interest is remote, or interspecific, hybridization.

Intensification of livestock raising and long-term unidirectional selection, including the use of related lines or crosses, lead to a decrease in genetic diversity and an increase in the negative consequences of inbreeding, in particular, to deterioration in the adaptive abilities of animals. In this case, wild forms are considered as unique reservoirs of genetic variability for their home relatives [12].

The interspecific hybrids are mostly sterile and unable to transfer their unique properties to the offspring [13]. However, there are exceptions. Thus, the descendants of the Pyrenean ibex (*Capra pyrenaica*) and the domestic goat (*Capra hircus*) retain the ability to reproduce [5]. Hybrids of bovine cattle (*Bos taurus*) with Indian cattle (*Bos indicus*) [14], American bison (*Bison bison*), bison (*Bison bonasus*)[13, 15], banteng (*Bos javanicus*) and gaur (*Bos gaurus*) are fertile [16, 17]. The offspring of crossing the domestic sheep with wild species, argali (*Ovis ammon*), mouflon (*Ovis orientalis musimon*), snow sheep (*Ovis nivicola*) is breedy and can be the basis for the creation of a new selection type of sheep, characterized by excellent adaptation to complex relief and natural climatic conditions and serving as a source of dietary meat [18, 19].

The fertility of offspring from the crossing of sheep of different breeds with mouflons has been shown in numerous experiments [20, 21] and in natural conditions, which casts doubt on the existence of genetically pure populations of this ungulate [22]. In 1934-1950 in Kazakhstan, a Kazakh arkharomeris was bred on the basis of interspecies hybridization of argali and domestic sheep of the Kazakh merino breed, which proved to be well adapted for breeding in mountainous areas [19, 23].

Obtaining objective data on the degree of introgression and changes in the breed allele fund under the influence of hybridization is primarily determined by an adequate choice of the type of DNA markers. Until recently, one of the most popular markers for detecting interspecific hybrids was mitochondrial DNA polymorphism (mtDNA) [1, 24-26].

However, this approach has significant drawbacks. First, the genetic contribution of the male to the introgression is not taken into account. Secondly, mtDNA analysis does not allow to estimate the degree and nature of changes affecting nuclear DNA (nDNA) in hybrids. Contrast genetic regularities for nuclear and mitochondrial markers are usually explained by the high genetic drift of mtDNA due to a reduction in the effective size of mtDNA compared to that of nDNA [24, 25].

Microsatellites, or STR (short tandem repeats) markers, are a widespread type of nuclear DNA markers [27]. Microsatellites which are co-dominant, having Mendelian inheritance and analyzed automatically have proved themselves for identification and characteristics of allele fond and genetic diversity in closely related species [28-32]. However, as per M.G. Sovic et al. [33], the use of microsatellites to evaluate the hybridization of more remote species is questionable.

With the development of modern high-performance genotyping technologies, SNP markers (single nucleotide polymorphisms) are becoming promising

tools for investigating introgressive changes in the genome [33-35]. The advantage of SNP is the ability to identify loci with fixed alleles specific for each of the parental species [36, 37]. The detection of such loci, as a rule, is a simple and fast process. The use of whole genome DNA platforms allows finding several hundred loci [38]. The results obtained by comparing two taxa and their hybrids using diagnostic loci are unambiguous and highly reliable [39].

At the L.K. Ernst All-Russian Research Institute of Animal Husbandry was created a unique model population of interspecific hybrids of domestic Romanov sheep (*O. aries*) and argali of the Pamir population (*O. ammon polii*), which to date includes two generations of descendants from the recurrent crossing of Romanov sheep with hybrid males. In the present work, the changes in genetic parameters in interspecific hybrids were studied in detail for the first time as compared to both parental forms in a population obtained under artificial rather than natural conditions.

Our goal was to assess the impact of introgression of the wild species [argali] on the genetic diversity of interspecies hybrids with domestic sheep, performed using SNP and STR markers.

Technique. The object of the research was the original parental forms - Romanov sheep (*O. aries*) (ROM, $n = 35$, maternal "domestic" form) and argali (*O. ammon polii*) (OAM, $n = 10$, paternal "wild" form]; hybrid male F_1 from crossing the Romanov sheep and argali, 50 % argali blood); backcrosses obtained by crossing Romanov gimmers with hybrid F_1 males (BC_1, 25 % argali blood, $n = 38$) and BC_1 (BC_2, 12.5 % argali blood, $n = 140$. The biomaterial was ear tissue samples.

DNA was isolated using Nexttec columns (Nexttec Biotechnology GmbH, Germany) and a DNA-Extran kit (ZAO Sintol, Russia) according to the manufacturer's protocols.

Genetic studies of STR markers were carried out by PCR amplification of 11 microsatellite loci of sheep as per ISAG (International Society for Animal Genetics) combined into two multiplex panels — panel 1 (BLT001B, CSRD247, FCB20, CSAP36, MAF65, McM147) and panel 2 (OarCP49, D5S2, HSC, BMS2213 and INRA23). Reaction mixture (final volume 20 μl) contained 1× PCR buffer, which included 16.6 mM $(NH_4)_2SO_4$, 67.7 mM Tris-HCl (pH 8.8), 0.1 (v/v) Tween 20, and 1.5 mM $MgCl_2$, 200 μM dNTP, 20 pmol of each of the primers, 1 U Taq DNA polymerase (Dialaz Ltd, Russia) and 1 μl of DNA (50-100 ng). After initial denaturation (95 ° C, 5 min), 35 amplification cycles were performed (95 °C, 20 s, 55 °C, 30 s, 72 °C, 1 min) for the panel 1 and 41 amplification cycle (95 °C, 20 s, 63 °C, 30 s, 72 °C, 1 min) — for the panel 2. The PCR products were separated on a PRISM 3131xl genetic analyzer (Applied Biosystems, USA). The fragment sizes were determined using the GeneMapper® Software v4 (Thermo Fisher Scientific, Inc., USA).

For a whole genome SNP scan, a medium-density DNA chip Ovine SNP50K BeadChip (Illumina Inc., USA] was used. With the PLINK v1.07 software [40], quality control was performed which included four steps. In the first stage, a GC score of 0.5 and GT score of 0.3 cutoff were applied. SNP localized on sex chromosomes and with unknown localization were excluded from the analysis. In the second stage, the SNPs polymorphic in the parent forms were determined at the frequency of minor alleles less than 10 % (-maf 0.01), the genotyping efficiency not less than 90 % of animals tested (--geno 0.1), and Hardy-Weinberg equilibrium test $p < 10^{-6}$ (-hwe 1e-6). After filtering, only SNPs common in both parent groups were selected. In the third stage, monomorphic SNPs unique for each group were identified, since in hybrid forms they could become polymorphic. The monomorphic SNPs were added to the poly-

morphs selected at the first stage. In the fourth stage, hybrids were added to the parent forms. Filtering was carried out according to GENO criteria (--geno 0.1) and LD linkage disequilibrium test (--indep-pairwise 50 5 0.5; for 50 SNP, one of the pair of markers for which LD was above 0.5 was deleted, and then the frame was shifted by 5 SNP). The MAF and HWE filters were not applied at this stage. Afterward the LD test, a set of SNPs for statistical calculations was formed.

The main population indicators, i.e. the observed (H_o) and expected (H_e) heterozygosity, inbreeding coefficient (F_{IS}), F_{ST} fixation index [41] and M. Nei distances (D_N) [42], were determined in GenAIEx software 6.5.1 [43] for STR markers, and in GENETIX 4.05 software [44] for SNP markers. The rarified allelic richness (Ar) in each animal group for both markers was calculated in the HP-Rare 1.1 program [45]. The identity state matrices (IBS, identical-by-state) for principal component analysis (PCA) were constructed in PLINK v1.07. R version 3.2.3 was used for creating input files and visualization [46]. Population structure was analyzed in STRUCTURE 2.3.4 [47] using burn-in period of 50,000, Markov chain Monte Carlo (MCMC) of 50000 repetitions, 10 iterations. The calculation was carried out for the coefficient k = 2 {the number of expected populations). For each of the groups, the average value of the membership ratio Q in the i-th cluster was determined for the total number of clusters k ($Q_{i/k}$).

Results. Loci BLT001B and CSAP36 were excluded from the STR set because of the lack of information in 70 % OAM animals. The final STR set included 9 loci. Primary analysis of SNP profiles in the parents showed the presence of 45155 polymorphic SNP in Romanov sheep and 9816 polymorphic SNP in argali. After MAF, GENO, and HWE filtering, 9002 polymorphic SNPs common for both groups were selected, which were supplemented with 231 unique monomorphic SNPs for each group. The resulting set consisted of 9233 markers. As a result of quality control throughout the sample using the GENO filter and the LD test, 61 and 581 SNPs were excluded. The final set of SNPs included 8591 markers.

Regardless of the type of DNA markers, a higher genetic diversity based on H_o and Ar was established in ROM compared to OAM (Table). Crossing led to an increase in this index in hybrids F_1. In both backcrosss groups (BC_1 and BC_2), the H_o values calculated for STR and SNP were higher than those in the parents. The Ar value calculated for SNP decreased in groups BC_1 and BC_2 compared to that in F_1 animals and was intermediate relative to those of parents. For STR, there were no clear changes in Ar in BC_1 and BC_2.

Parameters of genetic diversity of parental and interspecific hybrid forms of the genus *Ovis* on STR and SNP-markers

Marker	Groups	*n*	H_o	H_e	F_{IS}	Ar
STR	OAM	10	0.544±0.107	0.683±0.058	0.266	1.719±0.061
	ROM	35	0.656±0.040	0.775±0.028	0.154	1.778±0.147
	F_1	1	0.778			1.786±0.028
	BC_1	38	0.837±0.026	0.680±0.020	–0.242	1.689±0.021
	BC_2	14	0.745±0.074	0.757±0.025	0.027	1.785±0.027
SNP	OAM	10	0.270±0.002	0.280±0.002	0.038	1.296±0.002
	ROM	35	0.369±0.002	0.368±0.001	–0.004	1.458±0.005
	F_1	1	0.458			1.373±0.001
	BC_1	38	0.418±0.002	0.357±0.002	–0.172	1.362±0.002
	BC_2	14	0.433±0.003	0.344±0.002	–0.260	1.357±0.002

N o t e. H_o — observed heterozygosity, H_e — expected heterozygosity, F_{IS} — fixation index, Ar — rarified allelic richness; the values of H_o and Ar for SNP markers were significant at $p > 0.999$. OAM — argali (*Ovis ammon polii*), ROM — Romanov sheep (*O. aries*), F_1 — hybrid male from crossing of Romanov sheep and argali (50 % argali blood), BC_1 and BC_2 — backcrosses of Romanov gimmers to hybrid F_1 males (25 % argali blood) and BC_1 (12.5 % argali blood), respectively. The data for F_1 are not given, since the F_1 group was represented by one individual.

Similar trends for STR and SNP markers were noted when assessing the dynamics of heterozygotes in the generations. Thus, in the OAM, a heterozygote

deficiency was found which was notable for STR markers (13.9 %) and small for SNP markers (1.0 %). The ROM group was practically in equilibrium for SNP markers, while the analysis by STR showed a decrease in the heterozygote deficit to 11.9 %. Both types of markers indicated an excess of heterozygotes in BC_1 with reverse trends in BC_2 (a heterozygote deficiency estimated by STR and an increase in the excess of heterozygotes for SNP).

The PCA (Fig. 1) of DNA profiles for two types of genetic markers showed a more objective distribution of the animals according to their origin when using SNP markers. In summary, the first two components (PC1 and PC2) accounted for 25.8 7% of the variability of SNP markers and only for 12.46 % of the variability of STR markers. The third component (PC3) allowed to explain 6.16 and 4.45% variability, respectively.

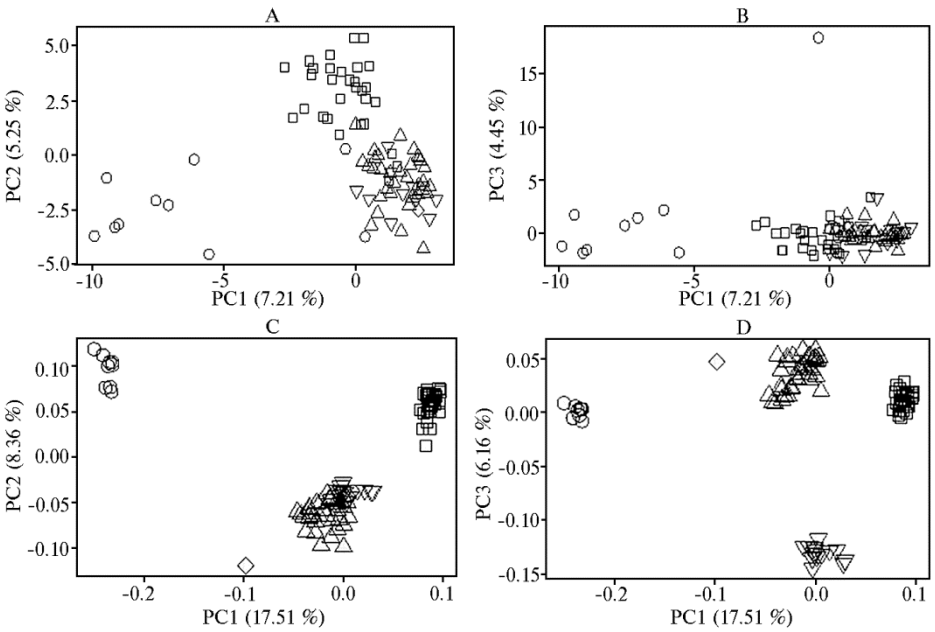

Fig. 1. PCA analysis for 9 STR loci (A, B) **and 8591 SNP** (B, D) **loci in the space of the components 1 and 2** (A, B)**, and1 and 3** (B , D) **for parents and their interspecific hybrids of the genus *Ovis***: ○ — OAM, argali (*Ovis ammon polii*), □ — ROM, Romanov sheep (*O. aries*), ◇ — F_1, hybrid male from crossing the Romanov sheep and argali (50 % argali blood), △ and ▽ — BC_1 and BC_2, backcrosses of Romanov gimmers with hybrid F_1 males (25 % argali blood) and BC_1 (12.5 % argali blood).

STR-based clusterization was fuzzy, and there were animals localized in the "alien" cluster (see Fig. 1, A, B). With SNP, already PC1 allowed to clearly differentiate OAM, ROM, F_1 and BC_1 + BC_2 (see Fig. 1, B). The equidistant position of the F_1 hybrid from the two parent forms was interesting and fully corresponding to its origin. PC3 made it possible to clearly separate the BC_1 and BC_2 (see Fig. 1, D).

When using SNP, a comparative analysis of genetic differentiation in the groups by F_{ST} and D_N showed a clear correspondence between the nature of genetic connections and the origin of animals. The maximum differences were observed between the parent species (F_{ST} = 0.280, D_N = 0.250). In hybrid individuals, as the blood content of the argali decreased, the genetic differences with respect to argali increased (F_{ST} = 0.223, D_N = 0.174 for BC_1, FS_T = 0.272, D_N = 0.218 for BC_2) which brought them closer to the domestic form. The nature of genetic linkages between groups, established by STR markers, did not correspond to the origin of the animals. Perhaps this is due to the insufficient information capacity of nine STR loci.

To date, many researchers [29, 47-49] consider the cluster analysis of admix models for multilocus marker in the STRUCTURE program as the most effective in detecting hybrids. In our work, the grouping of the studied individuals into clusters based on STR profiles did not correspond to their origin both for k = 2 (Fig. 2, A) and k = 3 (data not shown). This is due to two main reasons. First, the microsatellites used are discovered and recommended for the genetic analysis of domestic sheep, rather than argali. Secondly, the information capacity of nine markers could not be enough for such studies. So, according to J.P. Vaha and C.R. Primmer [29], at least 48 loci are required to adequately separate the backcrosses from purebred parental specimens.

STRUCTURE analysis for 8591 selected SNP marker showed that the clusters corresponded to the actual origin of individuals (see Fig. 2, B). The parents (wild and domestic forms) were grouped into two strictly consolidated clusters. The membership in the own cluster for argali was $Q_{1/2} = 0.94 \pm 0.04$, for Romanov sheep — $Q_{2/2} = 0.99 \pm 0.01$. Hybrid F_1 with 50 % of wild and domestic blood was approximately equal to the membership values in each of the two clusters ($Q_{1/2} = 0.54$ and $Q_{2/2} = 0.46$). In the BC generations the percentage of cluster membership specific for OAR decreased ($Q_{1/2} = 0.27 \pm 0.05$ in BC_1 and $Q_{1/2} = 0.22 \pm 0.04$ in BC_2) while that specific for ROM increased ($Q_{2/2} = 0.73 \pm 0.05$ in the BC_1 and $Q_{2/2} = 0.78 \pm 0.04$ in the BC_2).

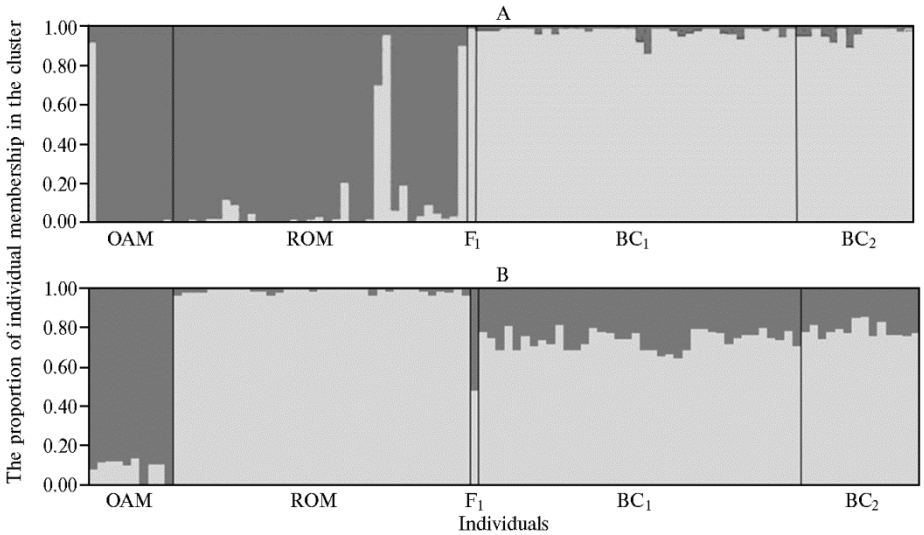

Fig. 2. Population assignment for parental and interspecific hybrids of the genus *Ovis* based on 9 STR loci (A) **and 8591 SNP marker** (B) **in the STRUCTURE program for k = 2:** OAM — argali (*Ovis ammon polii*), ROM — Romanov sheep (*O. aries*), F_1 — hybrid male from crossing of Romanov sheep and argali (50 % argali blood), BC_1 and BC_2 — backcrosses of Romanov gimmers with hybrid F_1 males (25 % argali blood) and BC_1 (12.5 % argali blood).

Thus, the change in genetic diversity in generations of interspecific hybrids due to the introgression of argali can be determined by both STR and SNP markers. Nevertheless, the significant advantage of using multiple SNP markers for differentiating hybrid animals from parental forms has been revealed. In the future, a more detailed search is planned to detect the diagnostic SNP loci required for a detailed study of introgression in generations more distant from the wild ancestor.

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ISOLATION, CULTIVATION AND CHARACTERIZATION OF QUAIL PRIMORDIAL GERM CELLS

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Abstract

The use of avian primordial germ cells (PGCs) for production of chimeric and transgenic poultry is regarded as an alternative way to traditional methods of selection and transgenesis. This approach involves the introduction of donor primordial germ cells in the dorsal aorta of the recipient embryo during their migration from blood to gonads. In case of recipient embryo gonads colonization by donor PGCs, the further differentiation of donor cells into mature germ cells becomes possible, for both males and females. A key factor for the effectiveness of this manipulation is obtaining of a pure population of embryonic cells. In this regard, the development of effective methods for isolation and maintenance of PGCs in culture is important. Our research was aimed at the improvement of methodical approaches for isolation and cultivation of quail PGCs. This type of cells was isolated and characterized. Five- to six-day embryos were used for obtaining PGCs culture. Isolation of PGCs from quail embryos was performed using two methodological approaches — mechanical dissociation and enzymatic treatment. Trypsin solution at concentration from 0.05 % to 0.25 % was used as proteolytic enzyme for enzymatic treatment. In order to obtain the most pure populations of PGCs, unequal ability to adhesion of different types of cells was taken into account. It was found that enzymatic treatment with 0.05 % trypsin is an effective method for embryos disaggregation preserving significant proportion of viability (94 %) of fetal cells. Separation of different cell types based on their different ability to adhesion allows obtaining PGCs culture maximally purified from other cell types. Moreover, single embryo fibroblasts, remaining in the PGCs cell suspension after separation from the other cell types are used as a feeder layer to which PGCs are attached at the subsequent cultivation. It was shown that own primary embryonic fibroblasts are optimal as a feeder layer for short-term PGCs culturing as compared to the use of STO cells and cultured embryonic fibroblasts. If using the growth medium based on DMEM with high glucose level (4.5 g/l) supplemented with 20 % fetal bovine serum, 2 mM glutamine, 10^{-6} mM 2-mercaptoethanol, 2 ng/ml LIF (leukemia inhibitory factor), 10 mM essential amino acids (MEM), the antibiotic gentamicin (50 ug/ml) the attachment of PGCs to the feeder layer was observed at day 1 to 2 of cultivation forming colonies at day 3 to 4. The presence of primordial germ cell colonies was confirmed by immunohistochemistry using specific primary antibodies to SSEA-1 (stage-specific embryonic antigen-1).

Keywords: primordial germ cells, quail, embryos

The production of transgenic and chimeric individuals based on primordial germ cells (PGC) is recognized as one of the promising areas of modern biotechnology, which are discussed as an alternative to traditional methods of genome selection and modification [1-5]. The use of this type of cells in selection and biotechnological programs opens wide opportunities for directed genome modification and the reconstruction of valuable breeds and lines preserved in cryobank conditions [6-12]. Peculiarities of biology of agricultural poultry allow us to consider PGC as a promising genetic material. The development of bird embryos outside

the female organism greatly facilitates the manipulations associated with the isolation of PGC from donor embryos and their transplantation into recipient embryos.

Primordial embryonic cells are the precursors of the germ cells. They are pluripotent, that is, in the process of embryogenesis they can differentiate into both male and female germ cells, which greatly expands the possibilities of realizing their potential when creating transgenic and chimeric individuals with given properties. The technology for obtaining such individuals involves the transplantation of donor cells into the gonads of recipient embryos [13, 14]. Subsequently, these individuals can be used as "bioreactors", which produce in the genital organs mature donor cells (sperms, ovules) serving as genetic material for obtaining descendants with given properties. When recreating the breeds and lines of agricultural birds, the use of this approach is more preferable in comparison to frozen-thawed spermatozoa, since it makes it possible to support the gene pool studs by male and female genetic material.

One of the key factors ensuring the effectiveness of the described technology is the production of a culture of PGC which is maximally purified from other types of embryonic cells. Much of the research was performed on chickens [15-20]. The identification and characterization of PGC of other types of agricultural poultry is topical.

Here, we reported the results of isolation and characterization of the quails PGC. It was shown for the first time that the preliminary removal of other cell types, in particular the largest population of embryonic fibroblasts, provides for a high purity of the quail PGC culture. The remaining fibroblasts form a feeder layer that has optimal properties for culturing the PGC as compared to other types of feeder layers.

The aim of the work was to optimize the methods of isolation and cultivation of primordial germ quail cells, as a step in development of technology for obtaining individuals with specified properties.

Tchnique. Primordial germ cells were isolated from 5-day-old quail embryos (*Coturnix coturnix*) of Estonian breed. For disaggregation of embryos, we used two approaches, i.e. mechanical cell dissociation and enzymatic treatment.

In mechanical dissociation, embryos were ground and pipetted for 5 min in DMEM (Dulbecco's Modified Eagle's Medium). In the enzymatic treatment, embryos were transferred to physiological solution supplemented with antibiotic-antimycotic, and homogenized. The resulting cell suspension was centrifuged for 5 min at 200 g. DMEM preheated to 37 °C was added to the precipitate, pipetted and centrifuged to separate the cells. Trypsin (0.05, 0.10, 0.15 and 0.25 %) heated to 37 °C was added to the precipitate and the cells were intensively pipetted for 5-10 min.

To increase the proportion of PGC in the resulting suspension, different cell types were separated by their capability of adhesion. A series of experiments was performed (in 3 replicates) with short-term cell culture. The proportion of PGC identified by staining for alkaline phosphatase was calculated.

DMEM HG (Invitrogen, USA) with 4.5 g/l glucose supplemented with fetal bovine serum (20 %), glutamine (2 mM), 2-mercaptoethanol (10^{-6} mM), LIF (leukemia inhibitory factor, 2 ng/ml), essential amino acids (MEM, Minimum Essential Medium, 10 mM), antibiotic gentamycin (50 μg/ml) was a growth medium in PGC culture. Cells were removed from the substrate with a 0.25 % trypsin solution.

To prepare feeder layers, the cells, after reaching 90 % monolayer, were exposed for 3 hours to mitomycin C (Sigma, USA), 30 μg/ml final concentration, washed thrice with Hanks solution (PanEko, Russia) and used for PGC culture.

Morphology of freshly isolated quail embryonic cells was studied under

a phase contrast microscope (Nikon, Japan).

Cultured PGC were identified by immunohistochemical and histochemical staining. The cells were fixed for 10 min with chilled methanol (–20 °C). The immunohistochemical study was performed with the avidin-biotin test system (Vector Laboratories, USA) [21]. Anti-SSEA-1 (stage-sPGCific embryonic antigen-1) served as the first antibodies. The antigen-antibody complex was detected by peroxidase with 3,3-diaminobenzidine tetrachlorate (DAB) (Vector Laboratories, USA). A BCIP/NBT kit (5-bromo-4-chloro-3-indolphosphate and nitrosine tetrazolium, Vector Laboratories, USA) was used in staining for alkaline phosphatase.

Results. Mechanical dissociation of quail embryos produced separate cells and their groups (Fig. 1, A), while enzymatic treatment (see Fig. 1, B) resulted in cell suspension consisting mainly of isolated cells. The cells practically did not aggregate. Single cell groups were detected only for low trypsin concentrations (0.05 and 0.10 %). Their proportion did not exceed 0.2 %.

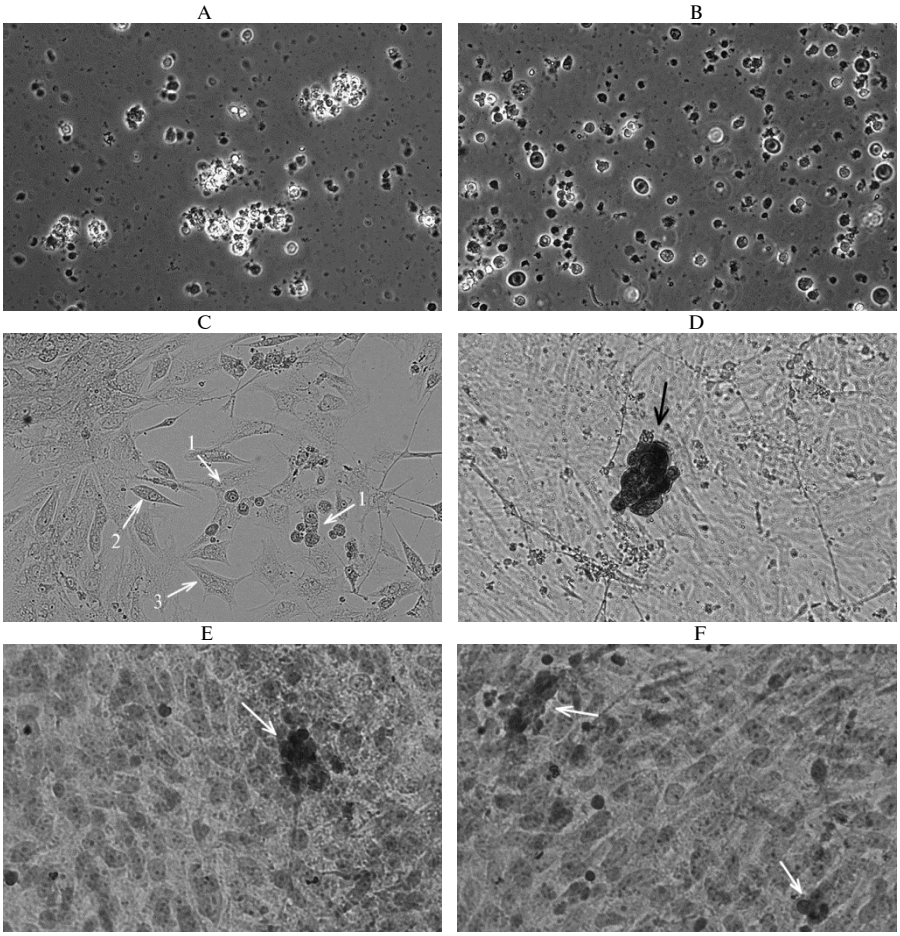

Fig. 1. Primordial germ cells (PGC) obtained from embryos of quails (*Coturnix coturnix*) of Estonian breed by different dissociation methods: A — suspension of embryonic cells after mechanical dissociation, small conglomerates of cells are seen (native preparation); B — suspension of embryonic cells during enzymatic treatment, isolated cells are seen (native preparation); C — 2-day culture, PGC (1), fibroblasts (2), epithelio-like cells (3) are seen (native preparation); D — 6-day culture, histochemical staining for alkaline phosphatase, arrow marks a colored colony of PGC; E — 4-day culture, immunohistochemical staining for SSEA-1 (stage-specific embryonic antigen-1), arrow marks a PGC colony; F — 4-day culture, absence of specific immunohistochemical staining for SSEA-1 expression in the control (without anti-SSEA-1), arrows mark the PGC colonies. Phase-contrast (A, B) and light (C-F) microscopy (Nikon, Japan), ×400.

However, the yield of living cells was 4-25% (depending on the concentration of the trypsin solution) less than in the case of mechanical dissociation, which was associated with the toxic effect of the enzyme on the cells (Fig. 2).

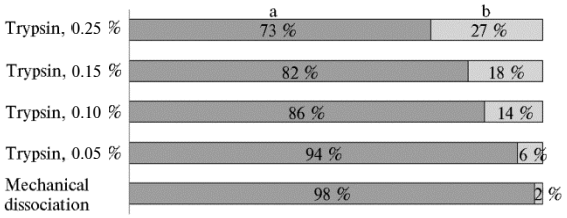

Fig. 2. The proportion of live (a) **and dead** (b) **primordial germ cells from Estonian quail (*Coturnix coturnix*) embryos produced by mechanical dissociation and trypsinization.**

Thus, in trypsinization, the maximum yield of living cells (94 %) was achieved using the minimum enzyme concentration (0.05 %), which was optimal to produce isolated embryonic quail cells while maintaining the high viability of the cell suspension.

The suspension of embryonic cells resulted from trypsinization was plated onto culture dishes. During 1 hour, a significant portion of embryonic fibroblasts were attached to the plastics. After 24 hours, these cells flattened and began to divide. The most PGC, which had the appearance of rounded formations, was in suspension, and only a few were attached to spreading embryonic fibroblasts, using them as a feeder layer. On the day 1 (see Fig., B), the PGC were attached to the feeder layer and acquired a spherical shape.

In the resulting cell population, several types of cells were identified. Numerous groups consisted of embryonic fibroblasts, epithelioide cells and PGC. PGC was characterized by a rounded shape, epithelioide cells were irregular in shape and had large nucleus, fibroblasts were spindle-shaped (see Fig., B).

To maximize the purification of the PGC population, we applied the method based on the unequal adhesive capacities of different cell types. After the embryonic quail cells were primary cultured for 1 hour, the supernatant with non-attached cells was removed and transferred onto fresh Petri dishes to continue culturing. The embryonic fibroblasts present in the suspension after collection of the culture medium served as the feeder layer on which the PGC were attached. After 1 hour, the supernatant was again removed for further culturing. After a three times repeated procedure, the proportion of PGC in the suspension increased to 79 %.

Isolated quail PGC were added onto STO (transplanted mouse embryonic fibroblasts) cells, primary embryonic quail fibroblasts, chick embryonic fibroblasts which served as the feeder layers, or onto plates with 0.2 % gelatin. The best results were obtained with primary embryonic quail fibroblasts (Table).

Efficiency of cultivation of primordial germ cells (PGS) **of quails (*Coturnix coturnix*) depending on the feeder layer**

Feeder layer	Attaching to the feeder layer	Colony formation
STO cell line	On days 1 to 2	On days 4 to 5
Embryonic fibroblasts of chickens	On days 1 to 2	On day 4
Primary embryonic quail fibroblasts	On days 1 to 2	On day 3

N o t e. STO — cell line of transplanted mouse embryonic fibroblasts.

On days 3-4 of the culture, PGC formed colonies the presence of which was confirmed by expression of stem cell markers, the alkaline phosphatase (see Fig., D) and the protein SSEA-1 (see Fig., E, F).

So, in quails, the uniformity of primary germ cells (PGC) culture depends on the method used for their separation from other cell types and the feeder layer. Separation of cells by adhesion ability allows the maximum purification of the PGC culture from other cells, in particular from embryonic fibroblasts. Single embryonic fibroblasts, remaining in the cell suspension, serve as the feeder layer during a subsequent cultivation on which the PGC are attached.

PGC culturing on the feeder layers with a suitable growth medium allows to obtain PGC colonies on days 3 and 4. Optimum feeder layer for quail PGC culture is formed from native embryonic fibroblasts.

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EFFECT OF CRYOPRESERVATION ON BIOLOGICAL PARAMETERS OF SEMEN IN ROMANOV BREED × ARGALI HYBRID RAMS

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Abstract

Cryopreservation of genetic material and artificial insemination is an important element of assisted reproductive technologies. Establishing cryobanks of biomaterial, derived of high-valuable breeding rams, provides the production of the maximal number of offspring. This technology allows more effectively use the genetic material with higher efficiency, save and restore the populations of rare and endangered species and carry out crossbreeding and hybridization between isolated populations. The most common biomaterials are the sperm, which are used in the programs for the conservation and restoration of wild and domestic genetic resources. This is associated with the availability and easiness of producing sperm. In sheep breeding, the use of reproductive technology has the local character in contrast to other livestock industries. One reason for this is the low efficiency of application of frozen-thawed ram semen that is caused by some complexities involved in sperm cryopreservation of this animal species. During the freezing and thawing process, the sperm undergoes significant technological impact. Some stages of this process are the shock to semen and lead to the destruction of a large part of cells or to the damaging their individual organelles or segments. Less is known about the freezing capacity of sperm derived from hybrid rams, which are produced by hybridization of domestic and wild *Ovis* species. Taking this into account the aim of our research was to study the effect of freezing and thawing process on the biological parameters of sperm derived from purebred Romanov ram and hybrid rams carrying argali (*Ovis ammon*) blood. As a material for our research, we used the sperm of hybrid rams of different origin: F_1 (n = 1, 50 % argali, produced by surgical insemination of Romanov ewe with frozen-thawed sperm of argali ram); BC_1 (n = 5, 25 % argali blood, produced by backcrossing of Romanov ewes with F_1 ram); BC_2 (n = 5, 12.5 % of argali blood, produced by backcrossing of Romanov ewes with BC_1 rams). The sperm of purebred Romanov ram (RAM) was used as a control. Qualitative and quantitative parameters of freshly derived and frozen semen of the experimental animals, the percentage of motile and immobile spermatozoa, their cryoresistance, the state of the acrosome and the degree of chromatin condensation were studied. The motility of spermatozoa was decreased significantly in sperm of both of hybrid and purebred rams (by 47 and 50 %, respectively). The cryoresistance of spermatozoa of F_1 hybrid ram was increased by 9 %, the osmotic resistance increased — by 20 % and dehydrogenase activity — by 44.5 % comparing to purebred Romanov ram. With the decrease of blood ratio of wild species, the decrease in differences for above-mentioned parameters was observed: the differences between BC_2 and ROM were 2.2, 4.7 and 10.0 %, respectively.

Keywords: argali hybrids, cryopreservation, sperm, freeze-thaw

Artificial insemination (AI) is one of the main elements of farm animal reproduction. At present, effective animal husbandry necessitates AI [1-5[. The cryopreservation of biological material (generative plasma, embryos, germ and somatic cells)is the most important auxiliary reproductive technology [6-8].

Spermatozoa are the most commonly used in conservation and restoration of genetic resources of wild fauna and domestic animals [9]. This is due to the availability and ease of obtaining sperm (5-6 billion germ cells per ejaculate).

In addition, for many animal species, artificial insemination technology using frozen-thawed semen has been developed.

Cryopreservation of sperm allows to accumulate viable generative material from high-value producers, to use it more efficiently, to obtain the maximum number of offspring, to preserve and restore rare and endangered species, and to cross and hybridize between isolated populations. Cryopreservation of spermatozoa consists of several treatments that lead to changes in individual structural units of spermatozoa, which is often accompanied by a decrease in their biological full-value [10-14]. The effect of the freeze-thaw cycle on the spermatozoa morphology is shown [15-19]. In all agricultural animals, when the cryopreserved semen is used, the yield of the offspring is reduced. According to a number of researchers, this is associated with a decrease in the motility of spermatozoa, since they must have a high speed of rectilinear motion to reach the ovum [20]. Spermatozoa with high activity, but ultrastructural, biochemical, or functional damage also have low probability of ovum fertilization [21, 22].

The use of assisted reproductive technology in sheep breeding is of a local character, due to the low efficiency of cryopreservation of ram semen. It is much more difficult to freeze it than the sperm of other mammalian species. About 10 % of the ejaculate obtained from rams using modern diluents and protocols does not undergo cryopreservation [23], and, therefore, to optimize cryopreservation of spermatozoa in this species is of importance.

Adding blood of wild species is one of the promising ways of genetic improvement of sheep breeds. At the L.K. Ernst All-Russian Research Institute of Animal Husbandry a model population has been created, including hybrids of domestic Romanov ewe (*Ovis aries*) and argali (*O. ammon*) with different ratio of the wild species blood. The change in biological and productive indices in individuals with the blood of wild animals compared to the original domestic species was shown [24, 25]. A semen bank of hybrid animals was created [26].

In the present paper, we first studied the indices of the biological full-value of the semen of hybrid rams with different percent of Romanov breed and argali as compared to their purebred analogues. The effect of cryopreservation on cryoresistance, osmotic resistance and dehydrogenase activity of the semen was shown. An increase in these indices in hybrid animals is noted, indicating a higher cryostability of their semen as compared to the semen of purebred rams.

The purpose of this work was to study the effect of freezing-thawing cycle on the biological parameters of spermatozoa in hybrid (with argali blood) and purebred Romanov rams.

Technique. The sperm was collected from the hybrid rams: F_1 (obtained by surgical insemination of the Romanov ewe with the frozen-thawed semen of argali, $n = 1$, 50 % argali), BC_1 from backcrossing Romanov ewe with F_1 ram ($n = 5$, 25 % argali), BC_2 from backcrossing Romanov ewe with BC_1 rams ($n = 5$, 12.5 % argali). The Romanov ram semen (ROM) ($n = 3$) was used as a control. Feeding and housing were the same for all the rams. The semen samples were collected in an artificial vagina. Dilution, deep freezing and thawing were carried out in accordance with the proprietary method of ram semen cryopreservation in granules. Quality indicators of freshly prepared diluted and frozen-thawed semen were studied.

For recognition and evaluation of sperm parameters, the software Zoosperm 1.0 (OOO VideoTesT, St. Petersburg, Russia) was used. The program is based on video image processing [27]. The integrity of the acrosome was studied by differential staining with Diakhim-Diff-Quick set (ABRIS+, Russia). The DNA fragmentation index in chromatin was determined by detecting DNA gaps in a test with acridine orange (AO-test) and fluorescence microscopy (Nikon,

Japan).

The data was processed statistically using the Microsoft Office software package. The tables show the mean *(X)* and their deviations (*x*).

Results. Analysis of the motility of spermatozoa in fresh and frozen semen of hybrid animals and their purebred analogues showed no significant differences. The proportion of spermatozoa with rectilinear motion (class A + B) in both experimental groups was almost the same reaching 90.0 % for freshly collected semen and 43.0 % for cryopreserved semen in hybrids, and 93.0 and 43.0 %, respectively, in purebred individuals. The proportion of spermatozoa of class C (with curvilinar movement) and class D (immobile) in freshly obtained semen did not exceed 4.3 and 5.7 %, respectively, in the hybrids, and 3.6 and 3.4 % in the purebred animals. In freezing-thawing, the number of immobile spermatozoa increased to 48.6 % in hybrid rams and to 51.2 % in their purebred analogues.

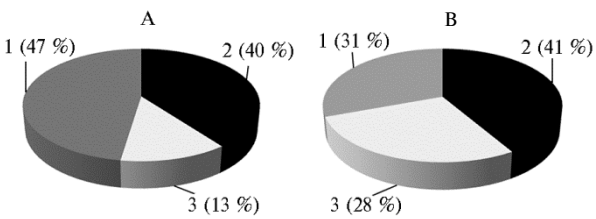
The indices of osmotic and hypoosmolar resistance, as well as dehydrogenase activity of spermatozoa in hybrid and purebred animals were within the established requirements (Table 1). At the same time, these in parameters were higher in hybrids. The differences between ROM and F_1 were 9.0 % for cryopresistance, 20.0 % for osmotic resistance, and 44.5 % for dehydrogenase activity. In consequent generations, the differences were leveled out: in the third generation, hybrids exceeded their purebred analogues in cryoresistivity by 2.2 %, in osmotic resistance by 4.7 %, and in dehydrogenase activity by 10.0 %.

1. The indices of the cryostability of spermatozoa obtained from hybrid and purebred Romanov rams (*X*±*x*)

Groups	Cryoresistance	Osmotic resistance	Dehydrogenase activity, min
ROM	0.44±0.02	0.21±0.06	7.82±0.65
F_1	0.48±0.03	0.25±0.08	11.31±0.38
BC_1	0.45±0.02	0.23±0.06	9.09±0.47
BC_2	0.43±0.03	0.22±0.01	8.61±0.22

N o t e. For description of the groups see the *Technique* section.

The study of the influence of cryopreservation on the morphometric parameters of spermatozoa revealed some differences between the experimental groups. The portion of spermatozoa with disturbed morphology in fresh ejaculates of hybrid rams was 10.7 %, which was 3.1 % higher than that for purebred animals. There was a difference between groups in the occurrence of abnormal morphology in segments of spermatozoa. A higher disorder percentage in the structure of spermatozoa in purebred producers was noted in the flagellum region, in hybrid rams — in the middle part (Fig.).



The frequency of morphological disorders in the segments of spermatozoa of purebred Romanov rams (A) **and hybrids with argali (*Ovis ammon*)** (B) **in frozen-thawed semen** (on average in three groups)**:** 1 — abnormal flagella, 2 — abnormal head, 3 — abnormal middle part.

When freezing and thawing, the number of spermatozoa with morphological disorders increased in both hybrid and purebred animals, however, the percentage of the frequency of abnormalities in spermatozoa segments within the experimental groups remained practically unchanged.

Sturdy of the acrosome and chromatin fragmentation in fresh and frozen semen revealed a decrease in the indices in the hybrid animals compared to the purebred analogues. The difference between the Romanov rams and BC_1 and BC_2 was 2.5-3.2 % for fresh semen and 3.9-5.2 % for frozen-thawed semen in the number of spermatozoa with intact acrosomes, and 25.8-43.9 and 0-21.2 %, respectively, in the chromatin fragmentation (Table 2). At the same time, we did not reveal a correlation between the changes in these indices and the percent of argali blood.

2. Estimation of spermatozoa in hybrid and purebred Romanov rams according to the state of the acrosome and the degree of chromatin fragmentation before and after cryopreservation ($X \pm x$)

Groups	Proportion of spermatozoa with intact acrosomes, %		Degree of chromatin fragmentation, %	
	FOS	FTS	FOS	FTS
ROM	92.4±5.2	90.3±8.7	11.6±3.9	12.7±1.7
F_1	96.5±6.7	88.3±4.7	8.6±2.8	12.7±3.5
BC_1	89.9±8.3	86.4±5.7	6.5±2.7	9.5±2.4
BC_2	89.2±1.6	85.1±4.6	8.2±3.8	12.3±3.2

Note. FOS — fresh semen, FTS — frozen-thawed semen. For description of the groups see the *Technique* section

In freezing-thawing, the proportion of spermatozoa with intact acrosomes decreased and the DNA fragmentation index increased in all groups. In hybrid animals, the differences were more pronounced and amounted to 4.6-8.4 % and 45.1-47.6 % respectively, in purebred — to 2.2 and 9.5 %, respectively.

Thus, after cryopreservation of the semen of both hybrid and purebred animals, the biological full-value of the spermatozoa decreased. The semen of the hybrids was more cryoresistant than the semen of Romanov rams. After cryopreservation, the motility of spermatozoa decreased by 47.0 % in hybrid animals and by 50.0 % in the purebreds. The spermatozoa cryoresistance indices in the hybrids also increased compared to the domestic parent. With the decrease in the argali blood percent towards the domestic form, differences in cryoresistance, osmotic resistance and dehydrogenase activity were leveled out and were 2.2, 4.7 and 10.0 % for the rams of maternal breed and hybrids BC_2, respectively. In the F_1 hybrid ejaculates, the content of sperm with intact acrosomes was 4.1% higher. In the fresh ejaculates of hybrids, the chromatin DNA fragmentation was lower than in the Romanov breed.

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COMPARATIVE STUDY OF THE STRUCTURAL INTEGRITY OF SPERMATOZOA IN EPIDIDYMAL, EJACULATED AND CRYOPRESERVED SEMEN OF STALLIONS

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Abstract

Cryopreservation of semen is an important way to preserve genetic resources. It is the most actual in horse breeding than in other livestock industries, as currently in many horse breeds, especially unique domestic breeds, the number is approaching a critical level. For many native breeds due to a specific management, year-round outdoors in herds, it is impossible to obtain sperm for cryopreservation by a traditional method using an artificial vagina, and the only cost-effective way to create cryobanks is getting epididymal semen. The technology for cryopreservation of stallion semen includes some critical steps that are characterized by a decrease in sperm quality. These are procedure for semen donation, dilution, temperature shock during freezing and thawing. For the first time a comparative transmission electron microscopy study of the ultrastructural integrity of spermatozoa was done for ejaculated and epididymal sperm in the same stallions thus avoiding the influence of different individuals on the compared parameter. Structural damage caused by cryoconservation of epididymal and ejaculated sperm was studied. It was found that acrosomes were the most susceptible and undergone the greatest impact. Its predominant pathology is the absence of internal contents (acrosome hypoplasia), resulted in enzyme deficiency. The frequency of this pathology was 12.4 % and 14.0 % for fresh epididymal and ejaculated sperm, respectively, and increased almost twofold after freezing and thawing, reaching 26.5 and 27.4 %, respectively. The rate of spermatozoa with the second most common pathology, acrosome degradation (premature release of sperm enzymes that dissolve the oocyte membrane, resulting in the loss of the ability to fertilize), after cryopreservation increased by 5.9 % ($p < 0.05$) and 8.9 % ($p < 0.01$) for epididymal and ejaculated semen, respectively. The nucleus of the sperm is one of the most resistant to cryopreservation among the organelles, though in two stallions we observed changes in the shape of the nucleus and vacuolation of chromatin after cryopreservation with a frequency of 1.6 to 6.1 %. Less than 10 % of the sperm had pathology of mitochondria. The axoneme of sperm is sufficiently resistant to cryopreservation. Outer dense fibers and fibrous membrane are almost not damaged during semen collecting as well as under semen freezing and thawing. Higher rates of ultrastructural integrity found for epididymal semen were not statistically significant. Thus, the sperm collecting results in minimal ultrastructural damage, whereas the main pathology is caused by cryopreservation. The ultrastructural integrity of spermatozoa in epididymal semen allow us to recommend this sperm collecting technique to organize cryobanks in case of impossibility of sperm collecting from stallions in traditional ways, or in need for early castration of stallions, in particular in sporting and productive horse breeding.

Keywords: stallion, electron microscopy, epididymal semen, ejaculated semen, cryopreservation

Artificial insemination of mares with freshly diluted, chilled and cryopreserved sperm makes it possible to make the most of the genetic potential of stallions [1]. The method of cryopreservation of sperm and technology to increase the reproductive status of stallions is important in maintaining genetic diversity and horse breeding. The advantages of cryopreservation are obvious: Its

use makes it possible to create sperm banks of high-value producers, for a long time to preserve genetic material for breeding, to transport semen over long distances [2].

Various technologies have been developed and successfully used for cryoconservation of the stallion ejaculated sperm [3-5]. Nevertheless, obtaining and cryopreservation of sperm from stallions includes a number of critical stages in which the quality of sperm is reduced [6]. These are the procedure for taking, dilution, temperature shock during freezing and thawing. In a number of cases, there is an objective need for cryopreservation of the epididymal semen, for example, when the death of highly valuable stallions or traumas with an unfavorable prognosis for recovery [7-9]. For some unique indigenous breeds with winter herd grazing, the use of epididymal semen is the only economically reasonable way to the cryobank formation.

The main problem with cryopreservation of sperm is its quality after thawing [10]. Basically, it worsens due to a decrease in the number of active spermatozoa with rectilinear movement, a decrease in the survival of spermatozoa and their structural disorders. The morphology of spermatozoa, along with mobility and survival, is one of the most important indicators of sperm quality [11]. The increase in the proportion of abnormal germ cells in the ejaculate is to a different extent due to the less fertility of stallions [12-14]. Due to a number of biological features of such spermatozoa, their fertility is reduced [15]. Usually, semen is studied by-light microscopy, which does not allow to reveal a significant part of ultrastructural damage in spermatozoa. At low stallion fertility it is recommended to conduct additional studies of sperm [16], in particular electron microscopy [17]. To date, this is the most accurate method of ultrastructural analysis [18] used to determine the etiopathogenesis of pathozoospermia, the spermatozoa function, and the diagnosis of disorders in the reproductive cells due to cryopreservation.

Spermatozoa from the deferent canals enter the epididymis, where their final maturation takes place resulting in the ability to fertilize [19-22]. A total of 15 to 25 billion spermatozoa are contained in the epididymis [23, 24]. Therefore, extraction of spermatozoa from the epididymis allows obtaining a sufficient number of gametes for artificial insemination of both freshly diluted and chilled and cryopreserved sperm.

Mobility, morphology, DNA integrity, viability of spermatozoa in ejaculated and epididymal sperm have been studied by many researches [7, 24-26]. The methodological approaches used by us for the first time made it possible to carry out a comparative analysis of the ultrastructure of germ cells (obtained by different methods and after cryopreservation) on biological material from the same stallions, which eliminated the influence of the individual characteristics of different individuals on the feature to be compared. This gives new knowledge about the state and safety of organoids in the freezing-thawing of epididymal and ejaculated spermatozoa.

The purpose of this study was to compare the ultrastructure of spermatozoa from epididymis and ejaculate subjected to cryopreservation and to diagnose the main pathologies of organoids.

Methods. Ejaculated sperm of 5 stallions of different breeds aged 5 to 8 years was obtained at 48 hour intervals using artificial vagina. In the data processing, the sperm indices of the two ejaculates produced after sexual rest were cutoff. The ejaculate volume, spermatozoa concentration, mobility, morphology [27], survival at 2-4 °C, and the time of mobility [3] were assessed. When diluting sperm, lactose-chelate-citrate-yolk (LCCY) medium (1:3, v/v) was used [3]. The sperm was frozen in liquid nitrogen vapor as per the standard technology of

the All-Russian Research Institute of Horse Breeding and stored in liquid nitrogen at –196 °C. After ejaculated semen collection, the stallions were castrated to obtain epididymal sperm which was diluted and frozen according to the same technology.

In electron microscopy, after a dilution (1:10) of native and cryopreserved samples of ejaculated and epididymal semen with isotonic NaCl, 2.5 % glutaraldehyde (Ted Pella Inc., USA), prepared with 0.1 M cacodylate Buffer (pH 7.2) (Sigma, USA), was added. The samples were centrifuged for 15 min at 1000 rpm, the supernatant was removed, The precipitate was treated with glutaraldehyde as described above, additionally fixed with 1 % osmic acid (Serva, Germany) and poured into epon (Fluka, Germany). Ultrathin sections were prepared on an UltraCut III microtome (Reichert Jung Optische Werke AG, Austria), dyed with an aqueous solution of uranyl acetate and lead citrate (Serva, Germany), and viewed in an electron microscope Hitachi 700 (Japan). General view of spermatozoa was studied at ×5000 magnification, acrosome, chromatin nucleus and mitochondria — at ×16000-18000, axonemal anomalies on transverse sections of flagella — at ×20000-25000. In each sample, at least 150 germ cells were analyzed.

The data was processed by conventional methods of variational statistics. The table shows the mean (X) and standard errors of the mean (x). The reliability of the differences was determined using Student's t-test. The differences were statistically significant when $p < 0.05$.

Results. On average, the ejaculate volume was 54.8±8.8 ml at spermatozoa concentration (184.5±23.7)×10^6/ml; the activity was 4.4±0.3 points, and a survival period at 2-4 °C was 125.2±8.4 hours. After cryopreservation the activity decreased to 1.8±0.2 points, the survival time — to 59.2±14.7 hours. The proportion of morphologically normal spermatozoa reached 71.4±4.4 % in native sperm and 68.4±4.0 % in cryopreserved sperm.

The proportion (%) of spermatozoa with normal morphology in freshly received and cryocon-served ejaculated and epididymal sperm of stallions (n = 5, $X \pm x$)

Morphological sign	Epididymal sperm		Ejaculated sperm	
	at collection	after thawing	at collection	after thawing
Intact head	76.1±4.2	64.4±6.1	73.6±4.2	57.2±7.3
The presence of the acrosome	98.5±0.5	92.6±1.3	98.0±0.7	89.1±1.9
Normal position of the acrosome	99.2±0.8	98.2±1.6	98.4±0.7	91.8±3.7
Normal form of the acrosome	94.4±1.6	87.8±2.9	91.6±1.5	87.0±2.9
Compact contents of the acrosome	87.6±3.3	73.5±5.2	86.0±3.7	72.6±6.7
Normal form of the nucleus	99.6±0.2	97.3±1.2	98.3±1.3	93.7±3.3
Normal mitochondria	97.1±1.3	90.3±1.4	93.7±2.3	88.0±2.1
Normal axoneme	91.5±2.3	84.7±4.5	88.3±2.9	84.8±4.1
Normal outer dense fibrils	99.5±0.5	98.9±1.1	98.8±1.2	98.7±1.3
Normal fibrous membrane	100	99.0±1.1	100	98.0±2.0

The use of ejaculated and epididymal sperm from the same stallions made it possible to compare the damage to germ cells after cryopreservation, depending on the method of sampling. One of the main indicators of sperm quality, determined by electron microscopy, is the number of spermatozoa with intact heads (Fig. 1), normal shape, acrosomes and chromatin [18]. In epididymal sperm, such spermatozoa amounted to 76.1 %, after ejaculation their number decreased by 2.5 % (Table). During freezing-thawing of ejaculated sperm, the number of spermatozoa with intact heads additionally decreased by 16.4 % and averaged 57.2 %. In cryopreserved epididymal sperm, the number of spermatozoa with intact heads (64.4 %) was 7.2 % higher than that in frozen ejaculates.

Of the pathologies of spermatozoa heads in collected and cryopreserved sperm, we noted an increase in the number of spermatozoa with acrosome degradation due to a premature acrosomal reaction, acrosome hypoplasia (incompact

acrosome contents), changes in the nucleus shape, and chromatin vacuolization.

Basically, during cryoconservation of both ejaculated and epididymal stallion sperm, the acrosome was damaged (Fig. 2, 3). In epididymal sperm, the acrosome was present in 98.5 % of spermatozoa, in the ejaculates and after freezing-thawing the proportion of spermatozoa with the reacted acrosome (with acrosome degradation) increased by 9.4 %. Upon freezing and subsequent thawing, the number of spermatozoa with acrosome degradation increased by 5.9 % ($p < 0.05$) in epididymal sperm, and by 8.9 % ($p < 0.01$) in the ejaculated sperm.

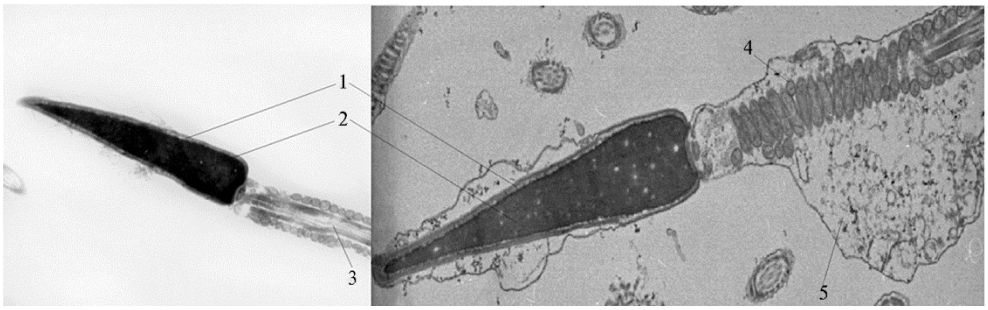

Fig. 1. Normal stallion spermatozoon (left) **and spermatozoon with asymmetric cytoplasmic droplet** (right)**:** 1 — acrosome, 2 — chromatin, 3 — longitudinal section through axonema flagellum, 5 — **cytoplasmic droplet**. Hitachi 700 (Japan), ×5000-25000 magnification.

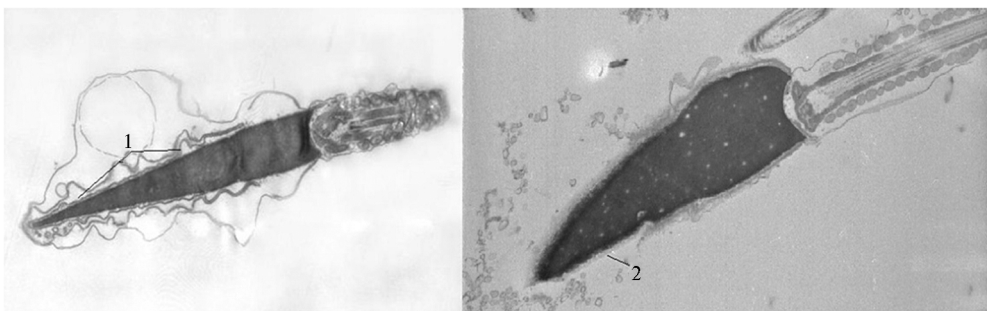

Fig. 2. Stallion spermatozoons with "empty" acrosome (left) **and reacted acrosome (after acrosomal reaction)** (right)**:** 1 — acrosome with transparent contents ("empty") and uneven contours, 2 — residual material of the reacted acrosome. Hitachi 700 (Japan), ×16000-18000 magnification.

The most common pathologies in cryopreservation were also the increase in the number of spermatozoa with acrosome hypoplasia and lack of content (acrosomes with electronically transparent contents). The percentage of the spermatozoa with incompact acrosome contents increased by 13.4 % in cryopreserved ejaculates, and by 14.1 % after freezing and thawing of the epididymal semen.

The effect of cryopreservation on the nucleus of spermatozoa was minimal. Changes in the shape of the nucleus were found in fresh and cryopreserved semen in not more than 5.9 % cells on average. In fresh sperm, spermatozoa with vacuolated chromatin did not occur, after cryopreservation, they were detected in a small amount (from 1.6 to 6.1%) in samples from two stallions.

The spermatozoa with normal mitochondria amounted to 97.1 % in epididymal sperm and 93.7% in the ejaculate sperm. Thus, at sperm collection and primary processing, the number of spermatozoa with damaged mitochondria increased by 3.4 %, after cryopreservation —by additional 5.7 %, as a result mitochondria were damaged in 9.1 % of the spermatozoa. The increase in the number of mitochondrial abnormalities in the cryopreservation of epididymal sperm compared to fresh one produced quite high ($p < 0.05$) significance of differences.

Morphological anomalies of the axoneme are closely related to low fertility and infertility [27]. Therefore, the study of axoneme integrity is one of the

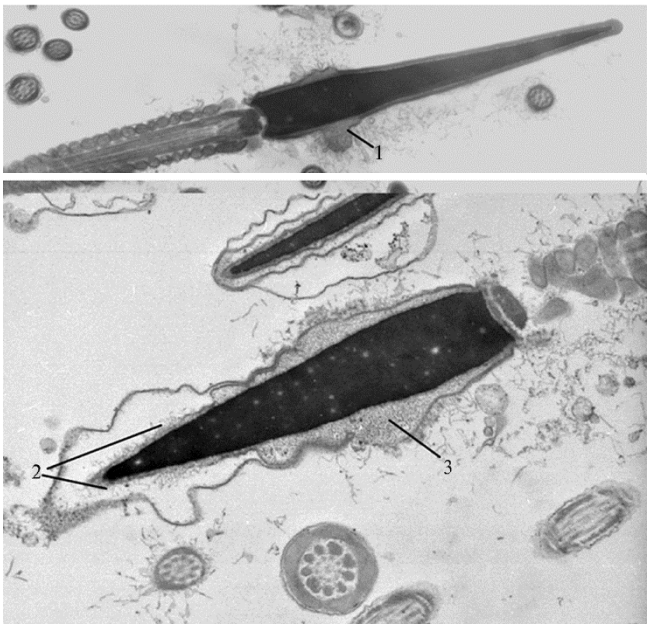

Fig. 3. The initial stage of stallion spermatozoa acrosome damage: 1 — diffuse expanded zones of an acrosome with fibrous contents, 2 — section of the expanded acrosome with electronically transparent contents ("empty" acrosome), 3 — the expanded acrosome with fibrous contents. Hitachi 700 (Japan), ×16000-18000 magnification.

mandatory stages in the electron microscopic study of ejaculate. The axoneme in both epididymal and ejaculated spermatozoa proved to be very resistant to cryopreservation. From the sperm collection to freezing and thawing, the number of spermatozoa with an abnormal flagellum increased by 6.7 %, which was significantly lower than the analogous index for mitochondrial and especially acrosomal damage.

External dense fibrils and fibrous membrane of the spermatozoa axoneme in stallions are also resistant to external influences. It was shown that they were practically not damaged either at sperm collection, or at freezing-thawing.

In general, the results of diagnostics of ultrastructural abnormalities in epididymal and ejaculated spermatozoa demonstrate that when semen is produced on an artificial vagina, the damage to germ cell organoids is minimal, and the main structural changes occur in cryopreservation. Confirmation of the high safety of epididymal spermatozoa, as well as their resistance to cryopreservation, allows us to make a positive forecast for the development and application of the cryopreservation technology for epididymal sperm of stallions.

So, the ultrastructural integrity of organoids in the germ cells of stallions in epididymal sperm is higher than in ejaculated one, but these differences are unreliable. To a lesser extent, ultrastructural damage to spermatozoa occurs when collecting sperm, and is mostly due to cryopreservation. When freezing and thawing both epididymal and ejaculated sperm, the spermatozoa acrosome is most vulnerable, mitochondria are less sensitive, whereas the axoneme, outer dense fibrils and fibrous shell of the axoneme are fairly stable, and the effect on the sperm nucleus is minimal.

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BIOLOGICAL INTEGRITY OF BISON EPIDIDYMAL SPERM UNDER CRYOCONSERVATION AND LONG STORAGE

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Abstract

Conservation of biodiversity is one of the global challenges of the modern world. The preservation of animal genetic resources is considered essential for the food supply, since sustainable food production appears to be the greatest problem due to the human population growth, depletion of the Earth's natural resources, and many species becoming endangered. In situ and ex situ methods of preservation of the species (i.e. in/out of their natural habitats, respectively) are two major approaches to animal biodiversity conservation. Ex situ strategy involves the techniques for the genetic material cryopreservation. Cryopreservation of the wildlife biomaterials allows to use these genetic resources not only for the conservation and the renewal, but also for the introduction into the genotype of the farm animals. The bison (*Bison bonasus*) is identified as the rare and endangered species. At present, the free-living bison population in Russia comprises more than 1500 animals. A research concept of the Russian bison gene pool preservation includes creating cryo-preserved pool of bison spermatozoa. In this paper we report findings on biological adequacy of the cryopreserved epididymal bison semen after the long storage (for more than 20 years). The sperm samples were collected post-mortem from the testicular appendages of four bison males sustained the injuries incompatible with life or culled and used for hunting. For the assessment of semen motility we used a computer-assisted semen analysis (CASA) device; the DNA fragmentation index was assessed in AO-test with the acridine orange staining. The acrosomal integrity was studied by Diff-Quik staining method. It was shown that the semen quality parameters differed significantly due to the individual peculiarities of the bison. The spermatozoa of A + B grade which performed good motility and rectilinear motion reached more than 28 % in the semen of the males Mutfil and Morus, while in the Avel's and Misir's semen over 67 % spermatozoa were non-motile and 12.1 % and 10.4 % spermatozoa exhibited rotational and vibrational motions, respectively. The frequency of spermatozoa with pathomorphological changes significantly varied depending on the individual properties of the bison, with the greatest and the lowest values of 14.6 % and 6.8 %, respectively. The DNA fragmentation index reflecting sperm chromatin integrity can depend on the numerous biotic and abiotic factors and may vary in great ranges. In our surveys, it varied from 7 % to 86 %. For all the morphometric parameters, except the head width, the bison spermatozoa were inferior to the spermatozoa of the bulls though the differences between animal groups were not statistically significant. However, the area of the spermatozoa head in bulls was 3.14 μm^2 larger than that of bison.

Keywords: European bison, *Bison bonasus*, cryopreservation, spermatozoa, acrosome, index of DNA fragmentation, chromatin

At present, preserving flora and fauna is one of the global challenges to

mankind [1, 2]. Due to unfavorable environment, anthropogenic, economic and other factors, most of which are also man-caused, some species are endangered. It is proved that the stability of the community is the higher the more the number of its constituent species, therefore, the conservation of biodiversity is the only mechanism for ensuring the stability of life on Earth [3, 4]. In addition, with a high rate of population growth and limited resources, the provision of food and raw materials for industry is becoming a serious problem. Its effective solution is in the breeding highly productive animals and the wild animal domestication. The genetic diversity of the source material affects the success of breeding. Long selection is accompanied by an increase in homozygosity, which, in turn, leads to a number of disorders, i.e. to a decrease in resistance, the appearance of hereditary diseases, etc. To increase the heterozygosity, a new genetic pool is often required, which could be find among wild relatives of domestic animals [5-9].

There are two main strategies for preserving gene pool: in situ (the management of species in the natural habitat or breeds of domestic animals under breeding conditions) and ex situ (conservation of components of biological diversity outside their natural habitats, e.g. in nursery-gardens, zoos, etc., or through cryopreservation of genetic material). The in situ method suggests the breeding and management of animals and poultry in special gene pool farms and is economically costly. The ex situ method is important for species whose reintroduction is not possible in the near future [10-14]. In many countries, ex situ conservation is an integral part of the environmental strategy [15]. Depending on the purpose and objective, the biological material for cryopreservation can be embryos, animal tissues, male (spermatozoa), and female (oocyte) germ cells [16-22]. Cryobank of genetic resources of wild fauna can be used to preserve species, as well as to create new selection types and breeds.

Bison (*Bison bonasus* L.), a wild forest bull, refers to rare endangered species. This is the largest ungulate animal, the only wild species of the subfamily *Bovinae*, inhabiting the European continent [23-25]. At the beginning of the 20th century, the free-living populations of bison were almost lost. As a result of prolonged scientific and selective work, the number of bison from 1927 to 2000 increased by more than 70 times and now amounts to more than 3,500 thousand individuals; in Russia, there are about 1500 free-living bison.

Cryotechnologies and artificial reproduction in vitro used in preservation and restoration of rare species are the methods recommended by the Convention on Biological Diversity and international organizations [26-28]. At the L.K. Ernst All-Russian Research Institute of Animal Husbandry, the technique of sampling and cryopreservation of bison epididymal semen has been developed, which made it possible to create a semen cryobank of the bison inhabiting the Prioksko-Terrasnaya and Oka state natural biosphere reserves [29]. Three generations of hybrid animals were produced using cryopreserved epididymal semen. In particular, four heifers were born after artificial insemination of black-and-white cows, and F_1 and F_2 hybrids from different crossing with dairy (black-and-white, Holstein) and meat (Aberdeen-Angus) breeds were studied [30, 31].

The epididymal and ejaculated semen are significantly different both morphologically and functionally. Epididymal spermatozoa are immobile, and have low metabolism. The motility of spermatozoa is initiated after ejaculation, as a result of which metabolic processes are activated. The cryoresistance of spermatozoa with a low metabolism is higher.

Cryopreservation of spermatozoa, including those extracted from epididymis, uses media that enhance metabolism. It is known that the last stage of condensation of chromatin in the nucleus of spermatozoa occurs after ejaculation, therefore, this parameter is lower in the germ cells extracted from the epi-

didymis than in ejaculated cells. That is, the probability of damage to genetic material during freezing-thawing in the nuclei of spermatozoa from epididymis is higher than that in ejaculated semen [32]. Cryopreservation affects the morphometric parameters of spermatozoa. As a result of freezing-thawing, the cell size decrease, especially the area and perimeter of the head [33-36]. Cryopreservation also affects the ultrastructure of spermatozoa [37-40]. Currently, common techniques are sperm cryopreservation in straws and in granules.

We first investigated the effect of cryopreservation and storage on morphometric and morphofunctional properties of bison semen.

The aim of the present work was to study biological usefulness of the frozen-thawed epididymal semen of the bison after a long (more than 20 years) storage.

Technique. Since 1998, epididymal semen has been sampled from four male bison in Prioksko-Terrasny and Oka state natural biosphere reserves. Postmortem Extraction was carried out from the appendage of testis in the animals with life-incompatible injuries or culled (intended for hunting). The contents of the epididymis tail were homogenized in a synthetic medium and filtered to remove cell admixtures. The content and mobility of freshly received epididymal spermatozoa in the sample was determined by the method of assessing semen quality in bull sires. The semen was frozen in granules as per the technology of bull sperm cryopreservation [41].

A routine evaluation of the frozen-thawed semen was carried out in CASA, a computer-assisted semen analysis system. DNA fragmentation was assessed with acridine orange (AO-test) and fluorescence microscopy. The acrosomes were studied using Diff Quik staining (42-44).

The of frozen-thawed spermatozoa motility was determined with Zoosperm 1.0 software (VideoTest, Russia), Makler counting chamber (Israel) and a Nikon Eclipse Ni microscope (Nikon Corp., Japan) based on video recording for 1 second of three fields as an average for 3 samples.

Data processing and classification of spermatozoa was performed in an automatic mode based on the following indices: VAP as the average velocity of the head moving along the average path of motion, μm/s; VSL as the speed of rectilinear movement of the head (the average velocity of the head of the spermatozoon along the straight segment between the start and the end points of the trajectory), μm/s; VCL as average speed of spermatozoa movement along the real trajectory, μm/s; ALH as the average deviation of the head (amplitude of lateral displacement of the sperm head from the trajectory), μm; BCF as frequency of oscillatory averaged movements (frequency of intersections), Hz; the average frequency of the intersection of the curvilinear trajectory of the sperm movement by its averaged trajectory per time unit.

The straightness of the mean trajectory (%) was STR = VSL/VAP × 100, a degree of undulation of the tracks (the magnitude of the variation of the true trajectory with respect to the average trajectory, %) was LIN = VSL/VCL × 100.

Depending on these parameters, the spermatozoa were divided into four classes, A (rectilinar speed not less than 25 μm/s, or a distance per second equal to their length); B (with slow rectilinear motion at less than 25 μm/s); C (with circus or oscillating motility); D (immobile).

Since it is impossible to obtain ejaculated bison semen in natural habitat of th animals, the bull semen (n = 4) was compared to the semen of their relatives, the Holstein bulls (n = 15), by morphometric parameters. The sperm of bull sires were cryopreserved in straws and stored no more than 2 years. Bison semen was stored over 20 years.

The data were mathematically processed using variation statistics in the

Microsoft Excel software package. The tables show average values (X) and standard errors (x).

1. The percentage of spermatozoa of different classes in the frozen-thawed bison (*Bison bonasus* L.) semen ($X \pm x$)

Bison name	Class			
	A	B	C	D
Mutfil	10.3±0.2	18.8±0.9	20.3±1.2	50.6±1.6
Avel	8.4±0.1	12.3±0.7	12.1±0.9	67.2±1.8
Morus	6.3±0.2	22.3±0.8	13.2±0.6	58.2±2.1
Misir	8.3±0.2	13.4±1.2	10.4±0.8	67.9±3.6

N o t e. For description of the classes see *Technique* section.

Results. The number of spermatozoa with rectilinear motion in native and frozen-thawed semen depends on a variety of factors, such as individual characteristics of the animals, species, age, cryoconservation technology, diluents and cryoprotectants used, thawing method [45-47].

2. Morphometric parameters of frozen-thawed spermatozoa of bison (*Bison bonasus* L.) and Holstein bull sires ($X \pm x$)

Parameter	Bisons (n = 4)	Bulls (n = 15)
Spermatozoon length, μm	65.90±1.57	68.3±0.98
Head length, μm	8.46±0.66	9.58±0.28
Head width, μm	4.72±0.42	4.60±0.12
Perimeter of the head, μm	23.95±1.55	24.61±0.36
Head area, μm^2	38.46±1.67	41.6±1.32*
Length of flagellum, μm	57.34±1.96	58.72±0.36

* $P \leq 0.05$.

The semen differed significantly in the number of A and B spermatozoa (Table 1). The main factors that influenced the activity of spermatozoa were the age, individual characteristics of the bison and the duration of semen storage, since the medium composition and cryopreservation-thawing technology for all samples were the same. In frozen-thawed semen of Mutfil and Morus, more than 28 % spermatozoa were of A + B classes; in Avel and Misir, more than 67 % spermatozoa were immobile, and those with circus and oscillatory motility amounted to 12.1 and 10.4 %, respectively.

In collecting and cryopreservation of the semen, different technologies were used in bulls and bison. Ejaculated spermatozoa and those extracted from epididymis significantly differed. For all the morphometric parameters, except for the width of the head, the bison spermatozoa were inferior to the bull spermatozoa. It should be noted that the differences between the groups in the indicators were not statistically significant, except for the area of the sperm head, i.e. in bulls, it was 3.14 μm^2 larger than that in bison (Table 2).

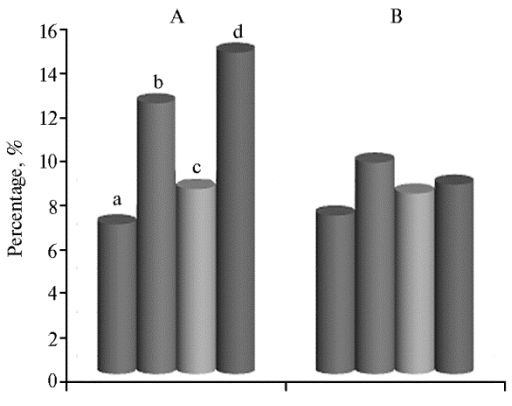

Fig. 1. The percentage of spermatozoa with morphological disorders (A) **and damaged acrosome** (B) **in the frozen-thawed semen of four bison (*Bison bonasus* L.):** a — Mutfil, b — Avel, c — Morus, d — Misir (stored for more than 20 years).

We also studied organelles and morphological structures of bison spermatozoa. The anomalous structure of spermatozoa is due to the genetic component [48], seasonal and environmental factors, individual predisposition [49-52], and age [53-56].

Chromatin is one of the most important structural elements of spermatozoa. The cause of idiopathic infertility may be a high index of DNA fragmentation in chromatin [57-61]. The DNA fragmentation in spermatozoa also depends on numerous biotic and abiotic factors [62-69].

A significant difference was found between the bison in the ratio of spermatozoa with morphological disorders and a damaged acrosome (Fig. 1).

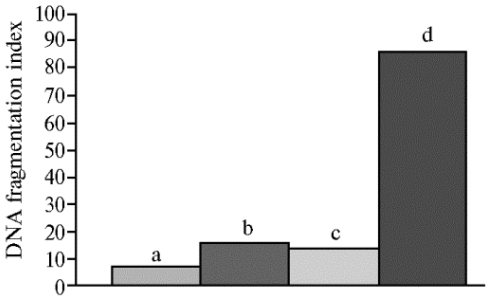

Fig. 2. The DNA fragmentation index in chromatin of spermatozoa in the frozen-thawed semen of four bison (*Bison bonasus* L.): a — Mutfil, b — Avel, c — Morus, d — Misir (stored for more than 20 years).

The frequency of spermatozoa with abnormal morphology was greatest in Misir (14.6 %), and the lowest in Mutfil (6.8 %). In the latter, the frequency of damaged acrosomes was insignificant compared to other bison and amounted to 7.2%.

The DNA fragmentation index in the chromatin of spermatozoa varied significantly among the samples (Fig. 2). High fragmentation was observed in Misir (more than 86 %), and the least was found in Mutfil (7 %).

Thus, the indices characterizing the biological integrity of spermatozoa in the frozen-thawed bison semen stored for more than 20 years depended on the animal individual characteristics. The number of spermatozoa with abnormal morphology varied from 6.8 to 14.6 %, the DNA fragmentation was from 7 to 86 %. The results of our studies show that the methods of sampling and cryopreservation technology affect the morphometric parameters of spermatozoa that were lower in the frozen-thawed epididymal semen as compared to ejaculated sperm.

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AN INCREASE IN THE VIABILITY OF RAM SPERM WHEN CULTURED IN MODIFIED MEDIUM FOR in vitro FERTILIZATION

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Abstract

Modern livestock necessitates controlled breeding, one of the main methods of which is a long-term preservation of animal reproductive qualities at cell level. In recent years, a considerable progress has been achieved in the development of techniques of animal sperm preparation for in vitro fertilization (IVF). In these, the main purpose is to obtain population of motile sperm with a suitable morphology capable of in vivo or in vitro oocyte fertilization. However, these methods are not always effective because of the lack of standards for the procedure. We first in Russia carried out a study on the viability of ram sperm cultured in the modified IVF medium. The semen was sampled from North Caucasian rams, 2-3 years of age, according to a routine technique, used for sheep and goats' artificial insemination, and domestic State Standards GOST 32222-2013. Sperm was evaluated on organoleptic and microscopic parameters, and cultured for capacitation to render the spermatozoa competent to fertilize an oocyte according to the prototype procedure, described by A.P. Gandhi (2000), and the method, developed by us. In the modified procedure, the sperm is brought to glucose-yolk-citrate diluent (GYC) and then transferred to medium SOF wash, prepared without glucose and supplemented with glutamine with 6 mg/ml bovine serum albumin, 0.2 mg ml caffeine and 50 mg/ml heparin. The developed technique increased the activity of sperm by 1.3 points compared to the commonly used method, the number of live semen increased by 43.7 %, their vitality increased 1.6 times and the number of spermatozoa with rectilinear movements was 1.6 times higher. Thus, the developed technique significantly improves ram sperm qualitative and quantitative characteristics and allows us to recommend this method of animal sperm maturation for in vitro fertilization procedure.

Keywords: sperm, capacitation, in vitro fertilization, rams, nutrient media

Key factors of animal productivity are genetic improvement and suitable conditions for phenotypic manifestation of the genetic potential [1]. Reproductive biotechnologies are being improved in Russia for many years [1-4]. There are reports of transplantation [5], producing embryos and the use of thawed semen [6], preparation of bull spermatozoa for in vitro fertilization and increase in their fertilizing capacity [7], morphological evaluation and increase in the boar semen capacitation [8, 9]. The experience of foreign researches is based on the recognition of the advisability of in vitro fertilization (IVF) fresh sperm [6, 10, 11].

However, to improve IVF, it is the sperm quality that remains critical. Auxiliary reproductive technologies (ART) include a variety of procedures for separating the most viable germ cells from the seminal plasma for ovum fertilization. The main purpose of these techniques is to obtain a population of mobile spermatozoa with a suitable morphology and ability to fertilize the oocyte in vivo

or in vitro. In capacitation (maturation, incubation), the movement of spermatozoa changes from regular wave-like to whip-like due to biochemical modifications of cell membrane (a similar process occurs in isthmus) [12]. In the acrosome, the glycoproteins and proteins of the seminal plasma are removed from the plasmalemma, which contributes to the acrosomal reaction [11, 13] and allows to remove dead spermatozoa, extraneous cells and their fragments. In vitro capacitation takes place in special media such as Krebs-Ringer and Tyrode, Brinster with high ionic strength, BW, TS-199 with fetal serum (10 %) or bovine serum [14]. The cells are separated in various ways — by once or twice washing the spermatozoa in a culture medium with further centrifugation; by flotation methods (including swim up) based on spermatozoa self-migration or sedimentation; by centrifugation in Percoll density gradient to separate different types of spermatozoa and choose those with appropriate morphofunctional properties, etc. [5, 15]. Filtration is the least common procedure, but it is worth noting that filtration is only applicable to samples of good quality, and because of the complexity this procedure is not applicable in veterinary [15, 16].

Despite the wide arsenal of technologies, the question of which method is most suitable for sperm in vitro processing remains discussible.

The novelty of our research is the development of an improved method for the preparation of ram sperm of for in vitro fertilization. The proposed procedure significantly improves the qualitative and quantitative characteristics of spermatozoa and can be recommended for practical use.

The purpose of this work was to study the viability of ram spermatozoa in a modified medium for in vitro fertilization.

Technique. In the experiment, North Caucasian elite rams (n = 6) aged 2-3 years were sperm donors. The animal weigh ranged from 107.5 to 110.6 kg. The ram sires productivity conformed to the requirements according to the practical guidelines ("Procedure and conditions for evaluation of fine-wool, semi-fine-wool of meat breeding sheep", Moscow, 2011). Sperm was taken urethrally 2 times a week at 2 day intervals in vivarium, using the artificial vagina (Minitüb GmbH, Germany) as per the descriptions [2, 17].

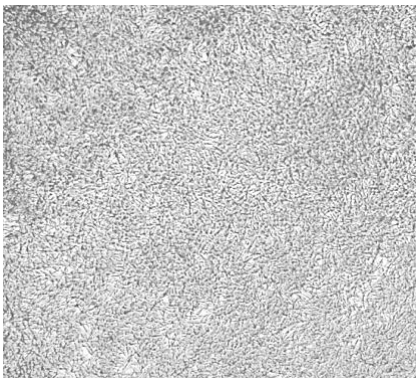
The samples were evaluated organoleptically and by microscopy (Mikmed-2, LOMO, Russia, ×100 magnification) [18]. Light microscopy was carried out in accordance with the WHO guidelines and RF State Standard (GOST 32277-2013) [15, 17]. Resistance of spermatozoa was assessed according to V.K. Milovanov and A.I. Korotkov as the change in activity in the presence of NaCl. The samples were stained with 10 % nigrosine (OOO TPO Lenpromchem, Russia) and 10 % eosin (OOO Chemical Line, Russia) [17].

As per a prototype-technique that we described earlier [19], fresh ram sperm was kept in a SOF (Synthetic Oviduct Fluide) wash medium without glucose with 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, pH 6.8-8.2), sodium lactate, sodium pyruvate (0.127 mg/ml) and bovine serum albumin (3 mg/ml) (OOO Origigio, Russia). To increase the activity and viability of spermatozoa before fertilization, we used the author's technique (hereinafter the developed technique), according to which the sperm was added to a glucose-citrate-yolk diluent (GCY), prepared according to RF State Standard (GOST 14746-69) which contained glucose medical anhydrous (30.0 g), sodium citrate $Na_2C_6H_5O_7 \cdot 5.5H_2O$ (14.0 g), egg yolk (200.0 ml), spermosan-3 (750-900 thousand units (ChPUP Gomel Plant of Veterinary Preparations, Belarus), and distilled water (1000.0 m)l. Then the sperm was transferred to the SOF wash medium without glucose and glutamine with the addition of bovine serum albumin (6 mg/ml), caffeine (0.2 mg/ml) and heparin (50 g/ml).

The swim up procedure was performed in the SOF wash and GCY cul-

ture media using centrifugation at 200 g for 8 min (Sigma, USA), after which the samples were placed into the CO_2 atmosphere at 37 °C for 1 hour (CO_2 incubator, BINDER GmbH, Germany).

The data statistical processing was performed in the Primer of Biostatistic 3.01 program (McGraw-Hill, Inc., USA), using Student's *t*-test. Differences were considered statistically significant for $p < 0.05$. The table shows the mean (*M*) and standard errors of the mean (*m*).



A smear of the North Caucasian ram semen after the swim up procedure when preparing a sample for the improved technology. Description of the groups is given in the *Tecnique* section. Staining with eosin and nigrosine, ×100 magnification, micrometer Mikmed-2 (LOMO, Russia).

Results. To determine the efficiency of sperm processing by the prototype technique and a modified procedure, we compared the most important spermatozoa indicators (activity, resistance and the number of active spermatozoa with rectilinear motion). Evaluation was carried out before and after the swim up procedure, as a result of which spermatozoa cells were capacitated (Fig., Tables 1, 2).

We found that with SOF wash medium prepared by the prototype method, the activity of spermatozoa before the swim up procedure decreased by 70.9 % compared to normal values, and after the swim up procedure increased by 14.5 %. Thus, after spermatozoa maturation in SOF wash medium the activity indices decreased significantly (by 47.6 % at $p < 0.05$).

The proportion of spermatozoa with rectilinear motion in fresh sperm placed only in the SOF wash medium decreased by 47.9 % before the swim up procedure and slightly increased (by 11.4 %) after the swim up, remaining, however, below the approved standards. The ratio of active spermatozoa after swim up was 53.2 % reduced. Since the number of germ cells in the sperm should be at least 80 %, low values (46.8 %) indicated the unsuitability of the sample for fertilization.

1. Some indicators of spermatozoa quality (North Caucasian rams, $n = 10$, $M \pm m$)

Animal No	SOF wash			GCY + SOF wash		
	activity, point	active spermatozoa, %	resistance, thousand units	activity, point	active spermatozoa, %	resistance, thousand units
	Before swim up					
1	5,5±0,5*	49,8*	18,45±0,20*	8,9±0,3*	95,2*	36,40± 0,20*
2	5,2±0,6*	50,7*	16,30±0,47*	8,9±0,3*	94,8*	33,24±0,30*
3	5,4±0,5*	51,2*	15,26±0,30*	8,9±0,3*	94,9*	32,60±0,46*
4	5,8±0,4*	49,0*	14,87±0,30*	8,9±0,3*	93,6*	31,59±0,30*
5	5,7±0,5*	48,2*	14,23±0,40*	8,8±0,4*	93,7*	34,74±0,20*
6	5,4±0,5*	49,9*	15,60±0,11*	8,9±0,3*	93,5*	32,10±0,12*
Total	5,5±0,5*, **	49,8*, **	15,80±0,30*, **	8,9±0,3*, **	94,3*, **	33,40±0,30*, **
	After swim up					
1	6,6±0,5*	47,3*	23,10±0,30*	8,6±0,5*	91,3*	31,60±0,20*
2	6,2±0,4*	48,2*	20,30±0,20*	8,7±0,4*	91,0*	33,20±0,20*
3	6,7±0,5*	46,6*	19,60±0,40*	8,9±0,3*	90,4*	29,30±0,30*
4	6,0±0,5*	46,0*	18,90±0,30*	8,7±0,5*	89,9*	27,90±0,30*
5	6,3±0,5*	45,6*	18,10±0,30*	8,6±0,5*	90,0*	31,10±0,10*
6	6,0±0,7*	47,0*	19,60±0,40*	8,7±0,5*	90,1*	28,20±0,20*
Total	6,3±0,5*, **	46,8*, **	19,90±0,30*, **	8,7±0,5*, **	90,5*, **	32,20±0,20*, **

Note. SOF wash — Synthetic Oviduct Fluide, GCY — glucose-citrate-yolk diluent, swim up — procedure of washing (for more details see the *Technique* section).

* Differences before and after swim up are statistically significant at $p < 0.05$.

** Differences in the parameters before and after the swim up procedure, carried out according to two methods, are statistically significant at $p < 0.05$.

A standard for spermatozoa resistance should be from 20 to 40 thousand

units. In our study, this parameter decreased by 54.06 % in the SOF wash diluent, and increased after the swim up by 21.00 % reaching 18.48±0.20 to 14.23±0.40 thousand units before the capacitation and 18.10±0.30 to 23.10±0.30 thousand units after the capacitation, but this did not correspond to the normative indicators.

Thus, the SOF wash reduces the viability of sperm and should not be used in processing sperm samples before in vitro fertilization.

2. Percentage of spermatozoa with rectilinear motility (North Caucasian rams, $n = 10$)

Animal No	SOF wash	GCY + SOF wash
Before swim up		
1	52,4	94,7
2	50,5	94,0
3	51,8	94,2
4	52,9	94,1
5	53,0	93,7
6	51,6	94,2
Total	52,0	94,2
After swim up		
1	59,4*	92,6*
2	59,0*	92,9*
3	60,0*	93,4*
4	57,9*	92,7*
5	58,4*	92,5*
6	57,8*	92,4*
Total	58,8*	92,7*

Note. SOF wash — Synthetic Oviduct Fluide, GCY — glucose-citrate-yolk diluent, swim up — procedure of washing (for more details see the *Technique* section).

* Differences before and after swim up are statistically significant at $p < 0.05$.

It was found that the index after introduction into the GCY was 5.6 % lower compared to the standard before the swim up procedure and significantly ($p < 0.05$) decreased by 2.1 % after swim up. In total, sperm processing according to the proposed method reduced activity by 7.7 %.

The number of spermatozoa with rectilinear motility in GCY, as compared to the initial values, decreased by 6.2 % before swim up and by 7.9 % after this procedure. The number of active spermatozoa in the sample after introducing an aliquot into the GCY medium significantly decreased by 5.7 % before the swim up, and by additional 9.5 % after the capacitation was compeered. These indicators are within the normative values for the number of active spermatozoa in the sample according to State Standard.

Spermatozoa resistance at the modified capacitation remained within the norm (i.e. decreased by 2.7 % before swim up and by 9.1 % after swim up).

In addition, we described in detail the viability of spermatozoa when applied SOF wash + GCY in norm, before and after the swim up procedure. A comparison of spermatozoa activity suggests that the significant difference between fresh sperm and that processed by the prototype technique is 1.4 points downwards, while in both methods the difference after maturation was 1.3 points. The proportion of spermatozoa with rectilinear motility in the samples prepared according to the developed method was 1.8 times higher before swim up procedure and 1.6 times higher after swim up compared to the samples processed according to the prototype technique. In the SOF wash + GCY media, before swim up, the number of active germ cells was 47.2 % (that is, almost 2 times) more than in the SOF wash. After the flotation procedure in SOF wash + GCY, the number of active cells increased by 43.7 %. Before the swim up procedure, the resistance of spermatozoa in the prototype technique was 2.1 times less than when using the developed technique. After the swim up of the spermatozoa in the SOF wash + GCY media, they were 1.6 times more viable than in the SOF wash without GCY.

In reproductive biology, the development of procedures for germ cell maturation and fertilization in vitro is an important fundamental and applied research area [20-22]. Admittedly, the processing of fresh sperm has not received sufficient attention, although this is an important factor in successful fertilization to produce healthy offspring (23). Papers in which the results of a study of the spermatozoa morphology during preparation of rams ejaculate for IVF are presented, are practically absent [24]. Currently, the researchers from

Stavropol State Agrarian University are the only ones in Russia who developed in vitro fertilization in ewes and the use of frozen-thawed ram sperm which results in embryos successfully produced in vitro. The technique developed for the ram sperm pre-IVF processing significantly improves qualitative and quantitative characteristics of spermatozoa, and, therefore, it may be used in assisted reproductive technologies for in vitro fertilization.

So, we showed that the proposed modification increased spermatozoa activity by 1.3 points compared to the existing technology, while the number of active spermatozoa increased by 43.7 %, their viability increased 1.6-fold, and the number of spermatozoa with rectilinear motility was 1.6 times higher, which is associated with a specific effect on the sperm of glucose, sodium citrate and egg yolk. An increase in the qualitative and quantitative characteristics, achieved due to the proposed method, makes it possible to increase the efficiency of in vitro fertilization and the production of healthy offspring.

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ULTRA-LOW CONCENTRATIONS OF BENZIMIDAZOLE DERIVATIVES CAN INCREASE BULL AND HORSE SEMEN RESISTANCE AT CRYOPRESERVATION AND UNDER THE INFLUENCE OF DAMAGING FACTORS

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Abstract

One of the possible ways to improve sperm cryopreservation is to find how to increase the resistance to damaging effects of low temperatures. Here we summarize our findings on the bull and stallion semen cryoresistance as influenced by ultra-low concentrations of biologically active substances, the ethyl-1-benzimidazol-2-yl-sulfanyl, 2-ethylsulfanyl-benzimidazol-1-yl and 2-benzimidazol-1-yl-acetic acid. It was found that these substances increased survivability of bull semen during storage in lactose-citrate semen extender. The best motility and vitality of sperm after freezing and thawing was observed when sperm was diluted by extender with added 2-benzimidazol-1-yl-acetic acid in the ultra-low concentrations of 10^{-13} to 10^{-15} M. The viability of sperm to 10 % motility was 73 % higher as compared to control. Similarly, freezing equine sperm in extender supplemented with 2-benzoimidazol-1-yl-acetic acid at 10^{-13} M was more effective: the semen survival after freezing and thawing was 8.1 % ($P < 0.01$) higher than that in the control, and the intactness of acrosome was 1.9±0.63 % higher ($P < 0.05$). 2-Benzimidazol-1-yl-acetic acids also improved semen vitality at 40 °C when different osmolarity and after cold shock. It can be assumed that the observed phenomenon is likely due to the protective effect of 2-benzimidazole-1-yl-1-acetic acid to plasma membrane and the mitochondria membrane structure of spermatozoa. Study of respiration in bovine sperm after freezing and thawing confirmed this assumption. Indeed, dinitrophenol almost equally increased cell respiration despite the presence or absence of 2-benzimidazole-1-yl-1-acetic acid in the semen extender while succinate, which penetrates through the damaged membranes, had less stimulating effect when 2-benzimidazole-1-yl-1-acetic acid added. The studies suggested the hypothesis that benzimidazole, a biologically active substance, at ultra-low concentrations can bind to a receptor on the sperm outer membrane resulting in the cell membrane restructuring. At the same time, the changes in viscosity of water associated with the membrane proteins may occur due to hydrogen bonds between water molecules and acid residues of benzimidazole molecules. As a result stability of the membrane structures to damaging effect of varying osmotic pressure increases. Possibly crystal formation of water associated with the cell membranes is decreasing during freezing that also reduces the damaging effect.

Keywords: sperm, freezing, benzimidazole, ultralow concentrations, cell membrane, mitochondria, bulls, stallions

Despite widespread use of cryopreserved sperm in breeding various types of farm animals, the death of up to 40-50 % of germ cells after freezing remains a problem for the practice of artificial insemination, so the search for ways to make spermatozoa resistant to the damaging effect of low temperatures is still relevant [1-5].

Nonspecific increase in cell resistance under the influence of chemicals of different nature in subthreshold doses and concentrations, several orders of magnitude lower than the sub-toxic, has been described for a long time. In the

domestic literature, early publications on this topic include studies that confirmed an increase in the time of spermatozoa survivability under the influence of subthreshold doses of a number of agentsю e.g. drugs, urea, inhibitors of metabolism, etc. [6-11]. Later, substances with the ability to cause an increase in cell resistance at concentrations several orders of magnitude lower than the subtoxic levels were found. Thus, it has been shown that benzimidazole and its derivative dibasol in concentrations of 10^{-3}-10^{-11} M promotes resistance of cells and tissues to the damaging effects of low and high temperatures [12-14].

In the last 20 years the attention of researchers has been attracted by the phenomenon of the effectiveness of ultra-low doses (ULD, 10^{-12}-10^{-15} M) substances with respect to biological objects. First of all, the reason is that many compounds in ULD can cause response reactions that are comparable and even more significant than at substantially higher concentrations [15, 16]. Attempts to explain the mechanism of the biological effect of physical and chemical factors in the ULD [17-21] have not led to a single opinion to date. Nevertheless, ULD in a number of cases found a successful application in medicine [22-24] and veterinary medicine [25, 26].

We first studied the effect of benzimidazole derivatives on the resistance of bull and stallion spermatozoa to the damaging effect of low and ultra-low temperatures during cryopreservation, and it was shown that the greatest activity and safety were noted when 2-benzimidazole-1-yl-1-acetic acid of at an ultra-low concentration of 10^{-12}-10^{-15} M was introduced into the medium for semen freezing.

The aim of this work is to study the effect of low and ultra-low concentrations of benzimidazole derivatives on the survivability of spermatozoa during dilution, freezing and thawing, their resistance to cold shock in the media with different osmolarity.

Technoque. Benzimidazole derivatives were synthesized at the Department of Organic Chemistry of the St. Petersburg State Agricultural University. Used concentrations of the substances in the diluent were 10^{-3}, 10^{-5}, 10^{-11}, 10^{-13}, and 10^{-15} M.

The experiments used sperm of black-and-white bulls (n = 11) of Leningrad type (FGUP Nevskoe, Leningrad region) and Trakehner, Hanoverian, Arabian and Holstein stallions (n = 10) (OOO Cowboy, Malanichevs farm and private owners, Leningrad region). The bull sperm had an initial mobility of 5-6 points (because of this values the samples were not allowed to freeze for production purposes; when measuring respiration rate, samples with greater mobility were used), stallion sperm had 7-8 points. To compare the experiment options, each ejaculate was divided into equal parts.

When determining the resistance of bull spermatozoa to changes in osmotic pressure and cold shock, an aqueous lactose solution was a diluent. Control was a solution with an osmolarity of 336 mosm/l, containing 11.5 g of lactose per 100 ml of water; to increase or decrease osmolarity in the series 246, 276, 306, 336, and 366 mosm/l, the amount of lactose was increased or decreased (by 1.02 g per 30 mosm/l). The procedure for sample freezing corresponded to the interstate standard (GOST 26030-2015) [27]. Cold shock of bull spermatozoa was caused by a decrease in the temperature of diluted semen from 20 to 4 °C for 2 min.

The sperm of the stallions were diluted in Kenney medium (49 g of D-glucose, 24 g of dried milk, 40 mg of gentamicin, 1 liter of distilled water) in a volume ratio of 1:3, then centrifuged for 8 min at 600 g. The spermatozoa residue was suspended in Kenney medium and diluted 100 million cells/ml concentration, the semen was packed into 0.5 ml straws, cooled at 4 °C for 90 min and

frozen in liquid nitrogen vapor for 12 min at 110 °C, then lowered in liquid nitrogen. The stallion sperm was thawed at 37 °C for 1-2 min.

The volume, number and mobility of spermatozoa (complete absence as 0 points, 100 % as 10 points) were assessed by conventional methods. Morphology and condition of the acrosome cap were studied with phase-contrast light microscopy. The time of the survivability of the bull sperm was expressed in hours to the preservation of 10 % of mobile cells and to the complete loss of mobility.

The respiratory activity of the cells was determined according to the description [28] on a polarograph LP 7 (Czech Republic) with a Clark platinum electrode. The incubation medium was 6 % glucose, in which sperm (50-100 million spermatozoids per ml) was successively added to final concentration, potassium succinate was a substrate (1.0×10^{-3}-2.5×10^{-3} M), and proton ionophore 2.4-dinitrophenol (DNP, 2.5×10^{-5} M) served as classical uncoupler of cellular respiration and phosphorylation.

Damage to the plasma membranes of stallion spermatozoa was assessed using Sperm VitalStain dye (Nidacon International AB, Sweden). Staining was performed in Eppendorf tubes (50 μl of sperm were mixed with 50 μl of dye) and smears were made on slide glasses. The preparations were examined with ×100 magnification (lens) and oil immersion, counting at least 200 cells in each sample (white cells mean no damage, red or pink were spermatozoa with damaged membranes).

The Axio Imager visualization system (Carl Zeiss Microscopy GmbH, Germany) was used for microscopy.

Data was processed in SigmaPlot 12.5 (Systat Software Inc., USA) and Micrisoft Excel programs. We performed a general statistical analysis and an estimation of the average difference between samples with pairwise coupled variants. The differences were considered statistically significant at $P < 0.05$. The tables show the mean (X) and standard error of the mean ($\overline{x}$).

Results. One of the possible approaches to the practical solution of the preservation problem of cryopreserved spermatozoa is to find ways to increase their resistance to the damaging effect of low temperatures. To date, enough data have been accumulated on the effect of cooling on cells [29, 30], which allow us to draw definite conclusions about the mechanisms of damage and approaches to preventing these damages. It is possible to distinguish two types of damage to cellular structures that occur during the action of cold, i.e. associated with cooling prior to freezing and resulting from crystal formation during freezing. Among the factors influencing the survival of cells and tissues during cryopreservation, the cold shock [31] and fluctuations in osmotic pressure during dilution, freezing and thawing of sperm are distinquished.

When comparing the time of survivability of bull spermatozoa at 20 °C in the presence in the diluent medium of three benzimidazole derivatives in concentrations of 10^{-3}-10^{-15} M, it was found that the survival of bull spermatozoa during storage in the lactose-citrate medium was increased. The greatest positive effect was given by ethyl-1-benzimidazole-2-yl-sulfanyl at a concentration of 10^{-5} M (46 % excess), 2-ethyl-sulfanyl-benzimidazole-1-yl at a concentration of 10^{-11} M (59 %) and 2-benz-imidazol-1-yl-1-acetic acid in concentrations of 10^{-11} M, 10^{-13} M and 10^{-15} M (by 88-90 %).

Comparison of the effect of the most effective concentrations of the studied derivatives at an elevated plus temperature (40 °C) (Table 1) showed that the greatest activity and survivability of bull spermatozoa after freezing-thawing was due to the presence in the medium for the semen dilution of 2-benzimidazol-1-yl-1-acetic acid in ultra-low concentrations of 10^{-13}-10^{-15} M, or

only 4-6 molecules of the test substance per spermatozoon. In this variant, the percentage of surviving spermatozoa (activity) was significantly higher (by 8-13 %, $P < 0.01$) than in the control. The time of survivability until the mobility of 10 % of the spermatozoa increased by 73 %, and the time until the mobility ceased completely exceeded the control by 85-90 %.

1. Survivability of bovine spermatozoa at 40 °C after freezing and thawing in a medium with ultra-low concentrations of benzimidazole derivatives ($n = 7$, $X \pm \bar{x}$)

Option	Activity after thawing, point	Survivability time, hour	
		up to 1 point (10%)	up to 0 point
Control	2,6±0,28	1,5±0,18	2,0±0,19
Benzimidazole derivative, M:			
ethyl-1-benzimidazol-2-yl-sulfanyl, 10^{-5}	3,2±0,01	2,3±0,22*	2,9±0,21**
2-ethylsulfanyl-benzimidazol-1-yl, 10^{-11}	3,0±0,23	2,0±0,23	3,2±0,18**
2-benzimidazol-1-yl-1-acetic acid, 10^{-11}	3,2±0,38**	2,0±0,21	2,9±0,24**
2-benzimidazol-1-yl-1-acetic acid, 10^{-13}	3,9±0,26***	2,6±0,21***	3,7±0,21***
2-benzimidazol-1-yl-1-acetic acid, 10^{-15}	3,4±0,07***	2,6±0,09***	3,8±0,15***

N o t e. Bull sperm with initial activity of 5-6 points were used.
*, **, *** Differences with the control are statistically significant, respectively, at $P < 0.01$.

Similar results were obtained by freezing and thawing of stallion sperm in a medium with 10^{-13} M 2-benzimidazol-1-yl-1-acetic acid. The number of mobile intact cells was 15.2±3.49 million/ml greater ($P < 0.01$) than without supplement, the survivability of spermatozoa after freezing-thawing increased by 8.1 % ($P < 0.01$), the preservation of the acrosome was 1.9±0.63 % higher ($P < 0.05$).

2. Survivability of bovine spermatozoa at 40 °C and different osmolarity under the influence of 2-benzimidazole-1-yl-1-acetic acid (10^{-13} M) ($n = 11$, $X \pm \bar{x}$)

Osmolarity, mosm/l	Mobile cells, %					
	after dilution		after an hour at 40 °C		after two hours at 40 °C	
	control	experiment	control	experiment	control	experiment
366	$28\pm4,8^{a}$	$42\pm5,8^{a}$	$10\pm0,9^{g}$	$14\pm1,6^{g}$	0	0
336	$60\pm3,2^{A}$	$60\pm3,2^{B}$	$29\pm3,7^{d}$	$41\pm3,3^{d}$	$11^{i}\pm2,4$	$26^{i}\pm3,0$
306	$24\pm2,4^{b}$	$40\pm4,4^{b}$	$11\pm1,6^{e}$	$19\pm2,7^{e}$	0	6±1,6
276	$15\pm2,2^{c}$	$31\pm4,0^{c}$	0	12±1,5	0	0
246	4±2,4*	20±3,2*	0	6±1,8	0	0

N o t e. Bull sperm with initial activity of 5-6 points were used.
* Differences in variants aa, bb, cc, dd, ee, ii, Aa, Ab, Bb and Ba are statistically significant at $P < 0.01$, in gg - at $P < 0.05$.

2-Benzimidazole-1-yl-1-acetic acid at the same concentration of 10^{-13} M positively influenced the stability of bovine spermatozoa as the osmolarity changed (Table 2). Immediately after dilution at room temperature (20 °C) in osmolarity isotonic for bovine sperm (336 mosm/l), 2-benzimidazole-1-yl-1-acetic acid did not affect the cell activity compared to the control. However, after 1 and 2 hours after storage (at 40 °C) in the presence of an additive, the motility of the spermatozoa in the isotonic medium turned out to be 12 % and 15 % higher, respectively ($P < 0.01$), than in the control. With a decrease or increase in the osmolarity by 30 mosm/l to the isotonic level, the proportion of mobile cells in the sperm decreased immediately upon dilution (see Table 2), but in a medium with 2-benzimidazol-1-yl-1-acetic acid (10^{-13} M) only by 18-20 % compared to 32-36 % in the control. In hypoosmotic conditions, a complete sperm death was observed after 1 h in the control, while in the experiment 6-12 % of the cells retained motility. After 1 h in hyperosmotic conditions (366 mosm/l), the percentage of mobile spermatozoa in the experiment was significantly higher (by 14 %, $P < 0.05$) compared to the control.

Phase-contrast microscopy revealed no morphological differences in the acrosome and sperm flagellum between the experimental and control variants

immediately after dilution, whereas different osmolarity of the medium led to a change in the safety and viability of the spermatozoa (see Table 2). After a cold shock in the control, 40 % of the spermatozoa lost their mobility, while in 10^{-13} M 2-benzimidazole-1-yl-1-acetic acid, the motility of spermatozoa decreased by 26 %. It can be assumed that the observed increase in cell resistance to osmotic influence and cold shock is associated with the protective effect that the benzimidazole derivatives have on the membrane structures of spermatozoa.

As is known, the mobility and time of sperm survivability depend on energy supply, respiration and phosphorylation, and are directly related to the functional state of the mitochondria. The mitochondrial membranes are most sensitive to damaging factors [28]. Succinate enhances cellular respiration, penetrating only through the damaged plasma membrane, and DNP serves as an uncoupler of tissue respiration and oxidative phosphorylation [28]. In our experiments (Table 3), the stimulation of the respiration of bovine spermatozoa by succinate after freezing and thawing in the presence of 2-benzimidazole-1-yl-1-acetic acid (10^{-13} M) was lower, and with DNP was higher than in control, that indicates a better energy supply when using an additive. This confirms the assumption that the ultra-low concentrations (doses) of the benzimidazole derivatives contribute to an increase in the stability of the spermatozoa membrane at an ultra-low temperature.

3. Change in respiratory rate in bovine spermatozoa under the influence of 2-benzimidazole-1-yl-1-acetic acid (10^{-13} M) **during freezing and thawing** ($X \pm \bar{x}$)

Sperm	Activity, point	Respiration, nmol O_2/ min		
		rate	stimulation	
			succinate K	DNP
Freshly diluted	7.0-8.0	130.0±1.56	1.06±0.012	2.20±0.050
After freezing and thawing:				
control	4.0-5.0	77.0±3.82	2.03±0.187	1.57±0.029
experiment	4.5-6.0	88.0±5.03	1.47±0.730*	1.84±0.021*

N o t e. DNP - 2.4-dinitrophenol.
* Differences with the control are statistically significant at $P < 0.01$.

It is known that the cooling and freezing of spermatozoa results in the release of K^+ ions into the medium, which adversely affects cell vitality [31]. Benzimidazole derivatives serve as plasma membrane H^+/K^+-ATPhase inhibitors and prevent excess K^+ release. Apparently, interacting with the receptor on the outer cell membrane, benzimidazole derivatives cause a cascade rearrangement of the membrane structures. At the same time, the viscosity of water changes due to the occurrence of hydrogen bonds between molecules with the formation of clusters. As a result, the resistance of membrane to the damaging effect of osmotic pressure fluctuations occurring during freezing and thawing can increase. Besides, it is possible that when the water freezes, the character of crystal formation changes towards decreasing crystal size, which reduces their damaging effect. The absence of a pronounced effect of the substances at intermediate concentrations agrees with the classical theory of R.P. Stephenson [32-34], according to which the maximum effect is achieved by binding the ligand to only a small part of the receptors.

So, when ultra-low concentrations (10^{-13}-10^{-15} M) of 2-benzimidazole-1-yl-1-acetic acid was introduced into the medium used in freezing bull and stallion sperm, the spermatozoa were the most active and safe after freezing and thawing, cold shock, elevated temperature (40 °C) and when osmolarity changed (as it could be concluded from the time of survivability and the proportion of cells that remained mobile). Dinitrophenol almost equally strengthened cellular respiration in the experiment and in the control, while succinate, which penetrates through damaged membranes, had less stimulating effect in the presence of 2-

benzimidazole-1-yl-1-acetic acid. The observed effect of 2-benzimidazole-1-yl-1-acetic acid is presumably associated with its protective influence on the plasma membrane and the membrane structures of mitochondria in spermatozoa due to interaction with the receptor on the outer membrane, and, possibly, may be caused by the effect on the state of water molecules associated with membrane proteins.

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CHOLESTEROL DOES NOT IMPROVE CRYOPROTECTIVE EFFICIENCY OF SOYBEAN LECITHIN-BASED EXTENDERS FOR BULL SEMEN

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Abstract

Design of plant phospholipid based extenders free of animal-derived components is urgent in farm animal semen cryopreservation. However, a complex of plant lipids, unlike the yolk complex, lacks cholesterol which plays an important role in cryoresistance of spermatozoa. Enriching spermatozoa membranes with cholesterol improves their cryotolerance (E. Mocé et al., 2010; E. Mocé et al., 2014; M.H. Fayyaz et al., 2016). We first estimated the impact of different cholesterol concentrations (0, 8, 18, 33 and 50 %) in soybean phospholipid mixtures both on morphology of obtained liposomes or lipid particles, and on their cryoprotective property as judged by the effect on motility when added to the extender at bull sperm cryopreservation. Liposome suspensions were prepared from commercial lecithin LeciPRO 90 («Unite-chem Co., Ltd», China) and purified cholesterol form lanolin («Sigma-Aldrich Co.», USA) by sonication at an ultrasound disintegrator UZDN-2T (NPP «Academpribor», Russia; 22 kHz, 60 W/cm^2, 5 min). It was found that cholesterol did not notably influence the obtained liposomes at low concentration (8 %). At 18 and 33 % cholesterol in the lipid mixture the size of the liposomes increased and averaged 66±6 nm and 62±11 nm, respectively. Fifty percent cholesterol changed the lipid suspension completely resulting in complex structures formed by phospholipid-cholesterol lamellae and crystals of cholesterol monohydrate. Eight percent cholesterol did not increase a cryorpotective efficiency of lecithin, while 18-50 % cholesterol decreased its cryoprotective capacity. An increase in cholesterol concentration in the lipid mixture up to 50 % (≈ 66 mol%) led to somewhat higher spermatozoa motility compared to a minimum score observed at freezing with 33 % cholesterol. The most probable cause of the negative impact of cholesterol on the cryoprotective effectiveness of lecithin suspension is impairment in liposome interaction with a sperm plasma membrane caused by condensation of phospholipid packing in a lipid bilayer.

Keywords: cattle, cryopreservation, sperm, cholesterol, phospholipids, liposomes

Cryopreserved sperm of elite producers is widely involved in farm animal reproduction and breeding. In Russia, media containing egg yolk which has high cryoprotective effect is traditionally used in bull semen freezing. In this, the complicating factors are volatility of the yolk, the high probability of contamination with dangerous pathogens, the rapid loss of protective properties during storage, the complexity of sterilization, etc. That is why much attention is recently being paid to the cryoprotective media without egg yolk or other components of animal origin [1].

It is known that the cryoprotective function of yolk is due to the presence of phospholipids of the light lipoprotein fraction [2, 3]. Plant-derived phospholipids, for example soy lecithin, could replace this component in cryoprotective media [4, 5]. However, in addition to phospholipids, the egg yolk contains a number of other substances with protective effect, in particular, cholesterol,

completely absent in plant lipids.

Cholesterol is an important component of cytoplasmic membranes and has a great influence on cell resistance to cooling and freezing. In mammals [6-8], birds [9] and fish [10] the spermatozoa with high content of cholesterol and lipids in the membranes are more resistant to cold shock than those with low cholesterol. Later, cyclic oligosaccharide cyclodextrin was used for artificial enrichment of cell membranes with cholesterol [11]. Numerous experiments confirm that the enrichment of cell membranes with cholesterol enhances the tolerance of bull spermatozoa to cooling and freezing [12-15]. Consequently, it can be assumed that the inclusion of cholesterol in the composition of yolk-free media based on plant lecithins can increase cryoprotective efficiency.

Soy lecithin is introduced into cryoprotective media as liposomes, the structure and size of which depend on the method of preparation and the composition of the raw materials. Since cholesterol has a strong effect on the structure and characteristics of the lipid bilayer, it will significantly change both the properties of liposomes, and their interaction with cytoplasmic membranes [16-18]. The effect of cholesterol on the bilayer membrane, in turn, depends on the phospholipid composition of the liposomes. Therefore, to obtain reliable data on the cryoprotective role of cholesterol-contacting liposomes of plant phospholipids, it is necessary not only to study their effect on spermatozoa, but also to track changes in the liposomes themselves.

The available data on how cholesterol affects the cryoprotective functions of complex liposomes are poor though this information is of special importance when improving yolk-free diluents. For the first time, we simultaneously evaluated the effect of cholesterol in different concentrations (0, 8, 18, 33 and 50 %) on the physicochemical properties of liposomes and lipid particles of soybean phospholipids, as well as on the protective effect of the resulting lipid suspensions in the cryopreservation of bull semen. It was revealed that cholesterol did not increase, and in the concentration of 18-50 % even reduced the cryoprotective efficiency of lecithin.

The aim of the study was to study the dimensional characteristics and cryoprotective properties of the suspensions resulted from a mixture of soybean phospholipids and different concentrations of cholesterol.

Technique. LeciPRO 90 (Unitechem Co., Ltd, China) commercial lecithin and purified cholesterol derived from lanolin (Sigma-Aldrich Co., USA) were used. Cholesterol fractions were 0; 8; 18; 33 and 50 %. The lipids in this ratio were dissolved in chemically pure chloroform. The chloroform was then removed with a rotary evaporator for 4-5 hours until a thin film of phospholipids was formed on the inner wall of the glass flask, after which a sucrose solution (242 mM sucrose, 10 mM HEPES) was added up to 1 ml of water per 15 μg of lecithin. The lipids were dispersed on a laboratory shaker (type 358S, ELPIN PLUS s.c., Poland) for 2 hours at room temperature. The resulting suspension, to prevent oxidation of lecithin, was purged with nitrogen, then cooled in an ice bath to 4 °C and treated for 5 min on an ultrasonic disintegrator UZDN-2T (NPP Akadempribor, Russia 22 kHz, 60/cm^2; an immersed probe of 2 mm in diameter).

The average hydrodynamic diameter of the liposomes or lipid particles in the suspension and their size distribution were evaluated using the dynamic light scattering method of the N5 Submicron Particle Size Analyzer (Beckman Coulter, Inc., USA) 24 hours after the suspension was prepared.

The particles were examined under an Axio Scope FL microscope (Carl Zeiss, Germany) at ×400 magnification with dark field contrast.

For cryopreservation of bovine semen and evaluation of the protective

efficacy of lecithin we used rejected ejaculates (OAO Head Center for the Reproduction of Farm Animals), in which the proportion of spermatozoa with rectilinear motility was at least 70 %. Lactose-glycerol diluent (242 mM lactose, 10 mM HEPES, 55 mM glycerol) was a cryoprotectant. Analyzed phospholipid or phospholipid-cholesterol suspensions were added to a final lecithin concentration of 3 mg/ml. Egg yolk (20 %) was added to the control samples instead of the suspension. The semen was diluted with the cryoprotective medium in two stages (depending on the amount in the ejaculate) and, after 4 hours of equilibration at 4 °C, frozen in open granules on plates of solid carbon dioxide (dry ice) by the standard procedure [19]. The granules were thawed in a water bath at 39-40 °C in a special solution containing 130 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 14 mM fructose, 10 mM HEPES (Merck KGaA, Germany), and 0.1 % bovine serum albumin (OOO NPP PanEco, Russia).

The cryoprotective efficacy of lecithin and a mixture of cholesterol and lecithin was determined as the survivability of spermatozoa during cryopreservation. The viability of the reproductive cells was assessed by the mobility immediately after thawing and after 5 hours of incubation at 38 °C. The mobility was determined on a SFA-500 sperm analyzer (OOO NPF BIOLA, Russia) as a proportion of spermatozoa with rectilinear motion at a velocity of more than 25 μm/s. A standard lactose-glycerol yolk medium was the control.

Statistical processing was performed in the program Sig-maPlot 12.5 (Systat Software Inc., USA). Differences were considered statistically significant at $P < 0.05$. The experimental data (except the particle size dispersion) are presented as mean ($\bar{X}$) with a standard error (±SE). For the sizes of particles produced by ultrasonic treatment, a size dispersion (SD) is shown.

Results. Dimensional characteristics of a suspension of lecithin obtained by sonication. Sonication is widely used for the preparation of nanosized liposomes. Unlike liposomes obtained by extrusion (punching a suspension through a polycarbonate membrane with pores of a certain size), in sonication the particle size essentially depends on the lipid composition and the properties of the lipid bilayer (Table 1).

1. Average size of phospholipid and phospholipid-cholesterol particles after sonication ($n = 4$)

Cholesterol content, %	Average size of particles, nm ($\bar{X}$±SE)	Dispertion of particles (SD) within the sample, nm
0	41±7	51
8	43±13	42
18	66±6	56
33	62±11	46
50	3476±532	2978

Note. In determining the average size, the difference between the mean values for particles in different samples is considered.

After sonication, the size of the liposomes formed in the suspension, depended on the lecithin/cholesterol ratio. Figure 1, A shows a typical histogram of the particle distribution in the sonicated LeciPRO 90 suspension. Ultrasonic treatment resulted in a polydisperse suspension with a two-, less often a three-modal distribution. There was a cluster with average liposome sizes of about 30 nm and a cluster of larger particles (about 100 nm). In some samples, up to 1.5 % liposomes were 300 to 1000 nm in diameter.

The increase in the duration of ultrasound treatment (from 5 to 20 min) did not significantly affect the average size of the liposomes and their size distribution. LeciPRO 90 is a complex mixture of soybean polar lipids and other fat-soluble compaunds characteristic for plant raw materials, for example, carotenes. The most common components of commercial soybean lecithins of a similar class are phosphotidylcholine, phosphotidylethanolamine and phosphotidylinositol [20]. Liposomes resulted from such complex mixtures after sonication quite often demonstrate a complex polydisperse distribution in size [21], which corresponds to the results obtained by us.

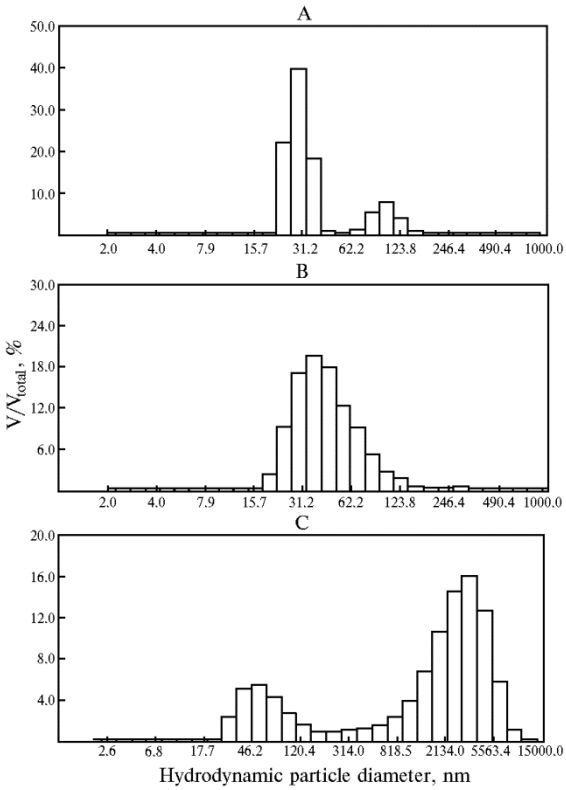

Fig. 1. Histogram of the size distribution of lipid particles formed from a mixture of cholesterol and lecithin LeciPRO 90 after sonication: A — without cholesterol, B — 18 % cholesterol, C — 50 % cholesterol; V/V_{total} — the ratio of the total volume of particles of the indicated size to the total volume of particles in the suspension. Ultrasonic disintegrator UZDN-2T (NPP Akadempribor, Russia, 22 kHz, 60 W/cm^2, 5 min).

The introduction of liposomes of LeciPRO 90 and low cholesterol (8 %) did not significantly affect the dimensional characteristics of the suspension. The average particle diameter did not change (see Table 1), and the type of their size distribution was similar to that observed for pure LeciPRO 90 (data not shown).

The increase in the cholesterol fraction up to 18 and 33 % led to an increase in the average particle size and changed their distribution. After sonication, the mixtures formed a monodisperse suspension with an average particle size of 66±6 and 62±11 nm, respectively (see Table 1, Fig. 1, C).

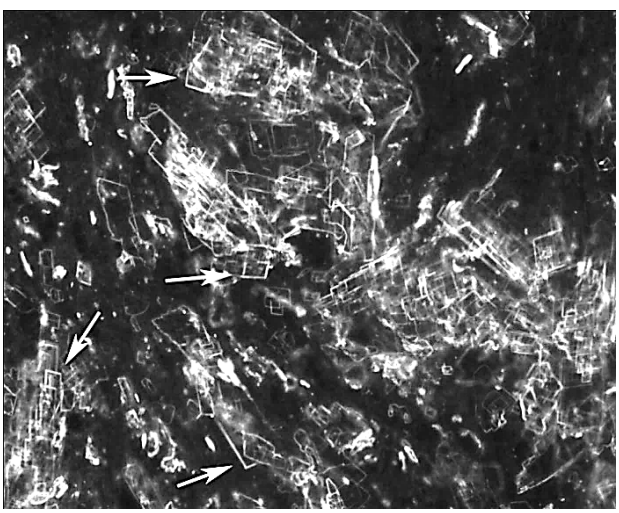

Fig. 2. Suspension obtained from a mixture of cholesterol and LeciPRO 90 (50:50 w/w) after sonication. The arrows indicate particles with a shape characteristic of cholesterol crystals. Microscope Axio Scope FL (Carl Zeiss, Germany), ×400 magnification, dark field contrasting.

The 50 % cholesterol corresponds to approximately 66 mole%, the limiting amount of cholesterol that can retain the lipid bilayer formed from phosphotidylcholine. A higher cholesterol/lecithin ratio results in high cholesterol phospholipid lamellas and cholesterol monohydrate crystals [22, 23]. The amount of cholesterol retained by the bilayer depends on the composition of the phospholipids [22]. For LeciPRO 90, this amount is unknown. From the data presented (see Table 1, Figure 1, C) it follows that the suspension of LeciPRO 90 and 50 % cholesterol (≈ 66 mole%) was fundamentally different from the others. The particle size in this mixture was more than an order of magnitude higher than that in suspensions from mixtures with a lower cholesterol concentration. In the suspension, two fractions of parti-

cles (of 40-120 and 2500-3500 nm) were observed. Among the particles that were larger and more clearly distinguishable under a microscope, those with a shape characteristic of cholesterol crystals were seen (Fig. 2). At that, the suspension was sufficiently stable. During 24 hours, no signs of stratification were observed, in contrast to a suspension of pure cholesterol crystals which precipitated fairly quickly. It can be assumed that the large fraction is formed by complex structures of cholesterol crystals and cholesterol-phospholipid lamellae, and the small fraction is a liposome from a mixture with the maximum possible cholesterol content in the experiment.

Cryoprotection effect of liposomes formed from LeciPRO 90 and cholesterol. Comparison of spermatosoa viability in the control (standard sucrose-glycero-yolk medium) and when adding liposomes or lipid particles from a mixture of soybean lecithin and cholesterol in different ratios did not reveal a positive effect of cholesterol. Moreover, the viability of bull spermatozoa cryopreserved in the presence of mixtures with high cholesterol concentrations (18, 33 and 50 %) was lower than when frozen with LeciPRO 90 without cholesterol or when adding egg yolk (Fig. 3). Interestingly, the viability of spermatozoa gradually decreased as the cholesterol concentration increased up to 33 % (≈ 50 mole%). It is in this range of concentrations that cholesterol positively affects lipid chains, making the lipid bilayer stable and reducing fluctuations in the mobility of molecules.

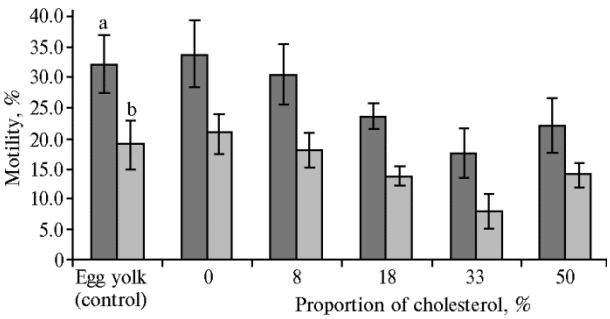

Fig. 3. Motility of bull spermatozoa frozen in the presence of mixtures of soybean lecithin and cholesterol 10 minutes (a) **and 5 hours** (b) **after thawing** ($\bar{X}$±SE, n = 10).

Further increase in cholesterol concentration to 50 % (≈ 66 mole%) was accompanied by a certain increase in the viability of spermatozoa relative to the minimum value with a cholesterol content of 33 %. Thus, a suspension of a lipid mixture with 50 % cholesterol provided significantly ($P < 0.05$) higher motility of spermatozoa 5 hours after thawing than a mixture with 33 % cholesterol (see Fig. 3). It is in this range of cholesterol concentrations that the structure and size of lipid particles in the suspension change radically (see Table 1, Fig. 1, 2).

From this it can be concluded that the addition of cholesterol impairs the interaction of the lipids of the suspension with the cytoplasmic membrane of the spermatozoon. A direct negative effect of cholesterol on spermatozoa should manifest itself in a monotonous decrease of cryoprotection across the whole range of concentrations, which does not correspond to our data. This contradiction may be removed by the explanation that cryoprotective efficiency changes due to weakening the interaction between the lipid particles and the cells.

An increase in the proportion of cholesterol in membranes often leads to a decrease in the fusogenicity of liposomes [16, 17, 24]. However, this trend is observed in the range from 0 to 50 mole% of cholesterol. With an increase in cholesterol content of more than 58 mole%, the interaction of cholesterol and lipid molecules becomes energetically less favorable [25], which can increase the fusogenic property of liposomes compared to a minimum at 50 mole%. Because of a complete rearrangement of the suspension structure which we observed in

our experiments (see Fig. 1, 2), we can expect a significant change in the interaction between the lipids of the suspension and cell membranes of spermatozoa.

Thus, cholesterol added at 18-33 % concentration to commercial soybean lecithin LeciPRO 90 increases the size particles after sonication of the mixture. At 50 % concentration cholesterol completely changes the lipid suspension leading to formation of complex structures of phospholipid-cholesterol lamellae, cholesterol monohydrate crystals and liposomes. At 8 % concentration cholesterol does not increase the cryoprotective effectiveness of lecithin, and at 18-50 % concentrations the cryoprotection decreases. The most probably, this is due to deterioration in the interaction of lipids from the suspension with the cytoplasmic membrane of the spermatozoa.

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EFFECT OF ATMOSPHERIC PRESSURE ON SEMEN PARAMETERS IN BULL SIRES OF MODERN SELECTION ON THE DAY OF COLLECTION

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Abstract

The publications concerning the effects of atmospheric pressure on the general patterns of metabolism, reproduction and adaptive capability in animals and humans from 1970 to 2015 are reviewed. The analysis could show that all those investigations were carried out on different mammalia species in different years and were fragmentary. Colossal changes in the Earth's atmosphere occurred over the recent decades, which were caused by the anthropogenic factors; considerable changes in the environment affected the habitats of living organisms. In addition, the rapid development in the livestock farming on the basis of the accelerated selection processes could contribute to the formation of animal breeds characterized by the changed metabolic functions, the affected stress tolerance, and the lower adaptive capabilities. That enabled us to conduct a survey to ascertain the effect of atmospheric pressure variability on the qualitative and quantitative semen parameters for bull sires of a modern selection. The survey was carried out in the Center for Animal Biotechnology and Molecular Diagnostics, the L.K. Ernst All-Russia Research Institute for Animal Husbandry, on the basis of the Head Center for Reproduction of Farm Animals. The collection, evaluation, freezing, thawing, and the use of the pedigree bull sire semen were carried out according to the National Technology (edited by N.M. Reshetnikov and A.I. Abilov, 2008). A total of 472 ejaculates of bull sires aged 2 to 10 years were analyzed in January, 2012. The atmospheric pressure varied in that period as follows: lower 755 mm Hg, from 755 mm Hg to 765 mm Hg, and over 765 mm during the periods of 7 days, 9 days, and 15 days, respectively. The ejaculate volume (ml), concentration (milliard per ml) and motility of spermatozoa with the progressive forward movements (PFM) (grades) were analyzed after the collection, freezing, thawing, and in 5 hours of incubation at 38 °C; the total number of spermatozoa per ejaculate (milliard), sperm defects (%), number of frozen semen doses per ejaculate, and the supposed loss of the semen doses caused by the sperm defects depending on both the variables of atmospheric pressure and the age of animals were examined comparatively. The obtained data were processed; the statistical reliability was calculated by the Stewdent's *t*-criterion. It is ascertained that the atmospheric pressure substantially affects the quantity and the quality of the obtained sperm products. These effects can be neatly traced by the number of sperm defects at sampling with defining a statistically valid value ($P > 0.001$). The bull sires aged 2.5 to 5 years are found to be more adaptive to the effects of various environmental factors. It is determined that the culling rate of the native semen samples by the activity parameter decreases with increasing the atmospheric pressure. The distinct dependency of the decrease in motility of sperm after the incubation at 38 °C from the increase in the atmospheric pressure was revealed. The best value for the number of the extracted spermatozoa was obtained at 760 mm Hg for each of the animal age groups; the lowest number of ejaculates having more than 5 milliard sperms was obtained at 775 mm Hg. Thus, the obtained data allow conclusion about the effects of atmospheric pressure variables on the sperm product parameters. In this case, the age of bull sires is of considerable importance. It is found that the bulls at the age of 2.5-5 years are more adaptive to the effects of various environmental factors. On the basis of the findings, it should be concluded therefore that in the breeding enterprises, the schedule for collecting the semen from the bull sires has to be corrected ac-

cording to the atmospheric pressure values. The use of bull sires should be limited or the schedule of their exploitation should be shifted at the atmospheric pressure of 775 mm Hg. The scientific novelty of the paper is in that the complex survey focused on the effects of atmospheric pressure variables on the qualitative and quantitative characteristics of the semen parameters in bull sires of the modern selection have been carried out for the first time.

Keywords: atmospheric pressure, bull sires, semen, age

In Russia, collection and use of semen of bull sires are regulated by the national technology [1], which currently does not consider the influence of a number of external factors on sperm production. At the same time, in many regions of the Russian Federation, including the Central Black Earth Region, due to long-term anthropogenic impact, an ecological situation is characterized by anthropogenic anomalies of different origin [2, 3]. Anthropogenic factors are related to realization of genetic potential in farm animals, including the inhibition of reproductive function in highly productive individuals, and a reduction in resistance to diseases and stresses. The adverse effects of environmental stress can occur at the genetic level. The precise identification of environmental factors affecting semen quality is important for improving bull reproductive function [4-6]. Morphofunctional abnormalities of the reproductive organs in cattle in a sharply continental climate reduce the reproductive capacity [7]. The season of the year affects the indicators of sperm production [8-13] and the proportion of cells with normal morphology due to changes in the hormonal background and the effects of ambient temperature [14, 15]. Winter and spring seasons are most favorable for sperm donation. In the summer period, stud bulls are relatively low sexually active. The total average monthly volume of sperm production indicates a decrease in activity in the autumn period, too [9, 16, 17]. The highest rates of ejaculate volume, semen count and sperm doses in spring are explained by photoperiodism and androgenic activity of bulls [10, 18]. To reduce the adverse effect of summer season factors on reproduction, it is proposed to reduce the frequency of semen taking and ensure better processing during cryopreservation [9].

In some studies, there was a positive correlation between atmospheric pressure and the birth rate of bull-calves [19]. There is evidence of the effect of high pressure on the sex ratio after insemination [20]. The effect of atmospheric pressure on the sex ratio was studied in Ecuador. Of 45 inseminated Holstein cows at an altitude of 2,750 m above sea level, 68.40 % have calved; 78.69 % of the calves from the heifers were also bull-calves ($P < 0.5$) [21]. In another experiment, 13 calves were born from cows inseminated with sperm which was exposed to reduced atmospheric pressure; 84.60 % of the calves were bull-calves. Of 12 calves born from cows inseminated with unprocessed sperm, 58.30 % were the bull-calves [21]. At 751.0-760.0 mm Hg atmospheric pressure, the time of manifestation of the copulation reflex in boars is much shorter than that at a higher or lower pressure, and the copulation and ejaculation reflex is longer. Atmospheric pressure exerted a significant influence on the biological indicators of boar semen quality. A larger volume of ejaculate and a higher concentration, an increase in resistance and an absolute survival rate of spermatozoa were noted in boars at 756 mm Hg and more [22]. It is reported that the sperm production of bulls was significantly influenced by atmospheric pressure and air humidity (along with other environmental factors) depending on animal breed, pedigree ine and age [23].

Sudden rain and strong winds act as negative factors in combination with low temperature, and solar radiation is unfavorable at high temperature [24]. Atmospheric pressure, solar activity, intensity of the Earth's magnetic field affect various physiological and biochemical indices of animals, e.g. the content of calcium, phosphorus, reserve alkalinity, carotene, and protein in the blood serum.

A significant decrease in dry matter digestibility was found in 58-83 % of cows [25]. If the atmospheric pressure deviates by 18-20 mm Hg from the mean normative index (750 mm Hg) and other conditions are equal, the enzymatic status of lymphocytes significantly changed [26]. In Hereford and Simmental cattle, feed intake increased with increasing atmospheric pressure [27]. There are data on the effect of atmospheric pressure on the duration of fruiting in cows and pigs [28]. When the intensity of abnormal meteorological factors over a long time exceeds physiological standards, the body's response becomes pathological, and an imbalance of the homeostasis is developed. Stresses result in a general adaptation syndrome, leading to hematological, morphological and clinical changes. Free radicals are intensively formed, oxidative stress and pathological processes develop [29, 30].

Protection of productive animals from impacts that disrupt the reproductive function and adversely affect growth and productivity are often associated with unprofitable costs. Therefore, study of sperm biochemical composition, the quality and fertilizing capacity of spermatozoa, as influenced by environmental conditions, age, breed and individual characteristics of the sire is relevant in improving reproduction [21].

In the present work, we conducted for the first time a comprehensive study of the effect of high atmospheric pressure over a long period on the sperm production in bull sires depending on their age.

Our goal was to assess the qualitative and quantitative indicators of semen in bull sires at different atmospheric pressures.

Technique. Semen from 35 Holstein bull sires aged from 2 to 10 years (breeding herd, OAO Head Center for the reproduction of farm animals, Moscow region, 2012) was collected in an artificial vagina with a doublet mounting with 10-15 min interval. The measured sperm parameters were the volume of ejaculate (ml), the concentration (bn/ml) and the motility (%) of native spermatozoa in the semen, the total number of spermatozoa in the ejaculate (bn), the number and percentage of the discarded semen, the rectilinear motility of the spermatozoa after freezing-thawing and after 5 hours of incubation at 38 °C (1). The main criteria for the semen quality were the number of frozen semen doses per ejaculate. All technological regulations were carried out in accordance with the national technology of freezing and using semen of breeding bull sires [1].

The atmospheric pressure records were obtained from meteorological service http://rp5.ru/Погода_в_Москве_(юг). The dates of the experiment were combined into conditional groups, i.e. with a pressure of < 744.9 mm Hg (1 day), 745.0-754.9 mm Hg (6 days), 755.0-764.9 mm Hg (9 days), 765.0-774.9 mm Hg (7 days), and > 775.0 mm Hg (8 days).

The data was processed in the Microsoft Excel program with confirmation of reliability by the Student's *t*-test. The mean (*M*), minimum and maximum values (min-max) of indicators, mean errors (*m*), and the levels of statistical significance of the differences (P) are presented.

Results. In total, 530 ejaculates were studied during the experiment, 132 of them were rejected as not meeting the technological requirements [1]. The greatest number of ejaculates was obtained at high atmospheric pressure (Table 1). It should be noted that a significant (2.5-3.0-fold, $P < 0.001$) decrease in culled out native semen occurred when atmospheric pressure was the highest, i.e. 755.0-764.9 mm Hg (9 days) and 775.0 mm Hg (8 days).

In young bull sires aged 1.5 to 2.0 years, a decrease in atmospheric pressure caused hypoxia which explains the significant amount (up to 50 %) of culled ejaculates (Table 2). With the increase in atmospheric pressure, the proportion of culled ejaculates lowered significantly.

1. Atmospheric pressure to the dates of the experiment and the rejection of native semen obtained from Holstein bull sires (Moscow region, January 2012)

Atmospheric pressure, mm Hg		Examined ejaculates		
min-max	$M\pm m$	total	culled	
			number	% ($M\pm m$)
< 744.9 (1 day)	744.0	46	13	28.26±4.69**
745.0-754.9 (6 days)	752.00±0.08	28	11	39.29±6.53
755.0-764.9 (9 days)	761.20±0.81**	57	31	54.39±4.66*
765.0-774.9 (7 days)	769.00±0.85**	188	40	21.28±2.11***
> 775.0 (8 days)	784.70±1.77*	213	37	17.37±1.84***

*, ** and *** mean P < 0.1, P < 0.01 and P < 0.001, respectively, as compared to 745.0-754.9 mm Hg

2. The percentage of the primary culled semen of Holstein bull sires of different age depending on the atmospheric pressure on the date of ejaculate collection ($M\pm m$, Moscow region, January 2012)

Age	Atmospheric pressure, mm Hg				
	< 745	745,0-754,5	755,0-764,5	765,0-774,5	> 775,0
1.5-2.0 years	42.31±6.85	50.00±17.68	33.33±4.81	16.87±2.91**	19.81±2.74*
2.5-5.0 years	0	0	35.29±8.20	25.00±12.10	12.90±4.26*
6.0 years and older	16.67±10.76	42.86±9.35	40.74±6.69	23.68±4.88*	0

*, ** means P <0.05 and P <0.01, respectively, between atmospheric pressure gradations.

The bulls aged 2.5 to 5.0 years were less dependent on atmospheric pressure which, in our opinion, is due to adaptation mechanisms. At a relatively low atmospheric pressure (< 755.0 mm Hg), the semen of these bulls was not rejected, but due to the lack of ejaculates, it is difficult to estimate the reliability of the results. In sires aged 6 years and older, with an increase in atmospheric pressure, a significant decrease in the number of culled ejaculates was also noted.

3. Activity (%) of spermatozoa with rectilinear motility in the frozen-thawed semen of Holstein bulls of different age after 5 hours of incubation (at 38 °C) **depending on atmospheric pressure on the date of ejaculate collection** ($M\pm m$,, Moscow Region, January 2012)

Возраст	Atmospheric pressure, mm Hg				
	< 745	745.0-754.5	755.0-764.5	765.0-774.5	> 775.0
1.5-2.0 years	18.5±10.5	Нет данных	17.22±12.20	13.66±6.50	16.81±9.10
2.5-5.0 years	23.33±5.80	18.33±10.40	15.00±7.10	15.91±8.90	14.17±6.40
6.0 years and older	21.67±17.60	21.00±7.40	13.12±11.90	12.00±8.10	16.92±9.50

As per the RF State Standard (GOST 26030-83) for frozen semen of bulls [1)], after thawing all series of cryopreserved sperm that do not meet the requirements are culled, and the cryopreserved sperm with a mobility of 40 % and higher is stored. In our experiment, the motility of the semen immediately after thawing averaged 40.0-43.5 % (regardless of age and atmospheric pressure on the date of collection). Samples that contained less than 40 % of spermatozoa with rectilinear motility after thawing were discarded and their results was not considered. Samples that meet the quality standards were taken for subsequent incubation.

Further, the viability of the thawed semen was studied after 5-hour incubation at 38 °C (Table 3). Quality was maintained in 18.5-23.3 % of spermatozoa ejaculated at low atmospheric pressure, and in 12.0-17.0 % of those obtained at 765.0-774.5 mm Hg. That is, with an increase in atmospheric pressure during ejaculation, the lifespan of cryopreserved spermatozoa during post-thawing incubation reduced.

There was a tendency to increase the loss of semen quality during incubation depending on atmospheric pressure (Table 4). Thus, for the semen obtained under low atmospheric pressure, losses during 5 hours at 38 °C were 44-48 %, reaching 50-54 % for 745.0-754.4 mm Hg, 58-67 % for 755.0-764.5 mm Hg, 61-71 % for 765.0-774.5 mm Hg and 58-65 % for > 775 mm Hg.

4. Loss of quality (%) **in the frozen-thawed semen of Holstein bull sires of different age after 5 hours of incubation** (at 38 °C) **depending on atmospheric pressure on the date of ejaculate collection** (*M*±*m*,, Moscow region, January 2012 of the year)

Age	Atmospheric pressure, mm Hg				
	< 745	745.0-754.5	755.0-764.5	765.0-774.5	> 775.0
1.5-2.0 years	47.47±11.65	Нет данных	57.83±8.23	66.46±6.72	58.12±5.81
2.5-5.0 years	44.01±15.70	54.17±17.62	62.50±10.32	60.94±6.77	64.91±6.38
6.0 years and older	48.00±15.80	50.00±15.81	67.20±10.50	70.73±8.60	57.70±9.69

The greatest volume of ejaculates from bull sires was obtained at normal atmospheric pressure (755.0-764.5 mm Hg). The lowest volumes were at the peak of atmospheric pressure (784.7±1.77 mm Hg) (Table 5). The decrease in volumes was observed above the level of 765.0 mm Hg and did not depend on the age of the sires.

In young bulls at 755.0-764.5 mm Hg the ejaculate volume was 4.21±1.36 ml, and with an increase in atmospheric pressure for every 10 mm Hg this indicator decreased by 15-20 %. In animals of active reproductive age (2.5-5.0 years) at 765.0-774.5 and > 775.0 mm Hg the ejaculate volume decreased by 34.39 and 28.32 %, respectively. In bulls over 6.0 years of age, the reduction was 4 % at 765.0-774.5 mm Hg and 28.9 % at 775.0 mm Hg and higher.

5. Average volume of ejaculate (ml) in **Holstein bull sires of different age depending on atmospheric pressure on the date of semen collection** (*M*±*m*, Moscow region, January 2012)

Age	Atmospheric pressure, mm Hg				
	< 745	745.0-754.5	755.0-764.5	765.0-774.5	> 775.0
1.5-2.0 years	3.85±1.60	Нет данных	4.21±1.36	3.32±1.16	2.82±0.77
2.5-5.0 years	6.33±0.58	4.83±1.61	6.92±2.69	4.54±1.60	4.96±2.60
6.0 years and older	4.67±2.08	6.20±3.19	4.89±1.34	4.69±1.85	3.82±0.88
Averaged	4.45±1.94	6.00±2.46	4.87±1.76	4.14±1.60	3.42±1.51

6. The number of spermatozoa (bn/ml) **in the semen of Holstein bull series of different age depending on the atmospheric pressure on the date of ejaculate collection** (*M*±*m*, Moscow region, January 2012)

Age	Atmospheric pressure, mm Hg				
	< 745	745.0-754.5	755.0-764.5	765.0-774.5	> 775.0
1.5-2.0 years	1.18±0.24	No data	1.15±0.21	1.16±0.33	1.19±0.37
2.5-5.0 years	1.13±0.35	1.23±0.38	1.23±0.31	1.05±0.25	1.24±0.32
6.0 years and older	1.23±0.32	0.98±0.29	1.25±0.22	1.20±0.23	1.12±0.38
Averaged	1.18±0.24	0.94±0.24	1.21±0.23	1.16±0.31	1.20±0.36

The effect of atmospheric pressure on the number of spermatozoa in the semen was not significant (Table 6). However, its increase in bull sires aged 2.5-5.0 and 6.0 years was observed. At 755.0-764.5 mm Hg this was 1.23-1.25 bn/ml being 8-15 % higher than at other atmospheric pressure. As the pressure increased, there was a clear trend towards a decrease in the number of spermatozoa in the ejaculate. The results obtained at low atmospheric pressure, because of the small number of samples, made it impossible to draw an appropriate conclusion.

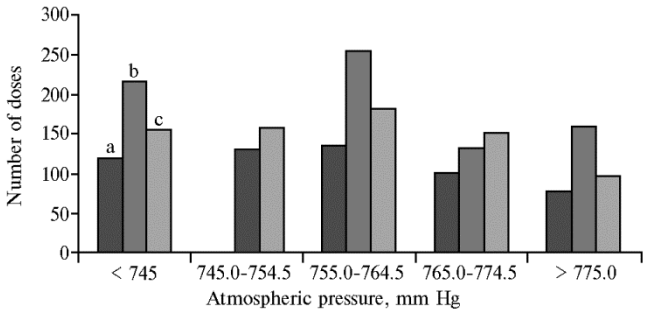
In young bulls still under the age of 2.0 years at 755.0-764.5 mm Hg the maximum number of spermatozoa (4.89 billion) was recorded in one ejaculate (Table 7). With an increase in atmospheric pressure, this indicator decreased by 22.7 % at 765.0-774.5 mm Hg, and by 33.95 % at < 775.0 mm Hg. In bulls aged 2.5-5.0 years, the greatest number of spermatozoa in the ejaculate (7.10 billion) was obtained at 755.0-764.5 mm Hg; this value decreased by 28 % at 769.0 mm Hg, and by 25 % at > 775.0 mm Hg. At low pressure, the reduction was 10-11 %. A similar trend was observed in bulls aged 6-11 years. The index was 6.02 billion at 755.0-764.5 mm Hg and decreased with an increase in atmospheric pressure to 765.0-774.5 and > 775.0 mm Hg by 8.5 and 32 %, respectively.

7. Number of spermatozoa (billion) **in ejaculates of Holstein bull sires of different age depending on atmospheric pressure at the date of semen collection** (*M*±*m*, Moscow region, January 2012)

Age	Atmospheric pressure, mm Hg				
	< 745	745.0-754.5	755.0-764.5	765.0-774.5	> 775.0
1.5-2.0 years	4.49±1.59	Нет данных	4.89±1.87	3.78±1.60	3.23±1.50
2.5-5.0 years	6.32±2.55	6.36±2.79	7.10±2.54	5.12±2.19	5.33±2.51
6.0 years and older	4.70±2.40	5.32±2.31	6.02±1.44	5.51±2.10	4.11±1.33

8. The percentage of ejaculates with sperm count > 5 billion in Holstein bull sires of different age depending on the atmospheric pressure on the date of semen collection (*M*±*m*, Moscow region, January 2012)

Age	Atmospheric pressure, mm Hg			Significance of the differences between the options		
	755.0-764.5 (I)	765.0-774.5 (II)	> 775.0 (III)	**I и II**	**I и III**	**II и III**
1.5-2.0 years	52.63±8.10	27.80±5.28	9.30±3.13	P < 0.05	P < 0.001	P < 0.01
2.5-5.0 years	90.90±6.13	46.15±6.91	54.10±6.66	P < 0.001	P < 0.001	P < 0.001
6.0 years and older	70.00±10.25	64.29±9.05	31.00±9.07	P < 0.05	P < 0.05	P < 0.05



The number of frozen semen doses per ejaculate in Holstein bull sires depending on the atmospheric pressure on the date of sperm collection: a, b, c — age of animals, 1.5-2.0 years, 2.5-5.0 years and over 6.0 years, respectively (Moscow region, January 2012).

When atmospheric pressure increased, the number of ejaculates with a sperm count > 5 billion (Table 8) significantly decreased. This was well traced in young bulls and animals older than 6.0 years. The sires of the active reproductive age (2.5-5.0 years) turned out to be less weather-dependent.

The peak of sperm production in bull sires of all ages fell on the days with atmospheric pressure of 755.0-764.5 mm Hg (Fig.). At its increase to 765.0 mm Hg and higher the output of qualitative doses in all age groups decreased more sharply, i.e. by 43 % for young males, by 37 % for bulls of active reproductive age, and by 45 % for animals aged 6 years and older.

Thus, bull sires, due to their adaptive capabilities, are able to cope with short-term changes in atmospheric pressure. However, with prolonged exposure to exogenous factors, the body adaptation is aimed at self-preservation. As a result, the number of spermatozoa in the ejaculate decreases (e.g., in bulls over the age of 6.0 years this was 6.02 billion at atmospheric pressure 755-765 mm Hg and 4.11 billion at 775 mm Hg), and the volume of the semen decreases by 21-34 %. As a consequence, the yield of qualitative sperm doses per ejaculate decreases by 37-45 % in different age groups. Bulls of active reproductive age (2.5-5.0-year old) are most resistant to changes in atmospheric pressure. Based on the data obtained, it is necessary to correct the schedule for semen donation from bull sires with regard to atmospheric pressure. If during an extended period (8 days or more) the atmospheric pressure is kept above 775 mm Hg, the use of animals should be limited or the schedule of their use should be shifted.

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METABOLISM AND REPRODUCTIVE FUNCTION DURING THE POSTPARTUM PERIOD IN FIRST-CALF COWS WHEN INTRODUCING THE PLACENTA EXTRACT

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Abstract

Most reproductive disorders in dairy cows are associated with postpartum metabolic state. One approach to normalizing the reproductive function is to use biostimulators, which have a modulating effect on the immune, metabolic, and endocrine systems. The aim of the present research was to study the action of the cattle placenta extract on the metabolic adaptation and reproductive system functioning during the postpartum period in first-calf cows. We have conducted for the first time monitoring of indicators of metabolism and the steroidogenic ovarian activity in first-calf cows, treated with the placenta extract, during the transition period and two months after calving and also assessed the reproduction indices. The raw material for the preparation was placenta separated within 4-6 hours after calving. The extract was isolated from placenta according to the procedure, described earlier (M.V. Varenikov et al., 2010), with the use of some modifications. Effects of the extract were evaluated in first-calf cows of the Black Pied breed (*Bos taurus taurus*) on the basis of ZAO PZ «Barybino» (Domodedovo Region, Moscow Province) in 2015-2016. Seven to fourteen days prior to calving and on the day of calving, cows of the group I (control, $n = 8$) received the saline, and cows of the group II (experiment, $n = 11$) received 20 ml of the placenta extract. Before the treatment and on days 3 to 5, 20 to 25 and 50 to 60 after calving, the animal blood was taken for biochemical analysis and enzyme immunoassay. In serum samples, the concentration of total protein and its fractions, urea, bilirubin, cholesterol, calcium, and phosphorus, the activity of aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2), and the content of progesterone and estradiol-17β were measured. Twelve months after calving, based on the analysis of zootechnical and pedigree records, the reproduction indices (the pregnancy rate, calving to conception interval, and service per conception rate) and the 305-day milk yield were determined. A rise ($p < 0,001$) in the blood content of total protein (by 24.9 %) and its globulin fraction (by 51.8 %) was observed on days 20 to 25 after calving only in animals of the group II. The cholesterol concentration in the blood of these cows remained unchanged, whereas it was reduced by 33 % ($p < 0.05$) in control cows from days 3-5 to days 20-25 after calving. During the studied period, an increase in the serum activity of ALT was more pronounced in cows of the group II, while a raise in the activity of AST was considerable only in control animals. On days 20-25, the progesterone concentration in the blood of experimental cows was 57.7 % lower than in the control group ($p < 0.05$). However, 50-60 days after calving, the level of blood progesterone in experimental animals rose sharply and was four times higher than in control ones ($p < 0.01$). The treatment of cows also resulted in a reduction in the calving to conception interval from 123.5±10.5 to 95.1±5.8 days ($p < 0.05$). Thus, the injection of the cattle placenta extract to first-calf cows exerts modulating effects on metabolism, primarily lipid metabolism, as well enhances the luteal activity of ovaries during the postpartum period. Normalization of the metabolic and hormonal status of cows is, obviously, related to a rise in the reproductive ability of the animals and leads to a reduction in the subsequent calving to conception interval.

Keywords: first-calf cows, placenta extract, metabolism, ovarian steroid hormones, reproductive ability

Low reproduction of the herd is one of the main problems of modern dairy cattle breeding both in Russia and abroad [1, 2]. Reduced reproductive

ability of dairy cows is due to prolonged postpartum anestrus, ovarian dysfunction, low fertility, high embryonic mortality, increased incidence of infectious diseases of the uterus because of weakened immunity and other disorders [1, 3]. As a consequence, up to 20 % of farm cows are subjected to culling already in the first lactation, and the calving interval is more than 400 days [4, 5].

The disorders in highly productive dairy cows are mostly associated with the metabolic state after calving [1, 6, 7]. In the early postpartum period, the animals lack the nutrients to maintain lactation. This leads to a negative energy balance which is compensated by mobilizing animal's own resources. In this, due to the limited supply of glucose, the main source of energy is fat depots [8]. The catabolic nature of metabolism during early lactation leads to a change in blood metabolites and metabolic hormones which affect animal fertility [1, 7, 9]. In addition, redirection of the main metabolic pathways for lactation causes a lack of energy and plastic resources to maintain other functions, primarily reproductive. The first-calf cows are in the most difficult situation, as their growth still requires additional resources [10].

To accelerate the restoration of the reproductive function of cows after calving, hormone preparations are widely practiced [11, 12]. An alternative approach is biostimulants with a modulating effect on the immune, metabolic and endocrine systems [13-15]. Such biostimulants can be extracts or tissue preparations of the placenta, which have a therapeutic and preventive effect on inflammation, oxidative and degenerative processes, resorption of exudates and scars, endocrine disorders, growth [16-20]. A homologous extract of the placenta injected before and after lambing stimulates udder development and increases the milk yield in sheep [21]. Data have been obtained on the reduction in the number of post-calving complications and obstetric gynecological diseases in cows after the application of human placenta [22, 23]. In the All-Russian Research Institute of Animal Husbandry a positive effect of cattle placenta extract on reproductive health and the reproductive function of cows has been shown [24, 25]. It was found that the maximum reduction of the open days is achieved after the administration of the placenta extract to animals with a higher milk yield (over 5000 kg), that is, with more pronounced postpartum metabolic disorders. These data suggest that the components of cattle placenta can normalize metabolism in cows post calving and thus modulate their reproductive ability.

In this work, we first studied the metabolism, steroidogenic activity of ovaries and reproduction indices in the first-calf cows after admiration of placenta extract during the transit period and 2 months after calving. placenta extract application led to a higher blood cholesterol level and a shorter open days compared to control animals. In addition, there was a marked increase in serum progesterone concentration 2 months after calving which indicates a faster recovery of the sexual cycle in cows with the placenta extract administered.

Our goal was to study the effect of cattle placenta extract on the metabolic adaptation and reproductive function during postpartum period in the first-calf cows with a milk production of over 6000 kg.

Technique. For extraction we used the afterbirth, separated within 4-6 hours after calving. A pure placenta without visible pathological changes in the tissues was collected from healthy black-and-white Holstein cows of the 2nd and 4th calving housed in OAO Rumyantsevskoye (Nizhny Novgorod region). The extract from the placenta was isolated according to the procedure described earlier [24] with modifications. After microwave exposure, the obtained extract was mixed with distilled water (1:3) and boiled in a microwave oven R-2471J (Sharp Corporation, Thailand) for 5 min. After cooling, the resulting suspension was centrifuged for 20 min at 1000 g in a GR 412 centrifuge (Jouan, France), the

supernatant was passed through a paper filter. For antibacterial treatment, 0.5 % alcohol solution of phenol (0.6 % of the total volume) was added to the filtrate, and the solution was filtered through a sterile filter with a pore size of 0.45 μm.

The cytotoxicity of the finished placenta extract was tested on infantile female laboratory Wistar rats. After subcutaneous injection of 0.5 ml of the extract the rats was observed for 1 week.

The effect of the placenta extract on the metabolism and reproductive function of Black Pied first-calf cows (*Bos taurus taurus*) was investigated at ZAO PZ Barybino (Domodedovo Region, Moscow Province) in 2015-2016. Animals were kept in loafing yard. The ration corresponded to zootechnical norms. All experiments were carried out in accordance with the Helsinki Declaration (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, 1964-2013) and the requirements of good laboratory practice (National Standard of the Russian Federation GOST R 53434-2009). One to two weeks before the proposed calving, according to the principle of analogues, 19 first-calf cows were selected, which were divided into two groups. The animals of group I (control, $n = 8$) were treated with saline. Animals of group II (experiment, $n = 11$) 2 times (7-14 days before the proposed calving and on the date of actual calving) were injected subcutaneously into the neck with 20 ml of the placenta extract.

Blood was taken before (7-14 days prior to calving) and after the introduction of the extract (on days 3-5, 20-25 and 50-60 after the actual calving) from the tail vein with an Apexlab vacuum system (Hebei Xinle Sci & Tech Co., Ltd, China). After receiving the serum, the samples were frozen and stored at –20 °C for subsequent analyses of biochemical parameters and concentration of ovarian steroid hormones.

In blood serum, the concentration of the total protein and its fractions, urea, bilirubin, cholesterol, calcium, phosphorus, the aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) activity were assessed on a ChemWell automatic biochemical analyzer (Awareness Technology, USA) with reagents of Analyticon Biotechnology AG (Germany). The estradiol-17β and progesterone level in serum was determined in enzyme immunoassay test with a Uniplan (Picon, Russia) plate-type spectrophotometer and commercial kit of reagents (NVO Immunotech, Russia) according to the company's instructions. The sensitivity of the test was 30 pmol l for estradiol-17β and 0.4 nmol/l for progesterone. All analyzes were performed in duplicate, the coefficient of variation in the assay did not exceed 15 %.

Twelve months post calving, after analysis of zootechnical data and pedigree records, the reproduction indices (i.e. the proportion of pregnant animals, open days, insemination index) and the yield for 305 days of lactation were determined in both groups.

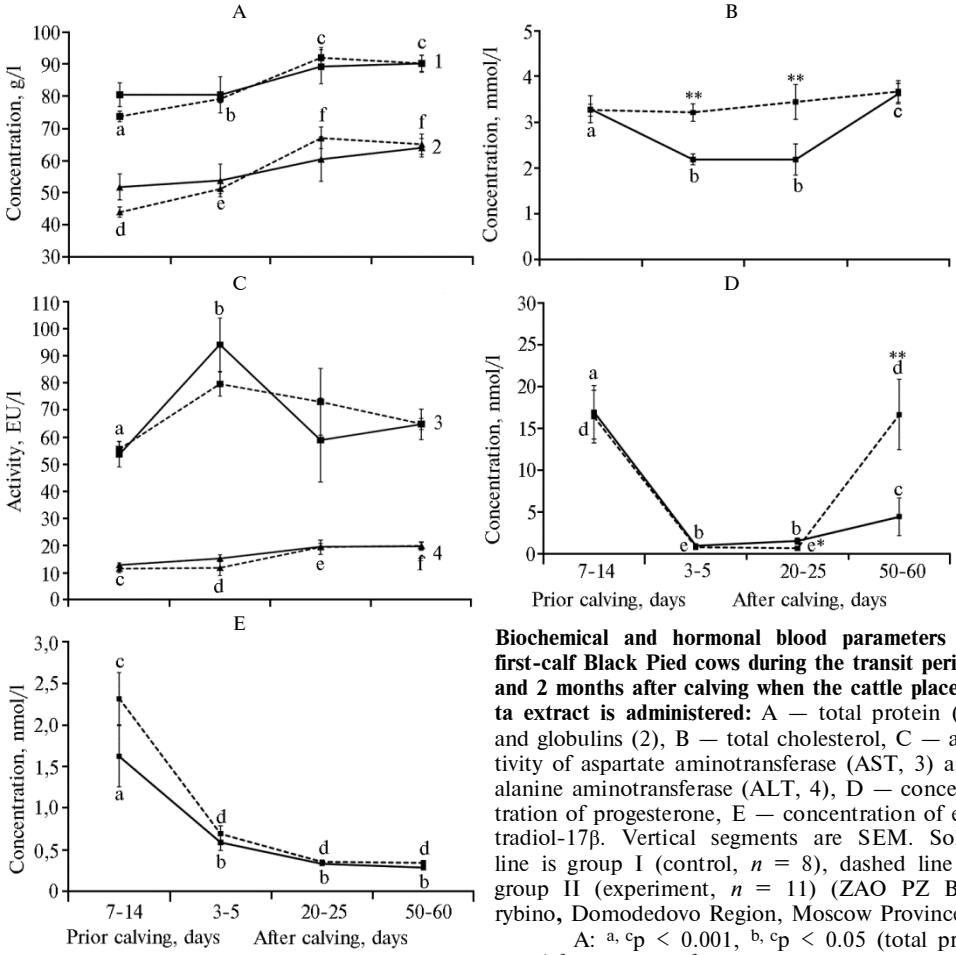
The data was processed using the one-way ANOVA method and the two-way ANOVA variance analysis with SigmaStat software (Systat Software, Inc., USA). The results are presented as mean values (X) and standard error (SEM). The significance of differences of the mean values compared was assessed using Tukey's test.

Results. Subcutaneous injection to rats of 0.5 ml of the placenta extract for 1 week did not cause an inflammation the site of administration. The general condition of the animals was satisfactory.

Before the introduction of the placenta extract to cows (1-2 weeks before calving), the biochemical blood indices and the content of sex steroid hormones in the compared groups did not differ significantly.

According to the widely accepted opinion, a transit period that covers 2-

3 weeks to calving and 3 weeks after calving [26] is critically important to determine the health, dairy productivity and reproductive potential of high-yielding cows. This time is characterized by the most pronounced changes in metabolism, which gradually disappear by the end of the first trimester of lactation.



Biochemical and hormonal blood parameters in first-calf Black Pied cows during the transit period and 2 months after calving when the cattle placenta extract is administered: A — total protein (1) and globulins (2), B — total cholesterol, C — activity of aspartate aminotransferase (AST, 3) and alanine aminotransferase (ALT, 4), D — concentration of progesterone, E — concentration of estradiol-17β. Vertical segments are SEM. Solid line is group I (control, $n = 8$), dashed line is group II (experiment, $n = 11$) (ZAO PZ Barybino, Domodedovo Region, Moscow Province).

A: $^{a, c}p < 0.001$, $^{b, c}p < 0.05$ (total protein); $^{d, f}p < 0.001$, $^{e, f}p < 0.01$ (globulins).

B: $^{a, b}p < 0.05$, $^{b, c}p < 0.01$ (total cholesterol); **$p < 0.01$ (between the control and experimental groups).

C: $^{a, b}p < 0.05$ (AcAT); $^{c, e}p < 0.001$, $^{c, f}p < 0.001$, $^{d, e}p < 0.01$; $^{d, f}p < 0.001$ (АлАТ).

D: $^{a, b}p < 0.001$, $^{a, c}p < 0.01$, $^{d, e}p < 0.001$ (progesterone); *$p < 0.05$, **$p < 0.01$ (between the control and experimental groups). E: $^{a, b}p < 0.001$, $^{c, d}p < 0.001$ (estradiol-17β).

Administration of the placenta extract modified a number of biochemical parameters during the transit period. In the group II between 7-14 days to calving and 20-25 days after calving, total proteins significantly ($p < 0.001$) increased by 24.9 % and globulin fraction increased by 51,8 % which was not observed in the control group (Fig., A).

The blood cholesterol level in control animals decreased by 33 % ($p < 0.05$) to day 3-5 after calving, persisted to days 20-25, and then increased ($p < 0.01$) to the pre-calving values to days 50-60 (see Fig., B). In the animals receiving the preparation, the indicator did not change during the whole period of the study. In the middle and at the end of the transit period, the concentration of blood cholesterol in the cows of the experimental group was 1.5-1.6 times higher than that in the control group ($p < 0.01$).

On days 3-5 after calving, a sharp increase in the activity of AST in the control (by 75.2 %, $p < 0.05$) occurred whereas in the experimental group an

increase was much less pronounced (see Fig., C). The activity of blood ALT in the cows of group II did not change until the middle of the transit period, but increased by 68.8 % ($p < 0.001$) to its end (see Fig., C). In the control, the ALT activity increased insignificantly and gradually. At the same time, we did not detect the effect of the placenta extract on the concentration of albumins, urea, bilirubin, calcium, phosphorus and on their changes in the postpartum period.

The placenta extract also had a long-term effect on hormonal status. In both groups, the progesterone concentration decreased almost 20-fold on days 3-5 after calving and remained low until the end of the transit period (see Fig., D). At the same time on days 20-25 after calving, this index in animals from group II was 57.7 % lower ($p < 0.05$) than in control. On days 50-60 after calving, the progesterone level in the experimental cows increased sharply (26-fold, $p < 0.001$) which indicated the beginning of luteal activity of the ovaries. In control animals, the progesterone concentration increased less significantly, remaining almost 4 times lower than in group II ($p < 0.01$). The estradiol-17β concentration and its decrease in first-calf cows in the postpartum period were similar in animals of both groups (see Fig., E).

Reproduction and milk productivity in Black Pied first-calf cows with subcutaneous administration of cattle placenta extract before and during calving (X±SEM, ZAO PZ Barybino, Domodedovo Region, Moscow Province, 2015-2016)

Parameter	Groups	
	I, control (n = 8)	II (n = 11)
Percentage of pregnant animals	75.0±15.3	90.9±8.7
Open days	123.5±10.5	95.1±5.8*
Insemination index	1.5±0.3	1.5±0.2
Yield of milk within 305 days of lactation, kg	7209±546	7256±251

N o t e. Description of the groups is given in the *Technique* section.
* Differences between the control and experimental groups are statistically significant at $p < 0.05$.

Administration of the placenta extract to animals resulted in a significant reduction in the duration of the open days (by 28.4 days, $p < 0.05$), and slightly increased artificial insemination (by 15.9 %), but did not affect the insemination index (Table). Milk productivity for 305-day lactation in the experimental and control groups was almost the same.

Consequently, the most significant metabolic changes in the first-calf cows caused by cattle placenta extract are associated with lipid metabolism, namely, with maintaining higher concentration of blood cholesterol during the transit period. The cholesterol remained within the limits of normal values for this indicator. A similar tendency towards an increase in cholesterol in the postpartum period was observed in red-and-white multiparous cows after application of extract from human placenta [22]. Previously, it was shown that the blood cholesterol concentration in high-yielding cows in the post-calving period is positively associated with the restoration of sexual circularity and a decrease in open days [27, 28]. These data are consistent with the results obtained by us.

It is known that the mobilization of fat depots in early lactation leads to an increase in the blood concentration of free fatty acids, and the β-oxidation of these fatty acids leads to an excess of acetyl-CoA and an increase in the synthesis of ketone bodies in the liver [7, 8]. Ketone bodies, in turn, adversely influence the reproductive function in cows [29]. Therefore, a positive relationship between the cholesterol concentration and the reproductive ability of cows may be due to a partial use of excess acetyl-CoA for cholesterol production and a corresponding decrease in the synthesis of ketone bodies. Besides, cholesterol is able to reduce the rate of metabolic clearance of progesterone necessary for adequate maintenance of pregnancy [6, 30].

Thus, the administration of cattle placenta extract before and during

calving has a modulating effect on metabolic processes (primarily lipid metabolism) in the first-calves in the postpartum period. The extract also causes an increase in the luteal activity of the ovaries 2 months after calving which indicates stimulation of the animals' transition from the state of postpartum anestrus. Normalization of the metabolic and hormonal status of cows is obviously associated with an increase in the reproductive ability of animals and reduces open days.

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THE EFFECT OF INJECTABLE VITAMIN E AND TRACE MINERALS (SELENIUM, CALCIUM, PHOSPHATE, COPPER, AND COBALT) ON REPRODUCTIVE PERFORMANCE DURING NON-BREEDING SEASON IN AWASSI EWES

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Abstract

Progesterone in combination with pregnant mare serum gonadotropin (PMSG) is widely used to synchronize estrus in ewes. It is also known that various minerals and vitamins are necessary to provide animal reproductive health, and their blood level is reliably associated with reproductive performance. The objective of our research was to determine the effects of supplementation of vitamin E and mineral mixtures during progesterone treatment on reproductive performance of estrus-induced ewes during the non-breeding season. The present study was carried out between May and June, which is the period accepted as non-breeding season for ewes in Sanliurfa province of Southeast Turkey. A total of 148 non-lactating adult ewes of Awassi breeds in good body score condition, ranging in age from 2 to 4 years and weighting between 45 and 60 kg were used in this study. Estrus was synchronized in all ewes using the intravaginal sponges containing 20 mg fluorogestone acetate. The intravaginal sponges were inserted into vagina of each ewe for 14 days. At the time of sponge withdrawal (day 0), all ewes were injected intramuscularly with 10 IU/kg of PMSG for stimulation of estrus and ovulation. Treatment group (n = 74) received orally supplementation of vitamin E and mineral mixtures (Bakosel® capsule; Ceva Dif, Istanbul, Turkey; a capsule contains 500 IU vitamin E acetate, 2.5 mg sodium selenite, 150 mg dicalcium phosphate, 10 mg copper sulphate, 12.5 mg cobalt sulphate) at the four times a week apart during progestagen treatment and at the time mated. The remaining ewes (n = 74) served as control group, and the each female in this group also received only placebo instead of vitamin and mineral supplementation. They were tested for pregnancy detection on day 40 after mating using real time ultrasonography with 5-7.5 MHz linear array rectal transducer. The estrus response was 87.8 % and 82.4 % for the treatment and control groups, respectively. The overall pregnancy rate was 71.4 % (90/126). The gestation length was similar between groups and it averaged 149.4±0.3 days. The lambing rate was 75.4 % (49/65) and 65.6 % (40/61) for the treatment and control groups, respectively. The number of multiple births (42.9 % versus 20.0 %) and prolificacy rate were higher ($P < 0.05$) for the treatment group than those of control group. It is concluded that vitamin E and the multi-trace minerals/bolus given to Awassi ewes 14 days before mating significantly increased the proficiency rate, the multiple births rates and led to stronger manifested estrus behaviors.

Keywords: ewes, progesterone, pregnant mare serum gonadotropin, PMSG, vitamin E, trace minerals, reproductive performance

The progesterone-PMSG based estrus synchronization protocols have been widely used in ewes, in especially, during non-breeding season [1-3]. The ultimate aims of any estrous synchronization method are to reduce the time used for estrous detection and, to provide acceptable pregnancy rates and high proficiency rate [4]. However, the long-term application of progesterone treatment has been associated with a lesser fertility [5].

In ewes, the ovarian follicle population is known to be rather sensitive to

dietary intake, and it is reported that both folliculogenesis and ovulation can be increased through nutritional manipulations [6]. In the other hand, various minerals and vitamins are essential for reproductive health of animals.

There is a significant relationship between the plasma levels of these substances and reproductive performance. In especially, the deficiencies of some trace elements such as vitamin copper, cobalt and selenium, suppress the expression of estrus behaviors, reduces ovulatory, induce embryonic loss and fetal death [7, 8]. Zinc, copper and manganese levels are higher in the conceptus, when compared to other reproductive tissues, which shows that the conceptus accumulates these minerals for its development, growth and vitality [7]. Vitamin E plays an important role in the management of oxidative stress. Oxidative stress adversely affects both ovarian activity and follicular development [9].

The objective was to determine the effects of supplementation of vitamin E and mineral mixtures during progesterone treatment on reproductive performance of estrus-induced ewes during in the non-breeding season.

Technique. The present study was carried out between May and June, which is the period accepted as non-breeding season for ewes in Sanliurfa province of Southeast Turkey located at latitude of 37 10′N, at longitude of 39 03′E and at altitude of 518 m above sea level.

A total of 148 non-lactating adult ewes of Awassi breeds in good body score condition, ranging in age from 2 to 4 years and weighting between 45 and 60 kg were used in this study. In addition, twelve Awassi breeding rams of proved fertility were used in the present study. All females were isolated from rams at least 4 weeks until the start of the experiment period. They were housed together in a commercial farm in Sanliurfa province. They were allowed to mixed graze at pasture of the farm throughout the day, and were kept indoors at night. When the ewes were kept indoors, they were given a diet composed of 0.5 kg of concentrate and 0.5 kg of alfalfa hay per animal per day during the experimental period. The fresh drinking water was available ad libitum. All procedures were conducted in compliance with National Animal Care and Use Committee guidelines.

At the beginning of the study, the females were divided at random into two equal groups according to age and body weight. Hormonal treatments for induction of estrus were the same in both groups. Estrus was synchronized in all ewes using the intravaginal sponges containing 20 mg fluorogestone acetate (Chrono-gest®; Intervet, Istanbul, Turkey). The intravaginal sponges were inserted into vagina of each ewe for 14 days. At the time of sponge withdrawal (day 0), all ewes were injected intramuscularly with 10 IU/kg of PMSG for stimulation of estrus and ovulation. Treatment group (n = 74) received orally supplementation of vitamin and mineral mixtures (Bakosel® capsule; Ceva Dif, Istanbul, Turkey; a capsule contains 500 IU vitamin E acetate, 2,5 mg sodium selenite, 150 mg dicalcium phosphate, 10 mg copper sulphate, 12.5 mg cobalt sulphate) at the four times a week apart during progestagen treatment and at the time mated. The remaining ewes (n = 74) served as control group and, the each female in this group also received only placebo instead of vitamin and mineral supplementation.

Following the termination of progesterone treatment, all ewes were checked for behavioral signs of estrus for 30 min at 8 h intervals (06:00 h, 14:00 h and 22:00 h) for a period of 96 h by visual observation using six rams equipped with an abdominal apron to avoid penetration. The onset of estrus was considered as the time the first permission to be mounted, and the interval from sponge removal to onset of estrus was recorded. Estrous duration was defined as the time elapsed between the first and last accepted mount within the same estrous

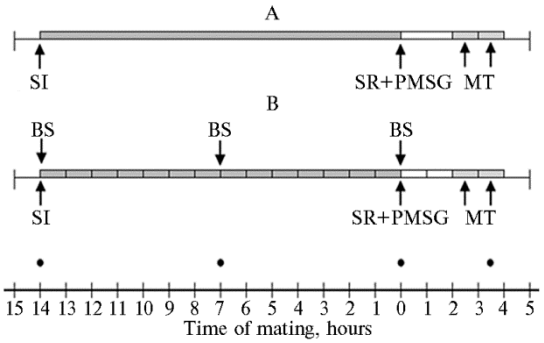
period. The intensity of estrus was recorded as attractive ewes that had very strong behavioral signs or receptive ewes that had normal behavioral signs regarding to visibility, intensity and frequency of occurrence of the expression of estrus behaviors as searching for the male and remain very close to it, restlessness, bleating, frequent urination, vaginal discharge, hyperemia and oedema of vulva.

All females in estrus were hand-mated twice at 8 and 16 h after onset of estrus by a ram with known fertility. They were tested for pregnancy detection on day 40 after mating using real time ultrasonography (Falco Vet, Pie Data Medical, Maastricht, and the Netherlands) with 5-7.5 MHz linear array rectal transducer. They were confirmed by the occurrence of parturition.

The following reproductive parameters were recorded; estrous response (number of ewes in estrus/number of treated ewes × 100), interval to estrus (the time elapsed from sponge removal to onset of estrus), estrous duration (the time elapsed from first to last mounting acceptances), estrous intensity (attractive or receptive), pregnancy rate (number of pregnant ewes/number of mated ewes × 100), gestation length (the time elapsed from breeding to lambing), lambing rate (number of ewes lambing/number of mated ewes × 100) and prolificacy rate (number of lambs born alive/number of ewes lambing).

Data are presented as mean±SEM. The differences in the average interval from sponge removal to onset of estrus, estrous duration, gestation length and prolificacy rate were compared between control and treatment groups using the Student's *t*-test. The proportions of ewe in estrus, conception rate and kidding rate were analyzed using Chi-square test. Differences were considered significant at a level of $P < 0.05$. The SPSS/PC program (Version 10.0; SPSS, USA) was used for all analyses.

Results. Design of the experiment is visualized in the scheme (Fig.).



Schematic presentation of the experimental design: SI — sponge insertion, SR — sponge removal, MT — mating, BS and • — supplementation of vitamin-mineral mixtures, PMSG — injection of PMSG.

The reproductive responses in ewes synchronized for estrus using intravaginal sponges/PMSG and received supplementation of vitamin E and mineral mixtures (treatment group) or placebo (control group) during non-breeding season are shown in Table 1.

The reproductive responses in ewes synchronized for estrus using intravaginal sponges/PMSG and received supplementation of vitamin E and mineral mixtures (treatment group) or placebo (control group) during non-breeding season are shown in the Table. The overall proportion of ewes exhibiting clinical signs of estrus was 85.1 % (126/148). Nine ewes from treatment group and thirteen ewes from control group did not show any overt signs of estrus during the observation period. There was no significant difference ($P > 0.05$) in estrous response between the two treatments.

The average time elapsed from sponges removal to onset of estrus were 43.2±1.1 h and 45.3±1.0 h for the treatment and control groups, respectively. The mean estrous duration was 30.9±0.7 h for treatment animals and 27.3±0.6 h for control animals. No significant difference was observed between groups regarding the interval to estrus and estrous duration. However, the intensity of be-

havioral signs of estrus was remarkably stronger ($P < 0.05$) for treatment group than those of the control group.

The overall pregnancy rate was 71.4 % (90/126). The gestation length was similar between groups and it averaged 149.4±0.3 day. The lambing rate was 75.4 % (49/65) and 65.6 % (40/61) for the treatment and control groups, respectively. The prolificacy rate was 1.45±0.08 (71/49) and 1.23±0.08 (49/40) for the treatment and control group, respectively. No statistically significant differences were observed between groups in terms of conception rate and lambing rate. However, the number of multiple births (42.9 % versus 20.0 %) and prolificacy rate were higher ($P < 0.05$) for the treatment group than those of control group.

The reproductive responses (mean±SEM) **in ewes synchronized for estrus using intravaginal sponges/PMSG and received supplementation of vitamin E and mineral mixtures** (treatment group) **or placebo** (control group) **during non-breeding season**

Reproductive parameters	Control	Treatment	Total
Estrus response, %	82.4 % (61/74)[a]	87.8 % (65/74)[a]	85.1 % (126/148)
Interval to estrus, hours	45.3±1.0[a]	43.2±1.1[a]	44.2±0.7
Estrous duration, hours	27.3±0.6[a]	30.9±0.7[a]	29.1±0.5
Estrous intensity			
attractive	52.5 % (32/61)[a]	73.8 % (48/65)[b]	63.5 % (80/126)
receptive	47.5 % (29/61)[a]	26.2% (17/65)[b]	36.5 % (46/126)
Number of ewes mated	61	65	126
Pregnancy rate	67.2 % (41/61)[a]	75.4 % (49/65)[a]	71.4 % (90/126)
Lambing rate	65.6 % (40/61)[a]	75.4 % (49/65)[a]	70.6 % (89/126)
Gestation period, days	148.9±0.4[a]	149.7±0.3[a]	149.4±0.3
The number of kids	49	71	120
including			
singleton	32 (32)	28 (28)	60 (60)
twin	7 (14)	20 (40)	27 (54)
triplet	1 (3)	1 (3)	2 (6)
Multiple birth rates, %	20.0 % (8/40)[a]	42.9 % (21/49)[b]	32.6 % (29/89)
Proficiency rate	1.23±0.08 (49/40)[a]	1.45±0.08 (71/49)[b]	1.35±0.06 (120/89)

Note. The values within a same line with different superscripts (a, b) are significantly different ($P < 0.05$).

This study demonstrated the effects of vitamin E and multi-trace element (selenium, calcium, phosphate, copper and cobalt) injection on the reproductive performance of seasonally anoestrous Awassi sheep, which were treated with a progesterone- and PMSG-based estrus synchronization protocol.

In this study, 87.8 % of ewes in the treatment group and 82.4 % of ewes in the control group were detected in estrus during the observation period. The mean estrous response was 85.1 %. These results are similar to the findings of other researchers [10, 11]. The ovarian sensitivity to hormonal manipulation may be different individually during anoestrous period.

An important requirement for successful estrous synchronization is uniformity of the time elapsed from the end of treatment to the onset of estrus. The overall average interval elapsed from sponge removal to onset of estrus was 44.2±0.7 in this study (see Table). The interval obtained in this trial was comparable to the findings of M. Ali [12] and I. Dogan et al. [13], and was longer than the 38.8±1.6 h reported by R. Ungerfeld et al. [14], but shorten than the 69.0±9.9 h reported by A. Ali [15]. It may be affected many exogenous factors such as nutrition, daylight, environmental factors, presence of male after sponge removal.

In our study, the estrous duration was similar between treatment and control groups, the mean estrous duration was 30.9±0.7 h for treatment and 27.3±0.6 h for untreatment groups and the overall estrous duration was 29.1±1.15 h in this study. M. Hashemi et al. [10] reported the ranges of duration of estrus period from 22 to 31 h using different progesterone treatments outside the natural breeding season in Karakul ewes. N. Ozyurtlu et al. [16], recorded as 29-30 h duration for induced estrous with various progestagens non-breeding

season. These differences might be due to breed, nutrition, stress, location, and present of male. It was observed that the Vitamin E and multi-trace minerals treatment did not showed any significant advantage with respect to the estrous response, the average elapsed time from sponge removal to onset of estrus and the estrous duration in this study. This may be explained that almost all of the ewes had normal ovarian cyclic activity in breeding season.

M. Hidiroglou [17] reported that trace elements have a major role in reproductive performance and fertility parameters in sheep. It has been ascertained that the administration of multi-trace element and vitamin ruminal boluses to ewes, in advance of mating, significantly increased both the lambing and twin-lambing rates [18, 19]. On the other hand [20], Se and vitamin E supplementation did not increase the reproductive or rearing performance of 2- and 3-year old ewes. Selenium is particularly significant for superovulation in cows as well as for sperm quality and multiple lambing in sheep, as it has a major role in sperm transport and the formation of ova [21]. The findings obtained in this study are similar to those previously reported [18] and demonstrate an increase in the multiple lambing rate of ewes supplemented with vitamin E and minerals in advance of mating.

Cobalt is a trace element required for the synthesis of vitamin B_{12}. It has been determined that sheep with cobalt deficiency show higher rates of stillbirth and neonatal death and lower lambing rates [22]. Cobalt deficiency is also known to be associated with multiple ovulation, reduced conception rates and a decrease in the manifestation of clinical oestrus signs [17]. In this study, it was observed that the ewes included in the treatment group, which received vitamin E and minerals, displayed the clinical signs of oestrus more strongly, attracted a greater number of rams and did not try to escape the rams during mounting. It was observed that these ewes remained still and allowed the rams to mount. On the other hand, the ewes, which had not received vitamin E and trace elements, displayed irregular estrus are weaker compared to other ewes.

It is concluded that vitamin E the multi-trace minerals/bolus given to Awassi ewe before14 days mating significantly increased the proficiency rate, the multiple births rates and stronger manifesto estrus behaviors.

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PATHOPHYSIOLOGICAL ASPECTS OF EMBRYONIC MORTALITY IN DAIRY COWS

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Abstract

Early embryonic mortality and its high frequency in lactating cows are among the causes for a decrease of animal performance, reproduction, and the effectiveness of modern dairy cattle industry as a whole. The aim of this research was to reveal pathogenic significance of maternal endocrine, metabolic and immune risk factors for occurrence of this pathology. The surveys involved black-motley cows with average annual productivity of 6.4-7.6 ths. kg. Pregnancy and embryonic mortality were diagnosed on days 19 to 23, 28 to 32, and 38 to 45 after artificial insemination by transrectal ultrasound examination with the use of an ultrasonic scanner Easi-Scan-3 (Great Britain). Venous blood samples were collected during the same periods. Blood progesterone, estradiol-17β, testosterone, proteins, total immunoglobulins, circulating immune complexes, urea, creatinine, cholesterol, glucose, vitamins E and C, total calcium, inorganic phosphorus, protein-bound iodine, magnesium, zinc, copper, manganese, selenium, middle molecular peptides, malonic dialdehyde, alkaline phosphatase activity, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, catalase, bactericidal and lysozyme activity of blood serum, morphological blood composition were assessed with the use of a biochemical analyzer Hitahi-902 (Japan), a spectrophotometer UV 1700 (Japan), an atomic adsorptive spectrometer Perkin Elmer-703 (USA), a hematology analyzer ABX Micros 60 (France), and spectrum analyzer Uniplan (Russia). The blood parameters were tested in 18 animals, 9 ones with physiological embryo formation (control) and 9 ones with embryo death. It was found out that embryonic death is firstly associated with an endocrine insufficiency of sex glands of the mother cows, as reflected by blood progesterone and estradiol-17β level which at various stages of gestation was lower by 12.0-43.3 % and 45.0-85.5 %, respectively, when compared to the animals of control group. Under embryonic death, the mother cows' metabolic profile was characterized by an increase in blood concentration of protein (by 3.2-5.4 %), urea (by 9.8-23.6 %), creatinine (by 10.1-13.5 %), cholesterol (by 10.9-17,1 %), middle molecular peptides (by 6.1-34.7 %). Blood alkaline phosphatase activity was higher by 12.8-36.2 %, alanine aminotransferase — by 3.6-13.2 %, aspartate aminotransferase — by 13.8-30.8 %, gamma-glutamyl transferase — by 45.4-77.5 %, and endogenous intoxication index increased by 13.0-40.0 % that was a reflection of liver and kidney insufficiency, cholestasis syndrome manifestation and endogenous intoxication. The pathology was related to the deficiency of essential bioelements, increased lipid peroxidation (LPO), decreased function of antioxidant protection (AOP) system, accumulation of LPO toxic products and oxidative stress development. Under embryo death, blood zinc concentration was 9.7-27.2 % less, copper concentration — 17.6-23.3 % less, manganese — 10.8-15.2 % less, selenium — 16.3-29.1 % less, protein-bound iodine amount — 7.3-33.4 % less, magnesium — 9.7-27.4 % less, glutathione peroxidase activity — 25.8-31.2 % lower, catalase — 26.5-51.2 % lower, vitamin E — 26.3-31.6 % lower, and vitamin C — 25.1-57.1 % lower, as compared to the control animals. The changes in immune status of mother cows with embryonic mortality manifested themselves by an increase in the number of blood leukocytes, their neutrophilic and eosinophilic forms, monocytes, by a decrease in phagocytic activity, the number of lymphocytes, immunoglobulins, bactericidal activity and lysozyme activity in blood serum, and also by vaginal dysbiosis. The conclusion is that diselementosis, oxidative stress and endogenous intoxication, endocrine and immune insufficiency are determinant pathophysiological factors in multiple-factor etiology of early embryonic mortality.

Keywords: cows, embryonic mortality, diselementosis, oxidative stress, endogenous intoxi-

cation, endocrine and immune insufficiency

In lactating cows, fetal mortality becomes one of the urgent problems of modern dairy cattle breeding. The frequency of this pathology varies from 20 to 45 % [1-5] and rises with the increase in milk productivity [6-8] accompanied by a profound rearrangement of endocrine, metabolic and immune homeostasis. According to some papers [6, 7], it can be 48-55 %. S.V. Belik (8) reported early embryo loss in 33 %, 43 % and 67 % cows with a milk yield up to 6 ths. kg, 6-7 ths. kg, and over 7 ths. kg, respectively.

Over the past decades, the dairy productivity in cows increased from 4-5 to 8-11 ths. kg, while their fertility in the first insemination, clinically diagnosed after 50-60 days, decreased from 60-65 to 32-35 % [9-12]. The increase in embryonic death leads to a decrease in fertility, the rate of reproduction and total profitability of dairy farming. Therefore, study pathogenetic significance of the maternal risk factors of this pathology has both scientific and practical importance.

In this work, for the first time we carried out a complex assessment of hormonal, metabolic and immune indicators in lactating cows at early gestation during normal embryo development and its death. It is shown that diselementosises, oxidative stress, endogenous intoxication, endocrine immune deficiency are of pathogenetic significance for embryonic development.

The aim of the work is to identify the role of endocrine, metabolic and immune disorders in lactating cows in embryonic mortality at early gestation.

Technique. Experiments were carried out in the winter 2014 at OOO SP Vyaznovatovka (Nizhnedevitsky Region, Voronezh Province) on black-and-white cows (a total of 32 lactating cows) with an average annual milk productivity of 6.4-7.6 ths kg at tethered farming technology. Animal diet included corn silage (25 kg), sainfoin hay (3 kg), straw barley (3 kg), grain mixture (7 kg), sunflower meal (1 kg). The daily intake of food was 17.6 kg dry matter (1 kg contained 5.5 g of calcium, 4.0 g of phosphorus, 2.3 g of magnesium, 7.2 mg of copper, 33.0 mg of zinc, 34, 5 mg of manganese, 0.18 mg of selenium, 0.37 mg of iodine).

Pregnancy and embryonic loss was detected on days 19-23, 28-32, and 38-45 after artificial insemination by transrectal echographic examination with an ultrasound scanner Easi-Scan-3 (BCF Technologi, Great Britain) with linear sensor 4, 5-8.5 MHz [13]. In the same gestation period, venous blood from the cows with physiological embryo formation (n = 9, control) and those with recorded embryonic death (n = 9) was collected for laboratory tests.

The concentration of sex hormones (progesterone, estradiol-17β, testosterone) was determined in the blood serum by solid-phase enzyme immunoassay using commercial test systems (OOO Hema-Medica, Russia) and an enzyme immunoassay analyzer Uni-plan AIFR-1 (ZAO Pikon, Russia) in accordance with the manufacturers' instructions.

Serum and whole blood levels of proteins, total immunoglobulins, urea, creatinine, cholesterol, glucose, vitamins E and C, total calcium, inorganic phosphorus, protein-bound iodine (PBI), middle molecule peptides (MMP), malonic dialdehyde (MDA), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), glutathione peroxidase (GPO), catalase were assessed by standard methods for studying metabolic parameters [14] on a biochemical analyzer Hitahi-902 (Roche Diagnostics GmbH, Germany-Japan) and spectrophotometer UV 1700 (Shimadzu Corp., Japan); zinc, copper, manganese, selenium, magnesium was estimated using an atomic adsorption spectrometer Perkin Elmer-703 (PerkinElmer, USA), blood morphology was studied on a hemoanalyzer ABX Micros 60 (ABX Diagnostics, France). Blood bactericidal (BA) and lysozyme activity (LA), circulating immune complexes (CIC) were determined according to guidelines for

assessing and correcting immune status in animals [15]. In cervical-vaginal mucus, general bacterial contamination, the titer of entero-, lacto- and bifidobacteria, and fungal infection were determined by common microbiological methods.

For processing the Statistica 8.0 software (StatSoft Inc., USA) was used. The data are presented as mean values (X) and the error (x). Differences were considered statistically significant at $p < 0.05$.

Results. The embryo death was recorded in cows with functional insufficiency of the gonads (Table 1). Thus, in cows that lost embryos at different stages of gestation the blood progesterone level was 12.0-43.3 % lower than in the control. In mother-cows this indicator, as compared to the initial values, increased by 28.8 % in normal gestation and decreased by 18.0 % at embryonic loss.

1. Concentration of blood sex hormones in black-and-white cows in normal gestation and at embryonic loss ($X \pm x$, OOO SP Vyaznovatovka, Nizhnedevitsky Region, Voronezh Province, 2014)

Hormone	Gestation, days		
	19-23	28-32	38-45
Progesterone, nmol/l	31.6±3.61/27.8±2.84	40.7±6.17/24.2±2.81*	40.2±5.06/22.8±3.33**
Testosterone, nmol/l	1.63±0.21/1.78±0.37	2.08±0.28/1.96±0.40	2.86±0.56/1.15±0.17*
Estradiol-17β, pmol/l	146.2±26.2/78.8±22.6	117.3±10.5/80.9±5.5*	142.1±14.6/40.4±7.0**

N o t e. Before and after the slash there are physiological formation and death of the embryo, respectively.
*, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ compared to control (physiological formation).

Developing hypoprogesteronemia entails not only the inferiority of the secretory transformation of the endometrium and the supply of nutrients to the developing embryo, but also an increase in the aggressiveness of peripheral mononuclear cells (lymphocytes, monocytes) and endometrial macrophages against embryonic tissues through Ig synthesis, oxygen explosion in macrophages and stimulation of the production of anti-inflammatory cytokines [15-20].

2. Blood biochemical indicators in black-and-white cows in normal gestation and at embryonic loss ($X \pm x$, OOO SP Vyaznovatovka, Nizhnedevitsky Region, Voronezh Province, 2014)

Parameter	Gestation, days		
	19-23	28-32	38-45
Total proteins, g/l	81.1±1.1/83.9±1.8	80.0±1.4/82.6±1.4	79.8±2.9/84.1±1.9
Urea, mmol/l	3.14±0.16/3.88±0.27	3.21±0.31/3.56±0.06	3.41±0.25/3.54±0.34
Creatinine, μmol/l	78.4±2.9/86.3±3.4	78.7±2.7/89.3±6.8	84.8±5.3/85.4±5.3
Cholesterol, mmol/l	6.15±0.16/7.20±0.39*	5.87±0.18/6.59±0.19*	5.99±0.27/6.75±0.29
Glucose, mmol/l	2.75±0.06/2.55±0.04	2.39±0.06/2.36±0.04	2.87±0.09/2.91±0.22
Alkaline phosphatase, U/l	60.6±5.2/82.6±6.5*	63.5±4.7/71.6±6.2	67.2±5.2/103.4±7.4**
Alanine aminotransferase, U/l	29.4±2.4/31.5±1.9	30.8±1.4/31.9±2.5	34.1±2.9/38.6±2.8
Aspartate aminotransferase, U/l	64.4±4.8/64.7±3.9	58.7±4.0/76.8±7.6	62.9±3.9/71.6±4.4
γ--Glutamyltransferase, U/l	22.5±1.8/31.6±2.9*	20.9±1.2/37.1±5.8*	18.5±0.8/26.9±1.8***
Middle molecule peptides, relative units	0.46±0.03/0.57±0.03*	0.49±0.03/0.52±0.04	0.49±0.05/0.66±0.05*
Index of endogenous intoxication, relative units	19.1±0.9/24.6±0.04***	18.4±1.2/20.8±0.9	16.6±0.7/22.9±1.2***
General calcium, mmol/l	2.76±0.09/2.56±0.09	2.84±0.09/2.60±0.10	2.80±0.08/2.48±0.11
Phosphorus inorganic, mmol/l	1.77±0.11/1.93±0.07	1.92±0.07/1.74±0.11	1.90±0.09/1.84±0.13
Magnesium, mmol/L	1.26±0.01/1.02±0.03***	1.36±0.03/0.99±0.02***	1.13±0.05/1.02±0.03
Zinc, mmol/l	52.7±2.51/34.0±3.24***	40.8±2.83/31.2±3.03***	41.2±1.91/31.7±2.42**
Copper, μmol/l	17.5±0.84/13.9±0.33**	18.7±0.71/15.4±1.44*	15.9±0.72/12.2±0.96*
Manganese, μmol/l	2.64±0.11/2.24±0.14	3.43±0.07/3.06±0.18	2.50±0.07/2.58±0.14
Selenium, μmol/l	1.27±0.08/1.00±0.11*	1.41±0.13/1.00±0.08*	1.23±0.10/1.03±0.09
Protein-bound iodine, μg%	4.10±0.17/3.80±0.24	4.58±0.14/3.05±0.15**	4.25±0.22/3.65±0.13*

N o t e. Before and after the slash there are physiological formation and death of the embryo, respectively.
*, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ compared to control (physiological formation).

The low concentration of estradiol-17β and testosterone responsible for protein synthesis in the embryo and proliferation in the uterus tissues in cows (see Table 1), also indicated a disorder in the hormone-synthesizing function of ovaries in the cows with embryonic death. In the first month of gestation, the

difference with control animals for estradiol-17β level was 45.0-85.5 %, reaching 352.0 % at embryo death. When embryos developed, the blood testosterone concentration gradually increased from 1.63±0.21 to 2.86±0.56 nmol/l, or by 75.4 % ($p < 0.001$). In case of embryonic loss, the testosterone level in the first month of gestation did not differ significantly from those in healthy animals but lowered 2.5 times by the time of the embryo death.

In assessing the metabolic status of cows, it was found that genetically programmed formation and development of the embryo in the first 1.5 months of gestation occurred without pronounced changes in blood level of total proteins, cholesterol, glucose, calcium, and AST activity (Table 2). However, there was a gradual increase in urea concentration by 8.6 %, creatinine by 8.2 %, phosphorus by 7.3 %, middle molecule peptides by 6.5 %, AP activity by 10.9 % ALT activity by 16.0 %, whereas GGT activity and endogenous intoxication index were 17.8 % and 13.1 % lower. These changes reflected the intensification of metabolic processes in dam cows already at the early stages of the embryo's placentation.

When the embryo died, we recorded an increase in blood protein concentration by 3.2-5.4 %, in urea by 9.8-23.6 %, in creatinine by 10.1-13.5 %, in cholesterol by 10.9-17.1 %, in middle molecule peptides by 6.1-34.7%, in alkaline phosphatase activity by 12.8-36.3 %, in ALT by 3.6-13.2 %, in AST by 13.8-30.8 %, in GGT by 45.4-77.5 %, and in the index of endogenous intoxication by 13.0-40.0 % as compared to the control. Together, these indicators reflected functional disorders in the liver and kidneys of cows with embryonic loss manifested by syndromes of cholestasis and endogenous intoxication.

Increased endogenous intoxication in the animals with embryo death was associated with a deficiency of pro-oxidant bioelements and a decreased activity of the antioxidant defense system (see Tables 2, 3). In these cows, the blood concentrations of zinc, copper, manganese, selenium and protein-bound iodine was below the control by 9.7-27.2 %, 17.6-23.3 %, 10.8-15.2 %, 16.3-29.1 %, and 7.3-33.4 %, respectively.

Zinc is essential in DNA synthesis and repair, growth, reproduction, cell differentiation and migration, embryogenesis and immunogenesis [21-25]. Its biological properties are associated with induction of zinc-copper-dependent superoxide dismutase, protection of DNA and transcription proteins from free radicals, inhibition of proteinases, neutralization of bacterial lipopolysaccharides and toxic metals. With a deficiency of zinc, the secretion of sex and corticosteroid hormones decreases, cytokine expression and inflammation in the uterus increase, cell proliferation and growth of the embryo are suppressed. Copper determines the activity of copper-zinc-dependent superoxide dismutase, is a part of ceruloplasmin and acts as an antioxidant, protecting cellular structures from oxidative stress. With its deficiency, free radical oxidation is activated, and the hormone-producing function of the hypothalamus, pituitary gland and gonads is reduced, which leads to an increase in death and resorption of embryos [23, 24].

Deficiency of selenium and iodine as constituent components of biologically active compounds (glutathione peroxidase, iodothyronine deiodinase, thyroid hormones) is accompanied by an increased lipid peroxidation, general metabolic disorder, lowered activity of the pituitary-gonad system, cell growth and tissue differentiation, hormonogenesis and immunogenesis [26-31].

There were no significant differences in manganese blood concentration between the groups of animals, but we do not exclude the role of the deficiency of this microelement in embryonic mortality reported in some papers [32, 33].

The magnesium deficiency in the body also led to early embryonic death. When the embryo died, magnesium concentration in the blood of cows was 9.7-27.9 % below control. Apparently, the lack of magnesium reduces the

energy potential of proliferating embryonic cells, metabolic activity and detoxification, enhances the synthesis of prostaglandins E_2 and F_2, causing endotheliosis and a decrease in the progesterone production in ovarium yellow body [34, 35].

A decrease in calcium blood level (by 7.2-11.4 % compared to the control) was characteristic of the cows with embryonic loss. Clear evidence of calcium specific effect on embryogenesis is lacking. Perhaps, calcium deficiency is manifested through the effect on the exchange of essential trace elements.

In clinical experiments [36, 37] it was shown that parenteral injections of zinc, copper, selenium and manganese salts contribute to 9-13 % higher survival of embryos. Biologically necessary accumulation of bioelements (as well as other nutrients) in the embryo is completely determined by the metabolic status of the maternal organism [32]. It can be argued that the deficiency and imbalance in the bioelement status of lactating cows with embryonic loss is associated with higher milk productivity and increased excretion of trace elements from the body with milk. The daily milk yield was 20.2±1.21 kg in cows with physiological development of the embryo and 24.6±1.03 kg in the cows with manifestation of embryopathies, which was higher by 21.8 % ($p < 0.05$).

Deficiency of essential microelements was accompanied by a weakening of antioxidant protection and increased activity of lipid peroxidation (LPO) (Table 3). A certain increase in free radical lipid oxidation, as it was evidenced by 6.4-8.9 % elevation in blood MDA concentration, was characteristic of cows with normal embryogenesis.

3. Functional state of antioxidant protection and lipid peroxidation in black-and-white cows in normal gestation and at embryonic loss ($X \pm x$, OOO SP Vyaznovatovka, Nizhnedevitsky Region, Voronezh Province, 2014)

Parameter	Gestation, days		
	19-23	28-32	38-45
MDA, μmol/l	1.24±0.11/1.79±0.14**	1.35±0.10/2.14±0.19**	1.32±0.10/1.83±0.11
Catalase, μmol H_2O_2/l · min	29.7±0.9/21.6±0.8***	34.6±0.5/16.9±0.4***	31.4±0.5/20.9±0.8***
GPO, mmol GSH/l · min	18.2±0.9/13.5±0.9***	15.2±1.2/14.4±1.2	17.3±0.5/11.9±1.1***
Vitamin E, mmol/l	36.9±3.3/27.2±2.2*	35.9±2.5/24.0±1.3***	34.8±2.9/23.8±1.9**
Vitamin C, mmol/l	19.8±1.4/8.5±0.9***	17.7±0.6/9.9±0.6***	17.9±0.8/13.4±1.1**

N o t e. Before and after the slash there are physiological formation and death of the embryo, respectively. GSH — reduced glutathione, MDA — malonic dialdehyde, GPO — glutathione peroxidase.
*, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ compared to control (physiological formation).

In cows with embryonic loss, MDA concentration exceeded the indicator in the control animals by 44.3 % on days 19-23 of gestation and by 58.5 % on days 28-32, and MDA level remained high after the death of the embryo, exceeding the control values by 38.6 % on days 38-45. Higher accumulation of toxic products of LPO appears to be associated with enhanced formation of reactive oxygen species (ROS), LPO activation, and insufficient antioxidant protection. Catalase activity in these animals, as compared to the control, was 26.5 % lower in implantation and 51.2 % lower in the early placentation. These differences were 33.4 % on days 38-45 in the cows with clinically confirmed fetal death. At embryonic loss, GPO activity were 25.8 % lower during implantation and 31.2 % at embryo death, vitamin E was 26.3 and 31.6 % lower, respectively, and vitamin C was 57.1 and 25.1 % lower than in healthy animals. Deficiency of enzyme and non-enzyme antioxidants should be associated with their high consumption for detoxification and elimination of actively generated aggressive free radicals and with the depletion of the reserves of the antioxidant protection system (AOPS) due to deficiency of essential bioelements. Violation of dynamic equilibrium in the LPO-AOPS, accompanied by excessive accumulation of toxic LPO products, leads to the development of oxidative stress characterized by irreversible oxidative modification and damage to proteins and DNA [38,

39], which should be considered as a sign of embryopathy development in cows.

The adverse effects of oxidative stress on embryo are related not only to the direct embryotoxicity of aggressive free radicals, but also to their negative effect on the hormone-synthesizing structures of gonads and the immune system.

Already on days 19-23 of gestation in the blood of cows with the risk of embryonic loss, an increase in the total number of leukocytes (by 12.9 %) and their neutrophil forms (by 14.0 %) was noted (Table 4), which could indicate intoxication or development of the inflammatory process in the genitals. The population of monocyte which are active phagocytes, precursors of tissue macrophages and producers of complement components also increased by 77.8 %.

The development of toxic, allergic and autoimmune response to embryo death showed a 2-fold elevation in the number of blood of eosinophils, which ensure the destruction of histamine, toxins of protein origin, foreign proteins and immune complexes. CIC (antigen-antibody complex) concentration in the animals of this group was 2.96 times higher than in cows with normal pregnancy. An excess in CIC accumulation, associated with allergic reactions, leads to destruction of the tissues in genitals and can affect the developing embryo.

4. Indicators of immune defense and natural resistance in cows of black-and-white breed in the physiological formation of the embryo and its death($X \pm x$, OOO SP Vyaznovatovka, Nizhnedevitsky district, Voronezh region, 2014)

Parameter	Gestation, days		
	19-23	28-32	38-45
Leucocytes, $\times 10^9$/l	8.5±0.50/9.6±0.44	9.0±0.28/10.0±0.62	8.5±0.34/10.0±0.46*
Neutrophils, %	28.6±1.8/32.6±2.1	30.8±2.1/27.8±1.4	29.3±1.7/35.0±2.9
Monocytes, %	2.7±0.2/4.8±0.2***	2.4±0.2/3.3±0.1**	2.9±0.2/3.3±0.2
Eosinophils, %	5.2±0.3/10.6±0.9***	6.4±0.3/14.0±1.2***	5.6±0.3/13.3±1.1***
Lymphocytes, %	63.2±2.1/52.0±3.5	60.4±2.3/54.9±3.4	62.1±2.8/48.4±2.6**
PAL, %	68.8±2.9/58.8±4.3	69.4±2.9/63.9±4.3	76.1±2.6/69.7±3.7
Immunoglobulins, g/l	29.3±0.7/21.8±1.1***	28.6±0.8/20.8±0.9***	27.0±0.7/20.4±1.4***
CIC, g/l	0.23±0.02/0.68±0.06***	0.29±0.02/0.61±0.02***	0.30±0.02/0.57±0.04***
BABS, %	82.2±1.9/61.4±5.3**	73.6±2.8/52.7±3.8***	79.0±2.9/57.5±3.2***
LABS, %	0.48±0.03/0.29±0.02***	0.40±0.03/0.28±0.02**	0.45±0.03/0.26±0.02***

N o t e. Before and after the slash there are physiological formation and death of the embryo, respectively. PAL - phagocytic activity of leukocytes, CIC - circulating immune complexes, BABS - bactericidal activity of blood serum, LABS - lysozyme activity of blood serum.
*, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ compared to control (physiological formation).

The blood CIC concentration was inversely related to the phagocytic activity of the leukocytes which was 14.5 % lower at the death of the embryo compared to the control. The high CIC level, together with a decreased activity of polynucleated leukocytes testified both to the congestion of the phagocytic system and to the increased migration of functionally immature neutrophils from the bone marrow under the influence of interleukin-1, the amount of which increases during pregnancy [18]. In cows with the manifestation of embryonic mortality, the relative amount of blood lymphocytes was 14.5 % lower, and the total immunoglobulin level lowered by 25.6 %. The latter can be related both to the oppression of their synthesis, and to the increase in CIC formation and utilization.

In animals with improper embryonic development, a deficiency in adaptation to stress caused by pregnancy was combined with a decrease in the indices of humoral factors of natural resistance. Blood bactericidal activity and lysozyme activity was 25.3 and 39.6 % less than in the control, which correlated with inhibition of phagocytic leukocyte reaction.

On days 28-32 of gestation, previously revealed differences in the indicators of cellular and humoral immunity in different groups, as a whole, remained unchanged. When the embryo died, the number of blood leukocytes increased by 11.1 %, the number of monocytes, eosinophils and CIC concentration were 37.5 %, 218.7 % and 210.0 higher, respectively, as compared to

the control, whereas PAL (phagocytic activity of leukocytes), number of lymphocytes, BA and LS lowered by 7.9 %, 9.1 %, 28.4 % and 30.0 %, respectively. Similar results were obtained on days 38-45. The observed changes in the immune status of mother-cows reflect a strong reaction of the body to endotoxins and embryo antigens, which testify to immunodeficiency and reduced adaptive abilities of animals in early gestation.

It is known that the microbiocenosis of the vagina plays a significant role in ensuring the homeostasis of the reproductive organs, their colonization resistance and protection of the reproductive tract [39-41]. The main representatives of its normoflora are lacto- and bifidobacteria, acting as antagonists of conditionally pathogenic and pathogenic microorganisms to suppress their growth and reproduction. The protective effect of lactobacilli and bifidobacteria is due to secretion of a significant amount of lactic and other organic acids, various bacteriocins, vitamins and other bioactive substances, and to a direct stimulating effect on immunocompetent structures of the reproductive tract, local and general immunoreactivity of the body. The stability of the vaginal microbiocenosis is ensured by the coordinated interaction of the endocrine and immune systems in which any violation leads to dysbiosis and a replacement of normal microflora by conditionally pathogenic and pathogenic microorganisms.

In normal gestation, the total microbial contamination of the vaginal mucus was quite stable and varied from 509.7±24.3 to 573.2±48.4 CFU/ml. The presence of lactobacilli was recorded in 66.6-83.3 % of animals at the titers of $10^{-4.08}$ to $10^{-3.38}$. Bifidobacteria, a part of normoflora possessing high colonization antagonistic and adhesive activity, were found in 100 % of animals with normal embryo formation. When embryo died, on days 28-45 of gestation the bifidobacteria were detected only in 50-75 % of cows with the titers 2.22-2.95 times lower than in healthy animals.

Reduction of colonization of the genital organs by normoflora was accompanied by an increase in the counts of enterobacteria (*Escherichia coli*, *Enterococcus faecalis*) and fungi in the mucus. The frequency of enterobacteria increased from 10.0-16.6 % to 33.3-70.0 %, while fungi were 1.4-2.0 times frequent. The pH of the vaginal mucus increased from 7.3±0.16-7.62±0.07 to 8.25±0.15-8.63±0.18.

Thus, in the polyfactorial etiology of early embryo loss in lactating cows, the pathophysiological determinants are the deficiency and imbalance of essential mineral elements, as well as oxidative stress and endogenous intoxication, which leads to endocrine and immune deficiency and is accompanied by a violation of immune and trophic relationships in the mother—embryo system. Means and methods for correction of diselementosis, antioxidant, endocrine and immune status should be developed to prevent embryonic loss and improve fertility in high-yielding dairy cows.

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FUNCTIONAL EGG PRODUCTION. I. THE ROLE OF ω-3 POLYUNSATURATED FATTY ACIDS
(review)

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Abstract

The world's market of functional foodstuffs is a permanently growing sector. Functional foods should meet the nutritive requirements of the consumers and render therapeutic and/or preventive effects on human health. Functional eggs enriched with different bioactive substances are one of the most voluminous segments of this market (N. Shapira, 2010). High rate and flexibility of avian lipid metabolism allow fast modifications in egg yolk composition via corresponding alterations in the diets of laying hens. In the last decades nutritionists pay increasingly close attention to ω-3 polyunsaturated fatty acids (PUFAs), primarily α-linolenic (ALA, C18:3), eicosapentaenoic (EPA, C20:5), and docosahexaenoic (DHA, C22:6) acids due to the benefits for human health and necessity for brain development, retinal function, prevention of cardiovascular diseases, etc. (A. Simopoulos, 2001); human diets in most countries including Russia are severely deficient in these essential fatty acids. Flexibility of avian lipid metabolism allows transfer of dietary PUFAs into eggs after 1-2 weeks of feeding PUFA-enriched diets (C.O. Leskanich, R.C. Noble, 1997). However, any increase in PUFA contents in dietary lipids can lead to definite changes in lipid metabolism in layers affecting productivity and egg quality: a decrease in blood level of total fat and triacylglycerols, an increase in hepatic level and catabolism of triacylglycerols which can cause the fatty liver hemorrhagic syndrome (FLHS), as well as the decrease in yolk and egg weight (M.E. Van Elswyk, 1997). Another common problem related to egg enrichment with ω-3 PUFAs is fishy taint phenomenon: panel tests often characterize enriched eggs as smelling fish-like, and this effect deteriorates market attractiveness of these eggs (F. Bubel et al., 2011). Diets for laying hens for the production of ω-3 PUFA enriched eggs usually contain one of the three types of dietary PUFA sources. The first is fish oil from different species; its advantages include higher levels of long-chain PUFAs (LC-PUFAs), primarily EPA and DHA, in resulting enriched eggs. Major disadvantages of fish oil, however, are instability of composition and high susceptibility to oxidation; frequent appearance of fishy taint even at the lowest levels of inclusion into the diets; relatively high price, market availability, and contamination with typical oceanic pollutants (I. Fraeye et al., 2012). The second type of additives is flax products, seeds, cake or oil containing substantially lesser amounts of LC-PUFAs compared to fish oil while being extremely rich in ALA (over 50 % of total fatty acids); ALA-enriched lipids in diets, layer body and eggs are more resistant to oxidation. The data of numerous studies suggests that reasonable level of inclusion of flaxseed products into the diets for layers are 5-8 % for seed and cake and 3 % for oil; these doses are reportedly beneficial for productivity and egg quality (E.M. Goldberg et al., 2013). In Russia flax products are available and inexpensive, and can therefore represent the most profitable dietary source of ω-3 PUFA for layers. The third type additives are macro- and microalgal species which are less available and still understudied, though the recent research data show that these additives can be the most promising dietary sources of ω-3 PUFA (J.H. Park et al., 2015). Enrichment of feeds and eggs with ω-3 PUFA requires additional dietary antioxidants to prevent lipid oxidation (Ch. Nimalaratne, J. Wu, 2015); the most efficient and well-studied antioxidant is vitamin E which is, after that, a valuable bioactive substance per se for egg enrichment. The data of

different experiments with different ω-3 PUFA sources are often inconsistent and controversial due to the close relation to the multifaceted avian lipid metabolism, and comparative analysis of these studies is further complicated by the lack of estimated parameters; it could be helpful, therefore, to launch an international database related to these experiments and containing raw datasets which could be statistically analyzed and compared in a more efficient way.

Keywords: functional eggs, polyunsaturated fatty acids n-3, lipid metabolism, flax seed and oil, fish oil

The term "functional food product" emerged in recent decades, means a product of everyday consumption that contains certain biologically active substances (BAS) in amounts significantly greater than those in non-functional analogs, and contributes to the improvement of health and (or) prevents the onset and development of diseases [1]. Enrichment of BAS should occur as naturally as possible, since the safety and naturalness of such products are important. In this connection, eggs and poultry meat are of interest because of the high (compared to other farm animals) efficiency of transfer of many substances from feeds to products [2]. In this, it is necessary to ensure both the well-being of livestock when feeding rations enriched with BAS, and the cost and availability of functional products for the population as in retail, they are 15-20 % more expensive than non-functional analogous [1]. Market research (for example, in Serbia) confirmed that this limits demand [3], however, consumption of functional products, including poultry, is expected to increase, as evidenced by the development of production of functional egg in such European countries as Serbia [4]), Macedonia [5] and Greece [6].

This review focuses on the biological and technological aspects of the production of edible egg enriched with various bioactive substances and their complexes.

Functional egg: general statements. By the end of the XX century, it was found that the amount of cholesterol consumed had only a negligible effect on its blood concentration [7], large-scale studies also did not reveal a significant relationship between egg consumption and the risk of cardiovascular disease [8], but "cholesterol disputes" in the press continues [9]. The accumulated data formed the basis for the concept of a functional (enriched, fortified, designer) egg. The first term implies a potential therapeutic and (or) prophylactic effect, the second and third mean directed enrichment with bioactive substances (BAS), the fourth mean the ability to create a product for different population groups and patients in accordance with the needs. The choice of BAS is determined by its deficiency in the human diet, the efficiency of transfer from the feed to the egg, the stability of the content during processing or cooking, the proportion of the daily requirement provided by a single functional egg. So, enrichment with vitamins B_1 and B_2 is inexpedient because of their high content in other foods, and for vitamins A and C it is impossible due to low transfer to eggs [10].

The lipid composition of the egg is largely determined by the fats in the diet (in contrast to the amino acid which almost does not depend on the diet) [11]. In addition, because of the high metabolic rate of lipids and lipoproteins in chickens, the fatty acid profile of the yolk varies rapidly depending on the diet [12, 13], which allows to modify the lipid composition of yolk and enrich it with fat-soluble BAS (for example, vitamin E, carotenoids, polyphenols), as well as selenium and iodine. For different target components of the yolk, the period of transformation and stabilization of the profile is 2-4 weeks [13-15], but the increase in content is noted in a week. This is consistent with the timing of the formation of the egg after the recruitment of the follicle (8 days, of which about 7 days accounted for the growth of the follicle before ovulation and about 1 day takes deposition of protein and shell) [16]; about 14 days passes from the onset of the growth of the primordial follicle to the prelaying of the egg [17].

The market of functional egg products (yolk, melange, etc.) is also beginning to develop, but in volume it is much inferior to the market of whole eggs [18]. In processing, bioactive substances that are not obtained or disadvantageously introduced into the egg can be added to egg products. In fact, the transfer of vitamin C and certain micro elements (Cu, Zn) from feed to eggs is ineffective and does not ensure their sufficient enrichment [10].

ω-3-Polyunsaturated fatty acids. Currently, the fatty acid composition of the diet, especially essential fatty acids (FA), primarily polyunsaturated fatty acids (PUFAs), is receiving increasing attention. Indeed, linoleic PUFA (LA, C18:2ω-6) and α-linolenic (ALA, C18:3ω-3), irreplaceable for humans and most animals, are not synthesized in the body from more saturated acids due to the lack of Δ^{12} desaturase [19]. LA and ALA from the diet can serve as precursors in the biosynthesis of PUFAs with longer carbon chains (long-chain PUFAs, LCPUFA). Some physiologically important PUFAs, for example docosahexaenoic (DHA, C22:6ω-3) or γ-linolenic (GLA, C18:3ω-6) acids, are conventionally indispensable, since they are not synthesized at a certain stage of ontogenesis or in some diseases [20, 21].

Physiologically significant PUFAS form ω-3 and ω-6 series (according to the position of the first double bond, counting from the final methyl group). The most important ω-6-PUFAs, in addition to LA, are GLA, long-chain dihomo-γ-linolenic (DGLA, C20:3ω-6) and especially arachidonic (AA, C20:4ω-6) acids. Long-chain DHA and eicosapentaenoic acid (EPA, C20:5ω-3) are distinguished in the ω-3- PUFAs group along with ALA. Physiological and biochemical functions of ω-3- and ω-6-PUFAs are individual, but competition is possible between them. For example, the use of elongase (elongation FA molecule) and desaturase (can catalyze the appearance of double bonds) in the formation of LCPUFA of both series from the corresponding PUFAs (LA or ALA), as well as in the biosynthesis of eicosanoids (prostaglandins, thromboxanes, prostacyclins, leukotrienes, resolvins, ect), the signaling molecules involved in processes associated with inflammation and other forms of immune response, regulation of cell growth, blood pressure control, etc. Often, the functions of eicosanoids synthesized from the FA series ω-3 and ω-6 are opposite [22], so not only the absolute level, but also the ratio of these acids in the rations are important. For example, with an increase in the fraction of ω-3- PUFAs, the biosynthesis of AA and its derivatives, pro-inflammatory eicosanoids [23], decreases.

As optimal, different ratios of ω-6 and ω-3-PUFAs (1:1, 3:1 and 6:1) are reported [24]. In the countries where the consumption of fish rich in ω-3-PUFAs is traditionally high (for example, in Japan), and the incidence of cardiovascular disease is very low, this ratio is close to 4:1 [25]. The 4:1 ratio in the diet leads to a 1:1 ratio in the cell membranes [26]. In developed countries, the indicator for the diet is excessively high reaching from 10:1 to 25:1 [27). Modern diets for poultry based on maize and other grains are also redundant in ω-6-PUFAs (the content of ALA is very small, and DHA and EPA are practically absent) [28]. The vegetable oils used (corn, soybean, sunflower, rapeseed) also contain significant amounts of ω-6-PUFAs (primarily LA) and few ω-3-PUFAs [29], except some rapeseed varieties [30]. Therefore, in the edible egg, the ratio of ω-6- and ω-3-PUFAs is much higher than the optimal one [31]. It was also noted that in white laying hens, ω-3-PUFAs are deposited more effectively than in brown eggs [32].

To successfully enrich in ω-3-PUFAs, it is required, first, to use a source with their high bioavailability, which does not have a significant negative effect on health, well-being of laying hens, egg production and egg quality, including

taste and smell; second, to control ω-6- and ω-3-PUFAs in both the diet and the final product. The problem is also the susceptibility of objects to oxidation with a high proportion of PUFAs (especially with 4-6 double bonds).

In addition, it is important to determine which ω-3- PUFAs are advisable to enrich the egg. In an adult human, the efficiency of bioconversion of ALA in ω-3-LCPUFA (in DHA and EPA) in the liver is less than 5 %; in chickens, it is also low [33, 34], and not only in the liver. Studying in vitro the formation of AA and DHA from ^{14}C-labeled LA and ALA (C18) upon incubation with fragments of the yolk sac membrane of the chick embryo, where the activity of Δ^9 and Δ^6 desaturase was detected [35], the authors expected to reveal the biosynthesis of LCPUFA in transport of yolk lipids through the perivitelline membrane to improve the supply of embryos. However, the bioconversion of both precursors was only 4-8 %, while the main amount of the label after 4 hours was in the fraction of triglycerides and phospholipids of the perivitelline membrane [35].

Intermediate products of conversion of ALA to DHA, the eicosatrienic ETA (C20:3ω-3) and docosapentaenic DPA (C22:5ω-3) acids, were found in eggs enriched with both ALA and LCPUFA, and of LCPUFA the DHA was most effectively deposited [36]. The high amount of LCPUFA in the diet reduced the effectiveness of bioconversion of ALA to DHA in the liver [36]. The affinity of the Δ^6 desaturase to substrate FA was enhanced with an increase in the number of double bonds [37], indicating the advantage of the most unsaturated LCPUFA (EPA, DPA) before ALA in competition for desaturase. Perhaps, therefore, reports of the simultaneous introduction of sources of ALA and LCPUFA (for example, a mixture of flaxseed oil and fish oil) into the diet are few, and their results are not of interest [38]. In a number of studies, the intermediate products of elongation and desaturation of FA were not determined and not taken into account in the total index for ω-3-PUFAs, although, in our opinion, a stepwise assessment of the change in the concentrations of such products would be informative.

Reduction of oxidative stability with increase in the percentage and unsaturation of PUFAs (including ω-3) in feeds and liver tissues can lead to the formation of oxidation products harmful for poultry and their transfer to eggs, and in eggs — to a faster lowering quality during storage and/or cooking. J.M. Miranda et al. [39] confirm that the oxidative damage of the yolk is associated only with the direct deposition of oxidized lipids from the diet (from the storage of eggs the lipids do not oxidize), and, however, refer to the paper [40], which shows a decrease in the concentration of substances reacting with thiobarbituric acid (SRTBA, the total concentration of oxidation products of lipids, especially malonic dialdehyde MDA) in eggs after storage for 60 days at 4 °C. The authors of the study [40] believed that the observed effect is most likely due to the reaction of MDA with other egg substances and, possibly, partial polymerization of MDA, which reduces SRTBA. A decrease in SRTBA during storage was reported in other publications [41-43], nevertheless, a steady increase in this indicator was reported as well [44-46]. Thus, the conclusion of J.M. Miranda et al. [39] on the unconditional oxidative stability of yolk lipids in stored eggs is not sufficiently substantiated.

The hypothesis of the transport of oxidized fats from the liver to the yolk has already been advanced [47]. In a number of cases, the values of SRTBA in the liver of layers were increased in response to feeding fish oil [47] or flaxseed [48]. The MDA in a fresh-laid egg in the presence of an antioxidant, the vitamin E, in fodder is also reported [49]. Indeed, the oxidation of yolk lipids is possible, since gas exchange through the shell [50] and the diffusion of water from the protein into the yolk [51] do not cease even at a lower storage temperature. On

other hands, liquid-phase extraction of lipids for the TBA test can cause oxidation upon solvent distillation, regardless of the presence of antioxidants [52].

The problem of oxidative stability in the enrichment of PUFAS is usually solved with the use of antioxidant stabilizers (selenium, vitamins E and A, carotenoids, etc.), which themselves are valuable BAC for humans and animals and can be added to feed (and, consequently, transferred to eggs). Another recommended technology is to reduce the storage of PUFAs sources and feed the poultry as freshly prepared products as possible with minimal oxidized lipids [53].

In rations, flax (seed or oil) and fish oil of a suitable fatty acid composition as available sources of ω-3-PUFAs [54] are most often used, and marine micro and macro alga have also been proposed. However, with the use of all additives, general (and specific for each) negative consequences for the health of the bird and the quality of eggs are possible.

Changes in the metabolism of lipids and a decrease in the yolk weight in the enrichment with ω-3-PUFAs. This effect was reported by many authors. Thus, in the groups receiving fish oil, the reduction was about 3 g, and it was evaluated as useful, especially for the laying hens post molting [55] because of a decrease in the percentage of large eggs with low quality shells, which in many countries are categorized as low-grade [56]. In other experiments, no decrease was observed. Indeed, on the ration with 3 % linseed oil, the analyzed indicator significantly ($p < 0.05$) increased (from 62.88 to 65.28 g), and the egg production was slightly below the control [57].

The decrease in yolk weight and blood lipid level in laying hens during prolonged feeding sources of ω-3-PUFAs is possibly associated with changes in fat metabolism. In mammals, an increase in the proportion of ω-3 PUFAs in the diet leads to suppression of lipid biosynthesis in the liver, enhancement of their β-oxidation in peroxisomes, and subsequent reduction in blood lipids of different fractions [58]. Poultry also had lipid lowering (a reduction in the amount of total fat and triglycerides in the blood) when feeding flaxseed [59] or fish oil [55, 60]. Later this hypothesis received a nutrigenomic confirmation. Cod-liver oil as a source of LCPUFA caused an increase in the expression of transcription factors involved in the biosynthesis and catabolism of FA in the liver, namely of the α-type receptor activated by peroxisome proliferators (PPARα), and the sterol regulatory element-binding proteins (SREBPs) [61], leading to lipid lowering and liver lipidosis. Another hypothesis [62] linked the regulation of observed changes in fat metabolism with the effect of endogenous estradiol, although the mechanism of such influence remained unclear for the authors. A hypothesis based on the effect of flax phytoestrogens on laying hens is partly associated with this assumption [63]. In mammals, phytoestrogens (secoisolariciresinol diglycoside and matairesinol) are metabolized by the microflora of the large intestine with production of mammalian lignans, the enterodiol and enterolactone, that exhibit estrogenic and anti-estrogenic effects due to structural similarity to endogenous estrogens [64]. In pullets who received flaxseed, these lignan metabolites were also detected and blood estradiol concentration decreased (up to –30 %) [65]. Perhaps this is why, when feeding flaxseed, egg production is reduced only at the beginning of oviposition, as the hormonal balance and lipid metabolism are not yet fully adapted to the requirements of laying and are particularly sensitive to any effects. Besides, using aminoglutethimide to suppress the biosynthesis of estradiol, it has long been shown that blood estradiol concentration affects the weight of eggs and yolk rather than egg production [66], which, with long-term feeding of flaxseed, generally remains lower compared to control.

Metabolic syndrome of liver fatness. Fatty liver hemorrhagic syndrome (FLHS) observed in laying hens at high energy rations is characterized by

a high level of different lipid fractions, especially triglycerides, increased fatty infiltration of hepatocytes, hemorrhages with consequences up to lethal. The etiology of the syndrome is not fully understood, although it is known that in birds predisposition to this is related to the fact that birds (unlike mammals) de novo FA biosynthesis occurs only in the liver, so the chylomicrons formed in the intestine are absorbed directly into the portal vein and practically do not enter other tissues [67]. Factors influencing the manifestation of this syndrome in laying hens are housing, genotype, diet composition, hormonal and antioxidant statuses [68]. The relationship between FLHS and the level of ω-3-PUFAs in the diet of laying hens [69-71] can not be considered clearly established, since such a relationship was not found in other studies either in visual assessment of liver status and hemorrhage index [48, 72] or in histological studies [59]. The available data indicate rather a relationship between the manifestation of FLHS and the amount of total triglycerides (regardless of their fatty acid profile) [73, 74].

"Fishy" smell of eggs. The appearance of eggs, which smell like stale fish, is associated with the inclusion in the diet of not only fish products, but also, for example, flax or rape oil or oilcakes. The unpleasant smell substantially reduces the demand for ω-3-PUFAs enriched eggs and egg products [54]. However, the organoleptic properties do not clarify either the chemical nature of the substances that cause the smell, or the cause of its appearance. A tasting assessment is more a marketing one than a biological or technological indicator that reflects changes that may or may not be related to PUFAs. Trimethylamine (TMA), which along with bioamines (putrescine, cadaverine, etc.) is released when the fish products are naturally spoiled, are the only well-studied substance that causes a truly fishy smell of eggs [75]. TMA is a product of some microorganisms of the distal part of the small intestine (including cecums) in chickens [76]. There are chicken genotypes predisposed to fishy smell of eggs due to the high frequency of A-T single-nucleotide polymorphism in exon 7 of FMO3 (flavin-containing monooxygenase 3) gene; interestingly, this recessive mutation is found almost exclusively in brown laying hens [77]. In humans and cattle, this mutation causes trimethylaminuria, i.e. a fishy smell of breathing and (or) the human body or milk of cows. Due to this mutation, the liver shows complete or partial deficiency in TMA oxidase, necessary for TMA oxidation to almost odorless and non-volatile TMA-N-oxide (TMAO). As a result, in chickens TMA secreted by intestinal bacteria is not oxidized in the liver to the N-oxide and is not excreted by the kidneys, which occurs normally, but accumulates in the ovarian follicles. Expression and heritability of the mutation is enhanced by prolonged diets with high proportion (12-24 %) of rapeseed oilcake acting as a nutriogenetic trigger, in this, choline, the main precursor in bacterial synthesis, unlike rapeseed, does not affect the deposition of TMA in the egg [78]. The observed nutriogenetic effect is explained by the action of the anti-nutrient factors of rapeseed glucosinolates that inhibit the expression of hepatic TMA oxidase and are also present (however, in much smaller amounts) in flaxseed. However, in none of the studies TMA has yet been identified as the main cause of fishy smell. It was reported that in hens with trimethylaminuria, breathing often have this smell [76], but this fact has not been noted in papers reporting findings in enriching eggs with PUFAS.

Thus, in most cases, for the time being, only the connection of the fish smell of eggs with the products of oxidation of PUFAs-rich lipids is assumed, and the presence of antioxidants does not always eliminate the problem. Earlier it was reported that fish oil in the laying hen's diet affects the content of 23 out of the 42 volatiles studied in the egg, and the possibility was discussed of the appearance of a fishy smell as a sensory resultant of quantitative change of several of these substances [79]. The appearance of fish smell in milk fat during oxidation is specifi-

cally associated with volatile compounds of aldoketone nature with conjugated diene fragments in hydrocarbon chains [80]. Similar compounds, for example 2,4-heptadiental, are found among products of spontaneous oxidation of fish oil during storage, and the authors also identify their smell as "fishy" [81]. Theoretically, the oxidation of PUFAS (and to a greater extent, LCPUFA) can give similar compounds and may be associated with biochemical mechanisms of fish egg odor, but this phenomenon and the development of countermeasures require special studies.

We note that only a very small number of papers contain a complex of such indicators as the fatty acid composition of sources and rations, the content of all target FA in blood, liver and eggs, the ratio of ω-6- and ω-3- PUFAs, organoleptic evaluation, etc., so the results, which are often contradictory, are difficult to compare. Data on the productivity of layers is also ambiguous. Therefore, it would be useful to create an international database of experimental data, which allows us to conduct statistical studies of information files.

Sources of ω-3- PUFAs in rations of laying hens. *Cod-liver oil.* This is the first and most studied target source of ω-3-PUFAS. Its main disadvantages are instability of composition, including that due to high oxidation ability, which is accompanied by a fishy smell of eggs and meat. The problem is overcome only partially, which has led to interest in the study of flax as an alternative source of less unsaturated lipids [82].

As the used additive, the fat from the American herring, menhaden (*Brevoortia tyrannus*), prevails. However, it is known that, due to instability in species composition of the catch, seasonal fluctuations in fish condition, high predisposition to oxidation due to a significant share of LCPUFA with a large number of double bonds (EPA, DHA), the composition of fish products (fat and flour) differs markedly not only between species of fish but also between batches from one supplier [83]. In Australia, a number of commercial fish oil preparations (even encapsulated ones) turned out to be highly oxidized (the concentration of oxidation markers exceeded the permissible levels), only 2 of 32 samples examined EPA and DHA met the specifications, and in the rest were lower than those declared by the manufacturers [84].

The next and main disadvantage of fish oil, even at low dosages, is fishy smell of eggs [82]. The deodorization of the additive did not improve the organoleptic characteristics of eggs, e.g. 2, 4 or 6 % of dietary fish oil led to significantly higher scores on the analyzed index, and no significant differences were found between the deodorized and non-deodorized forms [85]. At that, as in many other works, a significant ($p < 0.05$) linear decrease in the weight of eggs with an increase in the dose of dietary fish oil (by 3-4 % at a dose of 6 %) was noted. The use of deodorized fish oil significantly increased the deposition of the sum of ω-3-PUFAS in eggs (343 against 246 mg/yolk at doses of 6 % against 53 mg/yolk in the control). Perhaps, deodorized fat is more attractive for laying hens (the consumption of feed in such groups is 105 versus 101 $g \cdot head^{-1} \cdot day^{-1}$), which leads to a difference in the sum of ω-3- PUFAs in rations (17.82 and 15.88 g/kg vs. 2.39 g/kg in the control). Encapsulation of fish oil also does not exclude the appearance of a fishy smell. In White Leghorns, of 2, 4 or 6 % of dietary microencapsulated fish oil tested, even a 2 % dose significantly worsened the odor in 3 weeks compared to the control, although the total amount of ω-3-PUFAs significantly and linearly increased from 141 for control to 299 mg/yolk for 6 % of fish oil [86]. Do not forget that deodorization or microencapsulation of fish oil significantly increases the cost of the feed additive.

Finally, fish oil is produced mainly from commercial fish, whose stocks are falling in many countries, and additionally, contamination with heavy met-

als, polychlorinated biphenyls and other hazardous pollutants is increasing [87]. This leads to a reduction in market availability and a rise in the cost of quality fish oil for feed production.

Importantly, the data on the effectiveness of fish oil application are ambiguous. Thus, when 1.5 % of fish oil was added to laying hen ration, their productivity and the main quality parameters of eggs were kept within control limits [88]. In other experiments, with a fish oil dose of 3 %, the chick's productivity did not change, but the sum of ω-6-PUFAs decreased reliably ($p < 0.05$), the amount of DHA and EPA increased (from 0.19 to 3.21 % and from 0.00 to 0.18 %, respectively), and ω-6-PUFAs/ω-3-PUFAs ratio decreased from 33.52 to 2.55 compared to the control eggs [89]. It is now recognized that the proportion of fish oil in the diet for enriching eggs with ω-3-PUFAs should not exceed 1.5 % [90]. The same is confirmed for cod-liver oil. In ducks, 5-6 % of dietary oil led to a significant and reliable increase in the scores of eggs for fishy smell, but at a smaller dosage (2-4 %), this was not noted. Productivity of ducks remained within control, except for a small decrease in egg weight at 6 % dose [91].

Flaxseed oil and seed. More than half of FA in the flaxseed lipids is in ALA, and as a source, flaxseed has no equal [92]. Due to the lack of availability in the market and the relatively high price, the flaxseed oil are used less often in feeding birds than seeds or oilcakes [93]. The whole seed contains about 40 % fat, 20-25 % protein and 3-10 % sticky substances; the latter, along with the lignan phytoestrogens, linatinum (pyridoxine antagonist) and linamarin (cyanogenic glucoside), are among the main anti-nutrient factors of flax [94]. Producers usually recommend flax seeds as an additive to adult fodder at a dose not more than 10 % [93] and processed seeds after grinding, autoclaving, granulation, extrusion, microwave heat treatment, etc., which increases the availability of protein and ω-3-PUFAs [95, 96]. When feeding small gravel or shells, the bird very effectively grinds whole seeds in the muscular stomach [97].

The water-soluble seed adhesives, consisting mainly of neutral arabinoxylans and acidic pectin-like fractions, which are contained primarily in seed coats, can, like phytoestrogens, adversely affect productive parameters in laying hens. Arabinoxylans can significantly increase the viscosity of chyme, reducing the effectiveness of dietary nutrients [98]. It is known that the content of non-starch polysaccharides in linseed is higher than in rapeseed [99], and rations with rapeseed should be enriched with the enzymes that decompose non-starch polysaccharides [100]. Unfortunately, there are few such studies on flax, and they are not mentioned in the literature on egg enrichment with ω-3-PUFAs. However, it was reported that when feeding 15 % of linseed to laying hens from 39 to 63 days of age, the enrichment of the ration with the multi-enzyme Superzyme®-OM complex (Canadian Bio-Systems Inc., Canada) that cleaved non-starch polysaccharides significantly improved egg production (from 78.0 to 80.9 %, $p < 0.01$) and feed conversion (from 2.15 to 2.03 kg/kg, $p < 0.001$), while feeding the same dose of flaxseed without the enzyme reduced the egg production and shell weight compared to control without flax and the enzyme. When using the enzyme, a significant ($p < 0.01$) increase was recorded in total ω-3-PUFAs (from 546 to 578 mg per egg for an egg weight of 60 g) and in DHA (from 91.8 to 101.9 mg) [101]. It is not excluded that multi-enzyme additives in combination with high flaxseed ratio can improve production of eggs enriched with ω-3-LCPUFA, but so far the question of the organoleptic qualities of such a product remains open.

It is estimated that for every 1 % of dietary flaxseed, ω-3-PUFAs amount increases by about 40 mg/egg [102]. High doses of flaxseed can increase ALA up to 200 mg/egg, DHA up to 90 mg/egg [90]. Consequently, it is practically advisable a dosage of no more than 5 % for flaxseed and no more than 3 % for oil, because at

lower doses, the content of the target FA in the egg will be insufficient, whereas larger ones can adversely affect layer productivity and egg quality in the absence of a noticeable increase in total ω-3-PUFAs, since the plateau has already been reached [103]. A limit flaxseed dose of 8 % established in the 1990s [104] currently seems too high (due to the impact on productivity and fishy smell). According to most researchers, in moderate amounts flaxseed does not affect the egg production, except early productive period [69].

Comparing the effect of a standard diet rich in ω-6-PUFAs (especially LA) and the same diet enriched with ALA by adding 5 % of the extruded flaxseed, it was found [105] that total ω-3-PUFAs in the egg was 3.8 times higher than in the control (258.2 vs. 67.3 mg/egg), ALA was 6.4 times higher (156.7 vs. 24.5 mg/egg), and DHA was 2.4 times higher (101.6 vs. 42.8 mg/egg). A 3.6-fold decrease in ω-6-PUFAs/ω-3-PUFAs, a 5.7-fold decrease in LA:ALK, and 3.0-fold decrease in AA:DHA were also noted. After a week diet enriched with ALA, total ω-3-PUFAs increased 3.4-fold, being in 3 and 5 weeks 3.7 times and 4.0 times higher, respectively, compared to control. In introduction of 0, 5, 10, 15 and 20 % of unroasted or roasted dietary full-fat flaxseed [106], it was found that unroasted seeds declined egg production in chickens while the best (although unreliable) egg-laying rates were noted in groups receiving 5 or 10 % of roasted seeds (93.82 and 93.35 % vs. 92.71 % in the control). The weight and color of the yolk remained in all groups within the control indicators. The PUFAs content reached a plateau at doses of both additives of 15 %, with 2.9-fold increase for DHA, 4.0-fold increase for DPA, 5.5-fold increase for EPA, and 9.8-fold increase for ALA, and not 10 % reported in other works. The authors attribute this to the unusually low proportion of ALA (38.43 % of FA) in the flaxseed used and note that the flaxseed roasting did not significantly improve the deposition of PUFAs in the egg. Study of the rations with linseed oil (1.5, 3.0 and 4.5 %) [107] showed that its use at a dose of 3 % instead of corn, soybean meal and fish meal increased the deposition of ω-3- and ω-6-LC 2.54 times (0.2 mg/ml egg volume). ALA increased 1.55 times, EPA increased 0.15 times, docosapentaenic acid (C22:5) concentration increased 1.6 times, and DHA was 3.22 times higher. The protein quality (Haugh units) in the group with 3.0 % of dietary linseed oil was 81.3 % and was higher than in other groups, i.e. 80.8 % for the control and 4.5 % oil, and 80.0 % for 1.5 % oil (interest in the quality of the protein is not completely understandable).

Of the beneficial effects of dietary flaxseed, it should be noted the increase in IgY concentration in eggs: when feeding 10 % of linseed oilcake, it significantly ($p < 0.05$) increased from 10.4 to 12.0 mg/g yolk [108]. In this experiment, the egg production was significantly ($p < 0.05$) higher than in control, however, the egg weigh, relative shell weight (%) and shell thickness decreased with the same reliability. Recently, an increase in the counts of useful lactobacilli in the intestines of layers that received 0.5 or 1.0 % linseed oil was also reported [109].

Water plants. Alternative sea sources of ω-3-PUFAs attract researchers, since some auto- and heterotrophic micro-algae produce quite significant amounts of these FA, primarily EPA and DHA. The fatty acid profile in a number of species *Phaeodactylum* and *Nannochloropsis* (produce EPA up to 40 % of total FA), *Thraustochytrium* and *Schizochytrium* (synthesize significant amounts of DHA up to 30-40 % of FA), as well as some *Chlorella* species (produce significant amount of ALA) [110] are most studied. In aquaculture, it is possible to increase the FA deposition, including targeted ones, by controlling the composition of the growth media and conditions [111]. Water plants are a valuable source of protein for farm animals [112]. In addition, significant quantities of oily substances remained as a by-product after PUFAs extraction from algae

could be used in production of diesel biofuels [113]. Another important advantage of algae as a component of animal feed, including laying hens, is the high content of natural carotenoids (β-carotene, canthaxanthin, astaxanthin, etc.). In laying hens, they improve yolk color and antioxidant status of eggs [114]. Algae contain various trace elements, but the effectiveness of algae as dietary source of trace elements for poultry and the influence on the mineralization of egg shell have not been practically studied [115]). When feeding algae *Schyzochytrium* (0.5 % and 1.0 %) from week 40 of life for the next 6 weeks [115], the egg production approximated the control, and the quality of the shell and the color of the yolk improved. The blood triglycerides and total cholesterol significantly decreased in both experimental groups, DHA concentration in the eggs significantly increased (from 0.70 % in the control to 0.75 and 0.88 % for two doses of the additive), and ω-6-PUFAs/ω-3-PUFAs value significantly decreased (from 11.36 in the control to 8.20 and 6.08).

It turned out that the use of algae as a dietary source of ω-3-PUFAs is also associated with the problem of fishy smell. When comparing flaxseed and powdered dried algae *Nannochloropsis oculata* at equal dosages (8 %), it was found [116] that DHA level (111.62 mg/egg) was significantly ($p < 0.05$) higher for the algae than for flaxseed (98.66 mg/egg). However, with an organoleptic evaluation of boiled eggs, the scores for fishy smell, hardness and elasticity of egg yolk in the control were significantly better than in both test groups ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively), and more than half of the tasters preferred eggs from the control poultry.

Enrichment of eggs with ω-3-PUFAs to a large extent depends on the species of algae. It was reported that at 5 or 10 % of dietary dried biomass *Nannochloropsis gaditana*, the accumulation of ω-3-PUFAs (especially EPA) in the egg was low [117]. But in another study [118], when feeding the preparation of dehydrated whole-cell algae (All-G-Rich, Alltech, USA) for 70 weeks of the productive period, the DHA concentration in eggs linearly and significantly ($p < 0.05$) increased as the dosage grew (85, 187 and 240 mg/100 g for the groups receiving 0, 1 and 2 % additives). There were no influence of algae on the intensity of egg production (84 %), the egg weight (61.5 g), feed conversion (1.59 kg per 12 eggs), egg shell weight (9.9 % of the egg weight) and strength. In general, studies on the use of algae as a source of ω-3-PUFAs in egg enrichment have yielded rather encouraging results. Possibly, over time algae will become more important as a component of feed, including that in Russia, but so far the availability of these additives is limited.

The effectiveness of stabilizing ω-3-PUFAs with antioxidants. It is known that dietary ω-3-PUFAs together with antioxidants promotes oxidative stabilization of lipids in the liver, meat, and egg yolk. This method allows to avoid fishy smell, although in some cases not effectively enough, especially when using fish oil [63]. In the eggs, various natural and synthetic compounds and their mixtures, carotenoids, flavone and benzoquinolone antioxidants, vitamins C, A and E, selenium, iodine, gallates, butylated hydroxyanisole and hydroxyltoluol etc. were tried as antioxidants [119].

The most effective stabilizer of yolk lipids, enriched with ω-3-PUFAs, is fat-soluble vitamin E. Its antioxidant properties are associated with the ability to block lipid peroxidation by binding radicals of fatty acid hydroperoxides due to the formation of ether in phenolic groups and delocalization of the electron density along the aromatic structure of the vitamin fragment of the ether molecule [120]. When comparing the role of different doses of vitamin E (α-tocopherol, 0, 50, 100 and 150 IU/kg feed) and butylated hydroxytoluol (BHT, 0, 50, 100 and 150 mg/kg feed) in rations with 10 % flaxseed [40], vitamin E in all the doses, except for the lowest one, more effectively maintained the ω-3-PUFAs

concentration in eggs stored at 4 °C. At 50 IU/kg vitamin E and BHT doses of 50-100 mg/kg, the ω-3-PUFAs in eggs decreased by 13-17 %. It should be noted that egg concentration of vitamin E during storage also decreased. A relationship between productivity of the layers and the dose of dietary vitamin E was reported. With an increase in the latter from 27 to 50 IU, the egg production significantly ($p < 0.05$) increased from 94.3 to 96.1 % [53]. The addition of vitamin E at 100 IU/kg to a diet with 10 % flaxseed significantly ($p < 0.05$) increased the rate of SRTBA in liver of the chickens compared to those fed with flaxseed without vitamin E, providing SRTBA within the control values in the birds fed neither flaxseed nor vitamin E [59].

Interesting results were obtained in the study of accelerated oxidation of PUFAs in vitro [121], when the stability of ALA and DHA was evaluated in the presence of different doses of α-tocopherol (0.0, 0.25, 2.5 and 5.0 mg/mg of FA) at 70 °C. In the absence of vitamin E, DHA concentration decreased after 24 hours of incubation to 78.2 %, after 72 hours —to 43.0 % of the initial value, for ALA the indicators were 89.2 and 52.6 %, respectively. All the investigated doses of vitamin E increased the safety of both FA compared to the control without the vitamin, but the improvement was not as clear as could be expected, as in the experiment, unlike natural conditions, vitamin E could not restore its activity after neutralizing the oxidized FA. Thus, after 24 hour incubation, the best protection for DHA was observed at 2.5 and 5.0 mg/mg vitamin E (87.4 and 87.9 %), for ALA the doses were 0.25 and 2.5 mg/mg (99.1 % and 98.1 %), whereas 5.0 mg/mg vitamin E resulted in the percentage for ALA close to that in the control (only 90.4 %). After 72 hour incubation, the highest DHA protection with vitamin E was at 0.25 and 2.5 mg/mg (79.6 and 62.5 %), and the maximum dose (5 mg/mg) provided only 55.9 % protection. For ALA, the lowest dose of vitamin E was also more effective (94.2 % at 0.25 mg/mg and 87.4 % at 2.5 mg/mg), and at the highest dose, ALA protection did not exceed 77.0 %. These data reflect a non-linear relationship between vitamin E concentration and PUFAs stabilization (up to a decrease in stabilization of high PUFAs lipids under an excess of vitamin E), as well as a significantly higher oxidative stability of ALA in contrast to DHA.

Thus, enriching the diet of layers with sources of ω-3-polyunsaturated fatty acids (PUFAs) to increase their content in a functional egg can lead to hypolipidemia, lipidosis, a decrease in yolk weight and appearance of fishy smell. Additives based on fat of oily fish do not provide oxidative stability of long-chain (LC) PUFAs in feed, poultry and eggs, fishy smell often appears in products, and the availability and environmental safety of such additives are low. Flaxseed, oilcake and oil products are inferior because of anti-nutrients and low (compared to fish oil) LCPUFA deposition in eggs, but these additives are cheaper and more available, can provide higher oxidative stability of lipids, and caused less fishy smell of eggs. The micro-algae rich in PUFAs are perhaps physiologically optimal, but not universally available and insufficiently studied supplements. Long-term research allows to recommend maximum dietary dose in laying hen feeds of 1.5-2.0 % for fish oil, 5-8 % for flax seed or oilcake, 3 % for flax oil. An increase in the dosage can reduce productivity in laying hens. Enrichment with ω-3-PUFAs, which reduces oxidative stability of egg lipids, requires additional dietary antioxidants, of which vitamin E is most popular.

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MOLECULAR GENOTYPING OF CHICKEN (*Gallus gallus* L.) FEATHERING GENES IN CONNECTION WITH SEPARATION BY SEX

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Abstract

Traditional breeding is time and material consuming. Modern laboratory techniques significantly speed up and reduce the costs for breeding animals with the desired properties. A test based on the quantitative real-time PCR (qPCR) technology was developed to distinguish between homozygous and heterozygous state of the genes from alleles *K* and *k* which are responsible for the rate of wing feather growth in day-old chicks. The use of quantitative real-time PCR for the analysis of genotypes is aimed at the discrimination between one and two copies of the target gene in a genome. To obtain reliable results, certain rules must be followed when conducting the assay: the efficiency of the PCR should be close to the maximum; it is possible to obtain a significant number of false results without the appropriate statistical analysis. A new assay algorithm was proposed to overcome the limitations of qPCR: all samples are subjected to two successive independent analyses in parallel with the reference samples of both genotypes; if the two runs produce divergent results then the assay is repeated and the previous results are discarded. This approach allows to reduce assay error probability down to zero. The new system consists of three (instead of four) primers for amplification of two genes and two probes, allowing efficient analysis of various allele *K* genotypes. Quantitative real-time PCR data analysis was performed by ΔΔCt method using the statistics software package SPSS for ROC analysis. Using the method developed, the percentage of *KK*, *Kk* and *kk* genotypes was determined in 145 cocks of original lines B5, B6, B7 and B9 of domestic meat chicken of cross Smena 8. It was shown that 19 cocks of line B5 and 15 cocks of line B6 had *kk* genotype. From the 46 cocks of line B7, none had *kk* genotype, 17 cocks (37 %) had *Kk* genotype, and 29 cocks (63 %) had *KK* genotype. From the 65 cocks of line B9, none had *kk* genotype, 17 cocks (26 %) had *Kk* genotype, and 48 cocks (74 %) had *KK* genotype. Analyzed fragments were sequenced to exclude the effects of possible nucleotide sequence variability on the assay. The sequences did not contain any nucleotide substitutions in the sites of the primers and probes annealing. The data obtained will accelerate selection of new domestic meat chicken breeds with possibility of sexing based on feather length in day-old chicken. Further breeding work involves the assessment of the offspring using traditional and molecular genetic methods.

Keywords: real-time polymerase chain reaction, qPCR, genotype, gene copy number, autosex chickens, poultry selection, meat chickens

Gender identification (sexing), using simple phenotypic techniques (for example, differentiation according to the growth rate of feathers of the wing, according to the color of the feathers), gives a number of advantages in compari-

son to the traditional method, in which the presence of genital tubercles is determined. The solution of this urgent task will significantly reduce the requirements for personnel, the percentage of errors and allow rapid of sorting of chickens. For broiler sexing, a different rate of wing feathering is used in chickens of the opposite sex at 1-day age [1-3]. The genetics of this phenomenon is well studied [4-6]. The allele of slow feathering *K* is located on the Z chromosome and is linked to the gender. Cocks in the genome have two male sex chromosomes ZZ, and hens have one male Z and one female W. Sex of chickens at 1-day age can be determine by a development of the wing feathers, which is inherited through sex-linked alleles, dominant *K* and recessive *k* (rapid feathering) [7]. In 1-day-old chickens having the genotype *KK*, *Kk* or *Kw*, the formation of the feather cover of the wing is slow (the feathers are weakly developed, they are either the same in length or the covering feathers are longer than the flight feathers), in individuals with *kk* or *kw* genotypes, the flight feathers of the wing are well developed, and the covering feathers are shorter than the flight feathers. Crossing cocks homozygous for the fast feathering gene *kk* and hens with the gene of slow feathering *Kw* leads to offspring, among which all chickens with fast wing feathering are females *kw*, and chickens with slow feathering are males *Kk*.

A detailed study of the *K* gene located in the integration region of the endogenous retrovirus ev21 made it possible to establish a relationship of the doubling of the tandem partially deleted genes of the flagellar sperm protein *dSpef2* and the prolactin receptor *dPrlr* with the *K* genotype [8]. A study of the mechanisms determining slow feathering showed the importance of expression of the products of the *dSpef2* and *dPrlr* genes in the skin [9, 10). The prolactin receptor, whose ligand is the pituitary hormone prolactin, belongs to the family of receptors of cytokines and is expressed in all tissues of vertebrates. About 300 different functions are attributed to prolactin, including the maintenance of water-salt balance, participation in growth and development, metabolism, hormonal and brain activity, control of behavior, regulation and functioning of the immune system [11], reproduction, egg production in hens (12), induction of molting [13].

The peculiarity of the domestic cross Smena 8 is its universality. With a stable high rate of growth of young animals, which allows reducing the time of growing, the bird has a stable reproduction, is well adapted to Russian conditions (unlike foreign crosses) and both cage and floor housing technologies. The parent flock has a high egg-laying peak (85 %). The cross is also characterized by good viability, uniformity of the flock, high yield of pectoral and leg muscles, effective feed conversion (http://www.gossort.com/docs/rus/REESTR_SKOT2015.pdf).

The production of autosexing chickens with different feathering at 1-day-old age (by the example of the crossing of B7 cocks and chicks and B9 hens of the meat cross Smena 8) is illustrated by the scheme:

Line	B7 (♂) × B9 (♀)	
Genotype	*kk*	*Kw*
Trait	Fast wing feathering	Slow wing feathering
Offspring	B79	
Genotype	*Kk* (♂)	*kw* (♀)
Trait	Slow wing feathering	Fast wing feathering

Sexing is necessary for separated broiler farming. This technology provides more efficient use of feed, better survival, increased similarity in weight, that is, improves the output of broilers at lower economic costs.

We have proposed a new test system that allows to determine the number of copies of the gene responsible for the rate of feathering in chicks, as well as the algorithm for analysis of gene copy number by quantitative real-time PCR. This makes it possible to genotype *K* and *k* chicks effectively and with high throughput

to accelerated production of the original meat cross lines of the Smena 8 giving autosexing offspring.

The aim of the study was to develop a test for the molecular typing of fast and slow feathering genes in chickens using real-time PCR (PCR-RT).

Technique. For development of the test system and the genotyping algorithm we used 145 DNA samples of cocks of B5, B6, B7 and B9 domestic meat lines of cross Smena 8.

DNA was isolated from the selected feathers using the M-Sorb set (ZAO Sintol, Russia). For sample preparation, 0.5 cm tip of the quill was placed in a 1.5 ml test tube (OOO Tekhosnastka, Russia). A 400 μl lysis solution was added and incubated at 37 °C for 20 min with stirring, the lysate was precipitated in a high-speed microcentrifuge Cyclotemp-902 (ZAO Cyclotemp, Russia) for 3 min at 13,000 rpm. The supernatant was transferred to a 1.5 ml test tube and isolation continued according to the standard M-Sorb set protocol. The DNA concentration was determined on a NanoPhotometer spectrophotometer (Implene, Germany). About 3 μg of DNA was taken into the reaction ($A_{260}/_{280}$ = 1.8-2.0). The primers and probes for PCR-RT and sequencing presented below are based on known DNA sequences [9, 10, 14] and synthesized in ZAO Synthol; fluorescent labels were carboxyfluorescein (FAM) and 6-carboxyrodamine (R6G) with the 3′-terminal modification of phosphate (p):

LFBF1	5′→3′: CTCACTGAAACCATCCCTGGA
LFBR1	5′→3′: CTAACCTGAGACAAGTGTCGGA
LFBV1 (зонд)	5′→3′: (6FAM)-CCCCTTAAATGCC(dT-BHQ1)TGCTTTTCCATC-p
NFR	5′→3′: CTGTGGTTTGCTCGGTTTTGGA
NFP (зонд)	5′→3′: (5R6G)-CTCCATCTCTCC(dT-BHQ1)TGCTTTTCCATC-p
PROSF	5′→3′: GTTTGCCTATCACAGCATTGTAGA
PROSR	5′→3′: GCTGGTTCCTCATCCTGTCTGA
SPEFR	5′→3′: GTGCAATTTAGCAGTGCATGTGA

Multiplex PCR-RT (with FAM and R6G detection channels) was performed on an ANK-32M (Institute of Analytical Instrument Engineering, Russia) using the following temperature-time cyclogram (40 cycles): denaturation at 93 °C for 15 sec, annealing at 60 °C for 30 sec. A reaction mixture (20 μl) was used for PCR-RT (ZAO Sintol, Russia). The primer concentration in the reaction mixture was 450 nM, the probe concentration was 250 nM. The specificity of PCR-RT was confirmed by sequencing the amplification products obtained with primers PROSF, PROSR and SPEFR, on a Nanofor 05 genetic analyzer (Institute of Analytical Instrument Engineering, Russia) in accordance with the manufacturer's protocol.

Results. The primer system LFBF1, LFBR1 and the LFBV1 probe allowed the detection of the *dSpef2 dPrlr* gene, the primer system LFBF1, NFR, and the NFP probe revealed the reference *Prlr* chicken prolactin receptor gene. Primers PROSF, PROSR and SPEFR were used for sequencing fragments of the genes *Spef2* and *Prlr*.

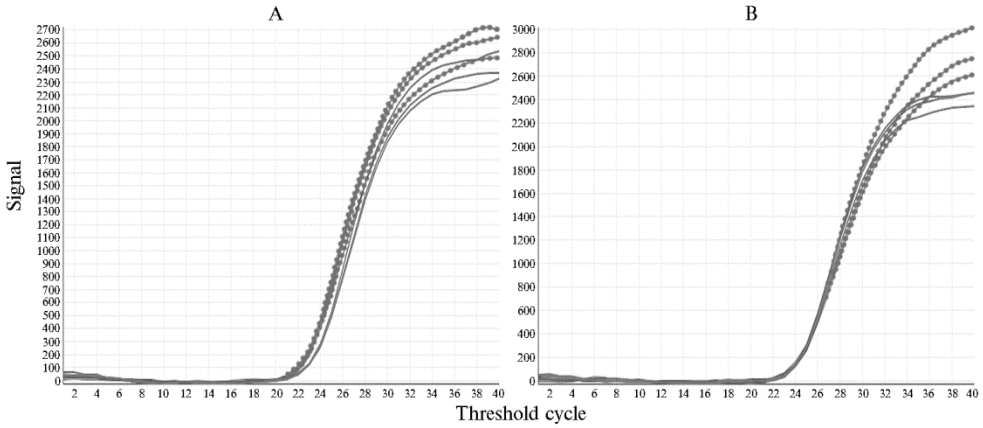
The determination of genotypes *KK* and *Kk* by PCR-RT is reduced to the problem of discrimination of two copies of a gene in the genome from one [8, 15, 16]. It can be solved by quantitative PCR [8, 17], as well as by hybridization of nucleic acids [15, 18, 19]. Each of these approaches has advantages and disadvantages. Southern hybridization is laborious, takes a lot of time and requires a variety of equipment. When quantitative PCR (PCR-RT) is used to solve the task, the results may be inadequate if the necessary conditions are not met during the test and subsequent statistical processing of the data [15, 18, 19]. However, with appropriate execution, this method is highly technological and allows for a short time to accumulate a large array of information. For reliable testing it is necessary that in one cycle of PCR the amount of DNA is doubled and

the amplification efficiency approaches the maximum [20, 21]. Also, you need to carefully monitor its probable fluctuations, which can lead to incorrect results.

The selected primers and probes were analyzed in multiplex PCR-RT for two samples (No. 64 and No. 65) in a series of five 2-fold DNA dilutions in two replicates. The amplification efficiency of the *dSpef2 dPrlr* gene was 92 % (R^2 = 0.9945) with 94 % for the reference prolactin gene (R^2 = 0.9991). The use of one common LFBF1 primer to amplify two genes makes the proposed system more robust by reducing the number of components and smooths possible differences in primer performance.

In preliminary experiments to determine the genotypes of chicks, the samples were examined in 2-fold replicates. Up to 10 % of the samples in one of the experiments yielded results not only lying in the so-called "gray" area, but also reliably opposite in the repetition of one sample. To increase the reliability of the test, we developed a new algorithm that differs from that described earlier [8]. For each batch of examined samples, it is mandatory to use at least four reference samples for each of those genotypes that are being analyzed. Then a re-independent determination is made with a similar set of samples. The results in each of the two tests are counted, and when any deviation is detected, a new definition with reference samples in two independent repeats is performed. Such an algorithm makes it possible to reduce the error probability to a minimum.

Using a new algorithm, 19 B5 samples of the DNA of the cocks from the line and 15 B6 were analyzed. In 100 % of cases, the genotype *kk* was confirmed, which completely correlated with the phenotype for these two lines.



The result of genotyping cocks of the B7 cross-line Smena 8 using real-time PCR: A — sample No. 64 (ΔCt = 0.8) for genotype *KK*, B — sample No. 65 (ΔCt = 0.02) for genotype *Kk*; « — » — detection channel FAM (*dSpef2 dPrlr*), « — » —detection channel R6G (*Prlr*).

To study the cocks of the lines B7 and B9, four reference cocks with the *KK* genotype and four with the genotype *Kk* were used. Examples of the kinetic curves of PCR amplification obtained for samples No. 64 and No. 65 of B7 are shown in the figure. For each analyzed sample, the difference in the threshold reaction cycles for the tandem *dSpef2 dPrlr* (detection channel FAM) and the prolactin gene (detection channel R6G) was calculated by the formula ΔCt = Ct (FAM) – Ct (R6G). As can be seen from the figure, the threshold cycles of amplification of the fragment of the reference gene of the prolactin chicks (detection channel R6G) are greater than the threshold amplification cycles of the fragment of the *dSpef2 dPrlr* gene (the detection channel FAM) (see Fig., A), or equal to them (see Fig., B). The difference is explained by the fact that DNA samples of individuals having the *KK* genotype contain twice as many copies of the *K* gene as in the case of cocks with the genotype *Kk*. The sample genotypes

were analyzed using the ΔΔCt [22] method in accordance with the description [8], the data was processed with the SPSS statistics package [23] using the ROC-analysis [24]. The use of other binary classifiers [25] and statistical methods [18] to increase the reliability of results can be promising in such studies.

The results of the study of samples from 46 cocks of the line B7 showed that none of the genotypes were identified as *kk*, 17 (37 %) had the genotype *Kk* and 29 (63 %) had the genotype *KK*. According to the analysis of samples from 65 cocks of the line B9, none has the genotype *kk*, in 17 (26 %) the genotype *Kk* was found and in 48 (74 %) the genotype *KK* was found. To exclude the possible influence of the variability of the investigated gene sections, sequencing of the DNA amplification products obtained with primer pairs PROSF and PROSR (*Prlr* gene), as well as PROSF and SPEFR (*dSpef2 dPrlr* gene) was carried out. It was found that the sequences in the analyzed regions are generally invariant and do not contain nucleotide substitutions in the annealing areas of primers and probes.

In the future it is supposed to estimate the population by traditional and molecular genetic methods. According to the results, the cocks of the B7 line with the genotypes *KK* and *Kk*, as well as the cocks of the B9 line with the genotypes *Kk* and *kk* and their descendants will be excluded from the selection, which will allow obtaining lines with a given genotype.

Thus, a test was developed and an algorithm was proposed for detecting in 1-day chickens the homo- and heterozygous state of sex-linked *K* and *k* alleles associated with the wing feather growth rate, using quantitative real-time PCR. With the help of the proposed test, the percentage of genotypes *KK*, *Kk* and *kk* was determined among 145 cocks of the lines B5, B6, B7 and B9 of the domestic meat cross Smena 8. Further selection work involves the evaluation of the bird by traditional and molecular genetic methods to exclude from the selection the cocks of line B7 with genotypes *KK* and *Kk*, cocks of line B9 with genotypes *Kk* and *kk*, and also their descendants as not corresponding to the target parameters.

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PANCREATIC SECRETION AND INTESTINAL DIGESTIBILITY OF AMINO ACIDS IN CHICKEN AT DIFFERENT DIETARY PROTEIN LEVEL AND QUALITY

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Abstract

The improvement of feed conversion into the products in poultry production is primarily related to the effectiveness of digestion and assimilation of dietary protein and therefore sets specific requirements on its nutritive value and profile of limiting amino acids, and on the availability of amino acids for absorption and subsequent metabolization. Digestibility and absorbability of amino acids were recently studied in pigs (E.N. Golovko, 2011) and poultry (P. Dalibard et al., 1995; A.E. Yapontsev, 2016) using the concepts of apparent and standardized ileal digestibility. Ileal digestibility is defined as the difference between consumed amounts of amino acids and the unabsorbed residues at the level of distal end of the ileum. Data on ileal digestibility rates allow optimization of dietary protein quality, digestive function and appetite (V. Ryadchikov et al., 2010). Our earlier studies showed that secretory function of the pancreas in adult chicken and broiler chicks can adjust to the changes in dietary amino acid balance; when dietary level of limiting amino acid (lysine) reached the recommended level the enzymatic activities in pancreatic juice grew substantially (Ts.Zh. Batoev, et al., 1990; V.G. Vertiprakhov, 2015). In this study we have experimentally found differences between enzymatic activities in pancreatic juice and ileal chymus and ileal amino acid digestibility in chronically fistulated chickens which were fed diets of different protein levels. Control diet was standard wheat-based, and experimental diet was supplemented with poorly hydrolysable ingredients (wheat bran, sunflower cake) contained lower levels of crude protein and energy. When chickens were fed with experimental diet instead of control one the decreases in proteolytic activity in pancreatic juice by 22-36 % and in ileal chymus by 34 % (compared to control) were found. Data on ileal digestibility of amino acids showed that intestinal digestion of amino acids is more effective at higher dietary protein level; this effect can be related to the increase in enzymatic activities of digestive juices in response to the increase in dietary protein, and to the concomitant improvement in feed palatability.

Keywords: exocrine pancreatic function, pancreatic enzymes, ileal chymus, amino acids, chicken

In the digestive tract, food is converted into substances able to be absorbed in the body, which ultimately determines the metabolic processes. Increasing the conversion of feed to livestock products, including physiological methods, is relevant. Effective use of fodder proteins by the bird puts forward certain requirements, i.e. usefulness, the optimal ratio of the amino acids that limit productivity, accessibility to absorption and involvement in metabolic processes. In recent years, there has been enough papers to consider amino acid assimilation in digestion of pigs [1-3] and poultry [4, 5]. For this purpose, methods are proposed that allow one to determine the apparent and true ileal digestibility of amino acids. It is known [1] that the traditional ways of determining the availability of amino acids in the terminal part of the digestive tract do not allow obtaining reliable indices because of significant qualitative and quantitative changes in the composition of nitrogen-containing substances by microorganisms

inhabiting the large intestine. Estimation of the availability of amino acids based on the difference in their input with food and undigested amount in ileum is performed on ileostomized animals by ileal method. This method showed that the animal appetite depends primarily on the balance of diets for essential amino acids [6].

Digestive enzymes of the small intestine adapt to the source of the consumed protein [7]. Previously, we found that the secretory function of the pancreas of broilers and hens respond to the balance of amino acids in the diet, and when the limiting amino acid is introduced to the norm of demand, the enzymatic activity of pancreatic juice significantly increases [8, 9]. In the study of microbial communities, their composition correlated to metabolism of essential amino acids in laying hens [10]. However, we do not know the papers considering a relationship between the availability of amino acids and the food taste.

In this paper, using the ileal method, we first showed that the assimilation of amino acids in the digestive tract depends on the secretory function of the pancreas, which adequately responds to the quality of protein nutrition.

Our goal was to study, in physiological experiment, the activity of enzymes in pancreatic juice and chyme of the ileum, as well as the content of amino acids in the ileum of hens when using compound feeds different in protein levels.

Technique. Pancreas secretion was studied in a chronic experiment on two fistulated 7-8-month old Leghorn hens according to C.Zh. Batoev et al. [11]. For the first 10 days the birds were fed with a control compound feed corresponding to zootechnical norms, and the next 10 days the compound feed containing hardly hydrolyzable components (wheat middling, sunflower oilcake) was given. Experiments started in the morning on an empty stomach. After the first 30 min the hens received 30 g of compound feed, and then pancreatic juice was collected for 2.5 hours with 30-min intervals. In the portions of the secret, the volume of juice and the activity of amylase, proteases and lipase were determined.

The enzymatic activity and the amino acid composition of the chyme were analyzed in three Leghorn hens aged 30-40 days with T-shaped cannula implanted in the ileum at 10-15 cm distance from the thick intestine. The experiment was started in the morning on an empty stomach, the birds received 30 g of compound feed, and within an hour the intestine chyme was collected. The sample was centrifuged for 5 min at 5,000 rpm, and the supernatant was diluted (1:50) with Ringer solution.

Activity of amylase estimated by Smith-Roy-Ugolov method [12] as the amount of starch (mg per ml juice) cleaved for a minute, and proteolytic activity expressed as the amount of casein (mg per ml) hydrolyzed for a minute were recorded at λ = 670 nm and λ = 450, respectively, on a photometer KFK-3 (OOO Zagorsk Optical and Mechanical Plant, Russia) [13], the lipase activity was assayed on a semi-automatic biochemical analyzer BS3000P (SINNOWA, China) using veterinary diagnostic reagent kit for the determination of blood lipase in animals (DIAKON-VET, Russia).

Free amino acids in the ileal chyme was determined by ion exchange chromatography with post-column derivatization with a ninhydrin reagent and detection at λ = 570 nm (for proline λ = 440 nm). The analysis was performed using a high performance liquid chromatography (HPLC) YL 9100 HPLC System (Young Lin Instrument Co., Ltd, Korea) consisting of a YL9110 gradient pump, YL9101 vacuum degasser, YL9120 UV/VIS detector, YL9150 autosampler (postcolumn derivatizer Pinnacle PCX, ion exchange column Na^+ 4.0×150 mm, 5 μm, pre-column Na^+ 3.0×20 mm, 5 m, Pickering Laboratories, Inc., USA).

The mean (X) and standard errors of the mean (x) are shown. The sig-

nificance of differences was evaluated by Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$.

1. Feed composition

Ingredients	Content, %	
	control	experimental
Wheat	33.6	44.7
Barley	45.1	22.9
Wheat middling	0	13.6
Soy bean meal	9.4	0
Sunflower cake	4.5	11.3
Lime (36 %)	2.3	2.4
Monocalcium phosphate	1.1	0.9
Maize gluten	1.0	1.0
Fish flour	1.0	1.0
Sunflower oil	1.0	1.0
Salt	0.25	0.25
Sodium sulfate	0.196	0.205
Mixture:		
mineral 0.08 %	0.080	0.080
vitamin 0.02 %	0.020	0.020
Methionine	0.066	0.091
Per 100 g of fodder		
Metabolic energy, kcal	270.0	260.0
Crude fat, %	3.57	4.98
Crude fiber, %	5.0	6.0
Crude protein, %	16.0	14.0
Calcium, %	1.20	1.20
Phosphorus, %	0.64	0.70

Results. The applied technique of collecting pancreatic juice is unique due to specific morphology of digestive tract in birds, with three pancreas ducts and two bile ducts joined to duodenum in the same place. When ileostomy, a fragment of the intestine (an isolated sac) is cut out, to which the main pancreatic duct is placed, and the intestine fragments are joined "end-to-end". The technique allows to obtain pancreatic juice during experiments, and in other the time juice is directed to the external anastomosis in the intestine. After the recovery period, the bird can be used for a long time.

Composition of the compound feed used is shown in Table 1.

Our experiments showed the exocrine function of chicken pancreas to be clearly adapted to the fodder (Table 2), and this was mainly due to a change in enzymatic activity, and not the amount of pancreatic juice.

2. Exocrine function of the pancreas in Leghorn hens depending on the consumed feed ($n = 20$, $X \pm x$)

Parameter	Hen 1		Hen 2	
	control	experiment	control	experiment
The amount of pancreatic juice per experiment, ml	5.4±0.49	6.8±0.50	7.0±0.31	8.8±1.15
Activity:				
amylase, mg · ml $^{-1}$ · min^{-1}	3400±240.8	3534±187.5	1984±86.1	2800±530.7
lipase, U/I	7792±396.5	9824±498.9*	11515±768.9	13518±74.6*
protease, mg · ml $^{-1}$ · min^{-1}	257±18.4	200±14.4*	164±11.4	105±5.3*
Amylase:protease	13:1	18:1	12:1	27:1

* Differences with control are statistically significant at $P \leq 0.05$.

The secretion of the great amount of enzymes necessary for digesting incoming food (and, as a consequence, an increase in their concentration in pancreatic juice) seems to be the most perfect type of specific enzymatic adaptations of the pancreas. An increase in the volume of secreted juice, a less effective and less specific mechanism, is used if specific adaptation is impossible for some reasons. In this, the amount of enzymes may not correspond to the diet so strictly. Adaptation of pancreatic enzymes can be studied in more detail by analyzing the dynamics of juice and enzyme secretion after ingestion [14, 15].

In our experiment there was a change in the activity of two enzymes, lipase and protease. In receiving experimental compound feed, lipase activity was 26.1 % ($P \leq 0.05$) higher in hen 1 and 17.4 % ($P \leq 0.05$) higher hen 2. On the contrary, proteolytic activity, with the replacement of compound feed containing 16 % crude proteins for those with 14 % crude proteins, decreased by 22.2 % and 36.0% for hen 1 and hen 2, respectively, compared to the control. The amylase:protease ratio also significantly changed and amounted to 12-13:1 in control period reaching up to 18-27:1 in experimental period. This indicates the adaptation of enzymatic activity to the low-protein diet.

Thus, when substituting more nutritious and energetically balanced feed

for low-protein diet with 10 kcal less metabolic energy, the exocrine function of the pancreas responded by 22-36 % decrease in proteolytic activity and 17-26 % increase in lipase activity as compared to control. This was due to the addition of wheat middling (13.6 %), sunflower oilcake (11.3 %), barley (22.9 %), which are difficult to hydrolyze, and 39.4 % more fat (see Table. 1). And the most significant differences occurred during the first hours after feeding because of the complex reflex regulation of pancreatic secretion and, thus, due to the fodder taste [14]. Given the fact that the pancreas is one of the central organs of the digestive system and its function correlates with the digestibility of the fodder [16], the observed changes should suggest a decrease in the digestion efficiency of the dietary protein.

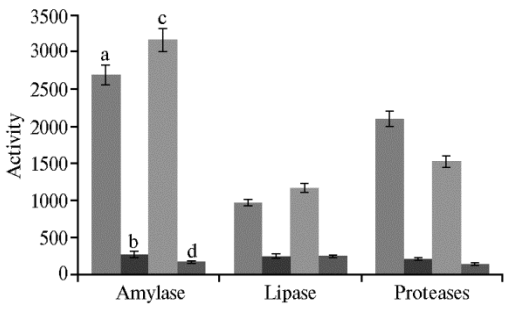
Activity of digestive enzymes in chyme of ileum, the main part of which are pancreatic enzymes, adapted to the nutrition (Table 3).

3. Enzymatic activity in chyme of the ileum in Leghorn hens depending on the consumed feed (average for 3 hens, $n = 20$, $X \pm x$)

Indicators	Compound feed		Vs. control, %
	control	experimental	
Amylase activity, $mg \cdot ml^{-1} \cdot min^{-1}$	269±44.1	167±16.4*	62.1
Lipase activity, U/I	2479±293.3	2435±185.8	98.2
Protease activity, $mg \cdot ml^{-1} \cdot min^{-1}$	21.0±1.39	13.8±1.55*	65.7

* Differences with control are statistically significant at $P \leq 0.05$.

The most significant changes were in the amylolytic and proteolytic after the replacement of compound feed containing 16.0 % of crude proteins for that with a reduced crude protein content (14.0 %). In this, the amylase activity was 37.9 % lower, and proteolytic activity was 34.3 % lower. The substitution of the control compound for the changed feed (see Table 1) did not affect lipase activity, despite the fact that the amount of fat in the fodders was somewhat different.



Amylolitic ($mg \cdot ml^{-1} \cdot min^{-1}$)**, lipolytic** (U/l) **and proteolytic** ($mg \cdot ml^{-1} \cdot min^{-1}$) **activity in pancreatic juice** (a — control, c — experiment) **and duodenal chyme** (b — control, d — experiment) **in Leghorn hens when using different compound feeds.** For a description of the composition of the fodder in control and experiment, see Table 1. The average values ($n = 20$) for 3 hens are given. The shown indices are 10 times decreased for lipase activity and 10 times increased for proteases.

Comparison of the activity of pancreatic enzymes in pancreatic juice and duodenal chyme led to the conclusion that when using a control compound feed, the activity of enzymes entering the intestine decreased 10 times for amylase and protease, and 4 times for lipase compared to the activity in pancreatic juice (Fig.). The replacement of the diet influenced the ratio of pancreatic juice enzymes entering the duodenum: the amylase activity decreased 19 times, lipase activity decreased 5 times, proteolotic activity decreased 11 times. Moreover, it should be noted that at experimental feeding the activity of amylase in pancreatic juice increased by 17.5 %, and in intestinal chyme decreased by 37.9 %, indicating dilution of duodenal chyme by other digestive juices, water, etc., or the formation of an enzyme-substrate complex in the cleavage of carbohydrates. This can be explained by experimental fodder composition with a higher content of non-starchy polysaccharides compared to control, which interfere with the interaction of digestive enzymes and substrates, and increase chyme viscosity [17, 18].

The activity of proteolytic enzymes in pancreatic juice and duodenal

chyme varied simultaneously, decreasing with the use of modified compound fodder (experiment). The ratio of lipase activity in pancreatic juice and duodenal chyme was the lowest (4-5:1), which is due to the relatively high activity of the enzyme in the intestine, since bile secreted into intestine along with pancreatic juice is the most powerful stimulant and fat emulsifier [19].

4. Amino acid composition of feed and chyme of the ileum in three Leghorn hens depending on the consumed feed (averaged samples)

Amino acid	In compound feed, %		Vs. control, %	In chyme, %		Vs. control, %
	control	experiment		control	experiment	
Cysteic acid	0	0		0.014	0.011	78.6
Aspartic acid	1.24	0.88	70.9	0.008	0.029	362.5
Threonine	0.55	0.45	81.8	0.015	0.047	313.3
Serine	0.69	0.60	86.9	0.007	0.026	371.4
Glutamic	3.17	3.15	99.4	0.025	0.057	228.0
Proline	1.18	1.17	99.1	0.015	0.037	246.7
Glicine	0.68	0.66	97.0	0.015	0.036	240.0
Alanine	0.75	0.70	93.3	0.026	0.025	96.1
Cisteine	0.30	0.28	93.3	0.011	0.005	45.5
Valine	0.75	0.67	89.3	0.017	0.034	200.0
Methionine	0.42	0.38	90.5	0.009	0.011	122.2
Isoleucine	0.61	0.51	83.6	0.012	0.027	225.0
Leucine	1.20	1.10	91.7	0.023	0.039	169.6
Tyrosine	0.49	0.44	89.8	0.013	0.033	253.8
Phenylalanine	0.77	0.65	84.4	0.017	0.031	182.3
Lysin	0.94	1.06	112.8	0.025	0.032	128.0
Histidine	0.43	0.34	79.1	0.006	0.016	266.7
Arginine	0.92	0.73	79.3	0.007	0.021	300.0
Total	15.10	13.77		0.270	0.520	

The amino acid composition of chyme in the ileum of chickens (Table 4) showed that the amount of proteins in the compound feeds varied by 1.33 %. Only for lysine the parameters for compound feed in the experiment exceeded the control ones by 12.8 %, and for the remaining amino acids a decrease was observed. When comparing amount of the basic amino acids to the norms recommended for laying hens [20], it can be noted that the control feed and the main ingredients of the experiment feed corresponded to these norms. In our experiment, during consumption of feed with hardly hydrolyzable components, the sum of amino acids in the chyme of the ileum increased, compared to the control period, to 3.77 % of the number of amino acids in the feed. In control period of feeding, 1.79 % of the amino acids in ileum remained undigested, which is almost 2 % less than during the experimental period. There is evidence that when the proportion of crude proteins in the diet decreases, the excretion of total nitrogen decreases as well, mainly due to an increase in the ileal digestibility of amino acids [21]. However, when using protein from a single source, the amount of protein does not affect the assimilation of amino acids in the ileum [22].

In our work, we was not motivated to establish the true availability of amino acids, but tried to connect the secretory function of the pancreas with assimilation of amino acids at different content and quality of proteins in the diet in order to understand the causes that affect the availability of amino acids for poultry. The question of the true availability of amino acids remains controversial which is caused by difficulties in determining endogenous amino acids, including limitations of almost all existing methods for their detection [2]. However, a comprehensive study of digestion processes on fistulated poultry, in our opinion, makes it possible to clarify and consider the processes of protein digestion and absorption, taking into account the neuro-humoral regulation. The advantage of this approach, laid down by academician I.P. Pavlov's in the physiology of digestion, is due to the opportunity to consider processes not fragmentarily [23], but in a body as a whole.

So, the feed most balanced in protein composition positively affects the

secretory function of the pancreas, stimulates the appetite of the poultry and contributes to an increase in ileal digestibility of amino acids.

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DIETARY PROBIOTIC *Lactobacillus plantarum* L-211 FOR FARM ANIMALS. I. THE ADDITIVE FOR BROILER CHICKS (*Gallus gallus* L.)

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Abstract

Lysine is an essential limiting amino acid in chick feeding. Its deficiency in feeds, especially of wheat-barley or corn-sunflower type, can reach 15-20 %. Dietary synthetic amino acids may negatively influence productivity due to imbalances caused by rapid amino acids entry into blood. This study continues a series of our experiments aimed at determining the efficiency of dietary synthetic lysine replacement by the microorganisms that synthesize L-lysine. Previous studies have shown a high positive effect of lysine producing *Escherichia coli* (Prolizer-BioR probiotic; JSC «Bioreactor», Moscow), however, seeking for similar producers among non-pathogenic microorganisms remains important. In this paper we present the findings in the support of *Lactobacillus plantarum* L-211 probiotic (JSC «Bioreactor», Moscow) ability to optimize the gut microflora in view to increase the productive performance in poultry. Using the T-RFLP (terminal restriction fragment length polymorphism), we compared the cecal bacterial community in four groups of Cobb 500 broiler chickens at 35 days of age. The diet in group 1 (control) was a balanced combined feed. The broilers of groups 2, 3 and 4 were fed with dietary domestic probiotics containing lysine-producing *Lactobacillus plantarum* L-211, *L. plantarum* which does not produce lysine, or *Bacillus subtilis* (a daily rate of 1 ml for lactobacilli and 2 ml for bacilli). The concertation of all live bacteria was 100 million CFU/ml. Lysine producing *L. plantarum* L-211 increased the level of lactobacilli by 3.88 times ($P < 0.005$), of cellulolytic and amylolytic *Clostridia* by 1.13 times, and of acid utilizing *Negativicutes* by 1.36 times ($P < 0.05$), whereas, on the contrary, reduced the portion of pathogenic peptococci by 1.35 times ($P < 0.05$), staphylococci by 1.46 times and enterobacteria by 2.33 times ($P < 0.005$). However, *L. plantarum* L-211, unlike *L. plantarum* or *B. subtilis*, did not affect the *Fusobacteria* or *Enterobacteriaceae* counts. Also, *L. plantarum* L-211 was not effective against pasteurella and actinomycetes which, on the contrary, increased in number 1.33- and 2.75-fold ($P < 0.005$) as compared to the control. The lysine-producing probiotic strain resulted in the highest average live weight in broilers at day 35 and the highest average daily weigh gain (5.01 % and 5.14 %, respectively). *L. plantarum* L-211 also led to the lowest availability of lysine in the diet.

Keywords: lysine, gut microflora, broilers, bacterial community, T-RFLP, probiotics, *Lactobacillus plantarum*, productivity, broiler chicken survival rates, feed conversion ratio

Ensuring high productivity in commercial poultry farming necessitates feeds which are balanced in all nutrients. Lysine, the essential limiting amino acid, is of particular importance. In vegetable feeds, its content is low and often not enough for animal and poultry diets, especially those with predominating cereals, sunflower meal and a small fraction (1-2 %) of ingredients of animal origin. In rations of wheat-barley and corn-sunflower type, the deficit of lysine can reach 15-20 %. Therefore, dietary synthetic lysine [1-3] is widely used for

balancing.

It is known that the introduction of synthetic amino acids in the diet must be limited because of their rapid absorption into the blood, as compared to amino acids of plant and animal origin metabolized in digestion, which leads to an imbalance of amino acids in the body and negatively affects productivity. Synthetic lysine monochlorohydrate leads to an excess of chlorine in combined feed and, as a consequence, to its imbalance, which also negatively affects productivity [4].

At present, feed additives based on bacteria that produce amino acids and other essential substances are used worldwide in poultry farming [5-7]. When studying the effect of the domestic bacterial preparation Prolisar-BioR, the efficiency of replacing synthetic lysine with its producer, thr strain *Escherichia coli* [8-10] was found. However, *E. coli* belongs to conditionally pathogenic microflora of the gastrointestinal tract and is a causative agent of colibacillosis in chickens when immunity is weakened [11-13]. Despite the fact that in our studies of lysine-producing non-pathogenic *E. coli* strain there were no cases of colibacillosis [14], searching for lysine producers of non-pathogenic microflora, e.g. lactobacilli, remains relevant.

The strain *Lactobacillus plantarum* L-211 was identified by analysis of 16S rRNA gene with a homology of 99 %. Phenotypically, strain *L. plantarum* L-211 is a small non spore-forming mesophilic facultative anaerobic gram-positive rod-like bacterium. According to biochemical signs, the microorganism is unable to metabolize glucose to CO_2 and to produce hydrogen sulphide and indole, is catalase negative, gelatin thinning, possessing nitrate reductase activity, reducing litmus milk, hydrolyzing L-arginine and fermenting most carbohydrates. Technologically, *L. plantarum* L-211 is characterized by acid formation of 80 °T in 24 hours, the limit of acid formation is 140 °T (7 days). The microorganism does not decarboxylate amino acids, therefore, the biogenic amines, histamine, tyramine, putrescine, cadaverine, are not forming. In mineral media, *L. plantarum* L-211 is capable of producing 148.4±4.45 mg/l lysine [14].

In this paper, we have shown for the first time the possibility to optimize the microflora of the gastrointestinal tract of the birds and their providing with amino acids, including lysine, using a *L. plantarum* L-211 based biological.

Our objective was to study the influence of the lysine producer *L. plantarum* L-211 on intestine bacterial community and productivity in broiler chickens.

Technique. Cobb 500 broilers (*Gallus gallus L.*) were divided into four groups, 35 heads each, and examined during day 1 to day 35 period of life in the vivarium FGUP Zagorskoye EPH VNITIP (Moscow Province). Chicks of the group I (control) were fed with combined feed balanced for all nutrients (basic diet, BD) as per the norms [1]. The bird in group II received the BD supplemented with a *L. plantarum* L-211 probiotic at a daily rate of 1 ml per head (OOO Bioreactor, Moscow), in group III the BD was supplemented with a developed domestic preparation based on *L. plantarum* at a daily rate of 1 ml per head dosage, in group IV a domestic probiotic additive based on *Bacillus subtilis* was used at a daily rate of 2 ml per head. In the preparations, the counts of living microorganisms were 100 million CFU/ml. The chickens were kept in Avi-Max cage batteries (Big Dutchman International GmbH, Germany) according to the technological parameters of VNITIP without separation by sex.

During the experiment, the main zootechnical indicators were recorded, i.e. live weight at 7-, 14-, 21-, 28-day-old age and by the end of experiment in individual weighing, survivability, daily weight gain, feed consumption and feed costs per 1 kg of live weight gain. In the physiological balance experiment on 35-day-old broilers, the digestibility of proteins, fat, the use of nitrogen, calcium,

phosphorus, the availability of methionine and lysine were determined [15, 16].

For molecular genetic studies, the cecum contents were collected at slaughtering on day 35 with strict sterility according to the requirements [16]. The composition of cecum bacterial community was examined by T-RFLP (terminal restriction fragment length polymorphism) method [17, 18]. Total DNA for T-RFLP analysis was isolated using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the manufacturer's protocol. PCR was performed on a Verity DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers 63F (CAGGCCTAACACATGCAAGTC) labeled at the 5'-end (WellFed D4 fluorophore, Beckman Coulter, Inc., USA) and 1492R (TACGGHTACCTTGTTACGACTT). Labeled amplicons of 16S rRNA gene DNA was purified by a standard procedure [18]. The resulting amplicons (30-50 ng) were restricted with HaeIII, HhaI and MspI endonucleases as per the manufacturer's recommendations (Fermentas, Inc., Lithuania). The restricts were examined using genetic analysis system CEQ™ 8000 (Beckman Coulter Inc., USA) according by the manufacturer's protocol. For phylogenetic affiliation, Fragment Sorter program (http://www.oardc.ohiostate.edu/trflpfragsort/index.php) was used.

For statistical processing, the variance analysis was performed in Microsoft Excel 2010. Mean (X) and mean errors (x) were calculated. Differences with the control were considered significant at $P < 0.05$; $P < 0.01$ and $P < 0.005$.

Results. Chicks were fed manually with dry feeds in full according to the norms of VNITIP for the cross (Table 1).

1. Composition of experimental combined feeds (base ration) **for growing Cobb 500 broilers**

Ingredient, %	Days 1-21	Days 22-35
Wheat	42.57	55.20
Corn	10.00	0
Sunflower fodder cake (protein content 34%)	9.86	7.18
Soybean oil	2.65	5.00
Choline chloride	0.07	0.07
Soy fatty fodder (protein content 34%)	25.00	23.94
Fish meal fodder (protein content 65%)	7.00	6.00
Methionine fodder (not less than 99%)	0.27	0.23
Lysine fodder (not less than 78%)	0.41	0.27
Monocalcium phosphate	0.47	0.59
Lime	1.36	1.13
NaCl	0.24	0.29
Premix (VNITIP, Russia)	0.10	0.10
Total per 100 g combined feed, %:		
dry matter	86.45	84.30
metabolic energy, kcal	310	320
crude protein	22.50	21.00
lipids	9.82	11.46
linoleic acid	4.68	5.52
crude cellulose	5.05	4.63
lysin	1.45	1.25
methionine	0.66	0.58
methionine + cystine	0.99	0.89
threonine	0.80	0.74
tryptophan	0.26	0.25
Ca	1.00	0.89
P	0.68	0.67
P digestible	0.39	0.39
Na	0.20	0.20
K	0.72	0.69
Cl	0.34	0.33
choline	0.05	0.05

T-RFLP analysis showed (Table 2) that the microorganisms of five phyla, i.e. *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria*, were identified in all groups, which was in line with our previous findings on cecum microbiome [19-21]. The representatives of phylum *Firmicutes* predomi-

nated, mainly cellulolytic and amylolytic bacteria of class *Clostridia* (families *Ruminococcaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Clostridiaceae*, etc.), acid utilizing bacteria of order *Negativicutes*, and families *Bacillaceae* and *Lactobacillaceae*. Bacteria of other phyla were less abundant.

Among conditionally pathogenic and pathogenic microorganisms, we found families *Campylobacteriaceae*, *Enterobacteriaceae*, *Pasterellaceae*, *Actinobacteriaceae*, phylum *Fusobacteria*, and the genera *Peptococcus* and *Staphylococcus*.

2. The bacterial taxa (%) in cecum of 35 day old Cobb 500 broilers fed with domestic dietary probiotics ($X \pm x$, vivarium FGUP Zagorskoye EPH VNITIP, Moscow Province)

Taxon	Group I (control, $n = 3$)	Group II ($n = 3$)	Group III ($n = 3$)	Group IV ($n = 3$)
Phylum *Bacteroidetes*	2.04±0.10	3.01±0.12**	2.76±0.14*	2.65±0.10*
Phylum *Firmicutes*	53.71±2.43	60.17±2.84	43.28±2.11*	63.80±3.01
class *Clostridia*	31.14±1.15	35.08±1.26	21.74±1.01**	40.14±1.98*
genus *Peptococcus*	2.36±0.14	1.75±0.05*	1.27±0.07**	1.94±0.08
genus *Lactobacillus*	1.72±0.08	6.63±0.31***	11.00±0.49***	10.60±0.60***
genus *Bacillus*	10.60±0.43	6.45±0.23**	4.40±0.21***	3.34±0.14***
genus *Staphylococcus*	0.67±0.23	0.46±0.21	0.38±0.14	0.26±0.11
order *Negativicutes*	7.22±0.29	9.80±0.42*	4.49±0.19**	7.52±0.39
Phylum *Actinobacteria*	2.82±0.13	7.76±0.30***	2.43±0.12	6.11±0.30***
семейство *Bifidobacteriaceae*	0.12±0.01	Brd	0.07±0.01*	0.04±0.01**
Phylum *Proteobacteria*	29.29±1.14	13.40±0.59**	25.47±1.02	15.06±0.62***
family *Enterobacteriaceae*	14.10±0.65	6.04±0.29***	15.70±0.78	1.07±0.04***
family *Campylobacteriaceae*	2.08±0.09	2.23±0.06	1.92±0.08	1.47±0.06**
family *Pseudomonadaceae*	9.43±0.58	1.71±0.05***	5.12±0.09**	8.12±0.27
family *Pasteurellaceae*	1.32±0.22	1.76±0.11	1.46±0.12	2.46±0.42
Phylum *Fusobacteria*	2.58±0.14	2.52±0.12	1.56±0.10**	2.18±0.17
Unclassified sequences	9.56±0.59	13.05±0.65	24.50±1.21***	10.20±0.52

N o t e. In the groups II, III and IV, the *Lactobacillus plantarum* L-211, *L. plantarum* and *Bacillus subtilis* probiotics, respectively, were added to the basic (control) diet (for more details, see the *Technique* section). Brd — below reliable determination by T-RFLP

*, **, *** Differences with control are statistically significant at $P < 0.05$, respectively; $P < 0.01$ and $P < 0.005$.

We found that the introduction of live probiotic bacteria into the diet of broilers led to a change in the number of identified taxa in cecum, which had similar tendencies for birds from experimental groups. Thus, in chickens, fed with probiotics, the proportion of genus *Lactobacillus* bacteria, which, as a rule, exhibits significant antagonistic properties against pathogenic species due to the synthesis of antimicrobial compounds [22, 23], increased 3.88 ($P < 0.005$), 6.40 ($P < 0.005$) and 6.12 times ($P < 0.005$) for groups II, III and IV, respectively, compared to control. The counts of other bacteria with similar activity against pathogens, the bacilli, decreased 1.13 ($P < 0.01$), 1.43 ($P < 0.005$) and 1.29 times ($P < 0.005$), respectively. In addition, in these groups, the bacteria of *Bacteroidetes* phylum involved in fermentation of carbohydrates due to amylolytic and cellulolytic activity, increased 1.48 ($P < 0.01$), 1.35 $P < 0.05$) and 1.30 times ($P < 0.05$), respectively, compared to control.

However, the effect of the preparations studied was not the same for bacteria of class *Clostridia* with amylo- and cellulolytic properties. Additives of the lysine producer *L. plantarum* L-211 and *B. subtilis* contributed to a 1.13-fold and 1.29-fold ($P < 0.05$) increase in the proportion of bacteria from the *Clostridia* class, respectively, whereas feeding with *L. plantarum*, not producing lysine, on the contrary, reduced this index 1.43 times ($P < 0.01$) compared to control. The effect of the *L. plantarum* L-211 preparation against *Clostridia* members is consistent with the data of foreign authors who showed that the lack of lysine in the diets reduces the number of genus *Eubacterium* in the cecum [24].

A similar trend was noted for acid utilizing bacteria of the order *Negativicutes*. Dietary lysine producer contributed their 1.36-fold increase ($P < 0.05$), whereas lactobacillus not synthesizing lysine caused a 1.61-fold decrease ($P < 0.01$) compared to control.

It should be noted that the additives contributed to a decrease in the number of pathogens in the cecum. All additives affected the proportion of pathogens causing purulent-necrotic infections. Counts of peptococci decreased 1.35 ($P < 0.05$), 1.86 ($P < 0.01$) and 1.22 times, of staphylococci was 1.46, 1.76 and 2.58 times lower in groups II, III and IV, respectively. Additionally, the lysine producer 2.33 times ($P < 0.005$) reduced the counts of enterobacteria, the causative agent of gastroenteritis. The *B. subtilis* proved its effectiveness against enterobacteria and campylobacteria, the causative agents of gastroenteritis, and fusobacteria, the causative agents of purulent-necrotic infections in animals and birds), resulting in a 13.17 ($P < 0.005$), 1.41 ($P < 0.01$) and 1.18 times decrease, respectively. Lactobacilli not synthesizing lysine, were effective against fusobacteria decreasing their counts 1.65 times ($P < 0.01$).

On the other hand, no effect of probiotics was observed against some pathogens. In groups II and IV there was a tendency to 1.33- and 1.76-fold increase, respectively, for pasteurellas which cause respiratory infections in chicks, and a 2.75-fold ($P < 0.005$) and 2.16-fold ($P < 0.005$) increase for undesirable actinomycetes of which some can cause actinomycosis.

Interestingly, in use of probiotics, the counts of *Pseudomonadaceae* representatives, which belong to transit microorganisms entering the body with food, decreased in cecum 5.51 ($P < 0.005$), 1.84 ($P < 0.01$) and 1.16 times, and of unidentified taxa increased 1.25, 2.18 ($P < 0.005$) and 1.08 times in groups II, III and IV, respectively.

3. Main zootechnical indices in 35 day old Cobb 500 broilers fed with domestic dietary probiotics ($X \pm x$, vivarium FGUP "Zagorskoye EPH VNITIP", Moscow Province)

Indices	Group I (control, $n = 3$)	Group II ($n = 3$)	Group III ($n = 3$)	Group IV ($n = 3$)
Stock preservation, %	97.1	94,3	100	97,1
Live bodyweight, g:				
on day 1	40.0±1.98	40,0±2,18	40,00±2,14	40,00±1,16
on day 7	120.22±2.88	112,22±3,25	117,44±2,77	114,22±2,84
on day 14	323.21±7.90	315,24±7,54	323,45±8,39	309,63±8,50
on day 21	708.42±13.72	668,42±14,44	698,16±14,95	671,58±14,35
on day 28	1128.0±36.89	1054,57±22,2	1110,14±38,30	1142,57±26,60
on day 35	1697.65±39.27	1739,68±33,17	1691,33±32,38	1687,20±39,85
including males	1882.50±38.97	1947,0±42,84	1900,0±26,42	1927,501±37,17
including females	1533.34±32.98	1640,95±23,06*	1587,0±22,74	1574,2±26,86
mean	1707.92±35.16	1793,5±33,19	1743,5±25,81	1750,85±31,45
Feed conversion rate per broiler, kg	2.748	2,779	2,681	2,635
Food consumption per kg of weight gain, kg	1.657	1,635	1,624	1,599
Average daily weight gain, g	49.05	51,57	50,10	50,32

N o t e. In the groups II, III and IV, probiotic preparations were added to the main (control) diet on the basis of *Lactobacillus plantarum* L-211, *L. plantarum* and *Bacillus subtilis*, respectively (for more details, see the *Technique* section).

* Differences with control are statistically significant at $P < 0.05$.

The poultry viability (Table 3) varied, with a difference of 3.7 %, depending on the probiotic used and was greatest in group III, where it was 2.9 % higher than in control and in group IV, and 5.7 % higher than in group II. The dynamics of live weight also differed depending on the group. Up to day 28 the control broilers had the largest live weight, on day 35 those of group II fed with dietary lysine producer *L. plantarum* L-211 was superior to other to other groups. The average live weight of the chickens of group II was 5.01 % higher compared to control, the males exceeded the control counterparts in live weight by 3.4 %, and the females exceeded by 7.0% ($P < 0.05$). Feed costs per 1 kg of live weight gain in group II decreased and was 1.635 kg vs. 1.657 kg in the control. Probably, the lower zootechnical indices for live weight in chickens from group II during the first growing period are associated with the use of the dietary

probiotic when lysine level in the diet was normative, which requires additional studies on its dosage.

It is necessary to note a slight lag in the live weight in group III, receiving *L. plantarum*, compared to control which was observed during the first period of fattening and was mainly due to the large number of females in the group. In the second period, the average live weight of broilers in group III exceeded the indicator in control by 2.08 % (by 0.93 and 3.50 % for males and females, respectively). At that, the feed costs per 1 kg of the live weight gain were below the control by 1.99 %.

In group IV with dietary *B. subtilis* the growth rate of the chicks on day 28 was higher, they were superior to group I in live weight with the control values exceeded by 1.30 % on day 28, and by 2.51 % to the end of fattening. Dietary *B. subtilis* probiotic also allowed to obtain the lowest feed costs per 1 kg of live weight gain, i.e. 1,599 kg vs. 1.657 kg in group I.

The results of physiological (balance) experiment (Table 4) were generally consistent with productivity. Thus, in all experimental groups the digestibility of the dry matter and the nutrients tended to decrease compared to control. We noted a lower digestibility of proteins, dry matter, fat and the use of nitrogen in chicks from groups II-IV, which indicates the need to adjust the way of application and dosage of probiotics (for example, use the intermittent supply with water). Note that not synthesizing lysine strain *L. plantarum* contributed to an increase in digestibility of cellulose by 0.5 % compared to control.

Higher availability of lysine was noted in chickens from groups III and IV who received bacteria not capable of producing lysine (93.8 % and 94.4 % vs 92.8% in control).

4. Use of feed nutrients in 35-day old Cobb 500 broilers fed with dietary probiotics ($X \pm x$, vivarium FGUP "Zagorskoye EPH VNITIP", Moscow Province)

Indices	Group I (control, $n = 3$)	Group II ($n = 3$)	Group III ($n = 3$)	Group IV ($n = 3$)
Digestibility, %:				
proteins	94.7±1.12	92.20±1.32	93.90±0.98	93.10±1.19
dry matter	73.7±1.26	67.00±0.67	71.00±1.24	69.60±1.14
lipids	83.2±1.01	80.20±1.85	81.30±2.13	80.10±0.95
cellulose	29.5±1.14	21.00±0.96	30.00±1.43	24.40±1.32
Metabolized elements, %:				
nitrogen	66.7±1.16	61.30±2.01	61.70±1.95	60.00±1.39
calcium	48.0±2.14	49.30±1.56	48.60±1.04	47.70±1.54
phosphorus	44.9±1.75	47.90±1.22	44.50±2.01	42.80±1.95
Availability, %:				
lysine	92.8±1.13	90.10±0.95	93.80±0.68	94.40±1.16
methionine	84.1±1.05	80.10±1.65	77.10±1.25	78.80±1.82

N o t e. In the groups II, III and IV, probiotic preparations were added to the main (control) diet on the basis of *Lactobacillus plantarum* L-211, *L. plantarum* and *Bacillus subtilis*, respectively (for more details, see the *Technique* section).

Thus, the use of dietary *L. plantarum* L-211 had a pronounced effect on the cecum bacterial community. Unlike the two other bacterial probiotics studied, the *L. plantarum* L-211 promotes a simultaneous increase in of cellulolytic and amylolytic *Clostridia* and *Negativicutes* bacteria. In addition, it has probiotic activity, affecting positively the number of lactobacilli and reducing opportunistic and pathogenic microflora including peptococci, staphylococci, and enterobacteria. It should be noted that dietary *L. plantarum* L-211 did not reduce the number of fusobacteria which were suppressed by the lactobacilli non synthesizing lysine, and campylobacteria which were suppressed by dietary *B. subtilis*. Nevertheless, the probiotics were not effective against some pathogens, and, moreover, *L. plantarum* L-211 resulted in an increase in pasteurellas and actinomycetes compared to control.

So, our studies have confirmed the possibility of using a probiotic strain

Lactobacillus plantarum L-211 to improve the productivity of broilers. This dietary additive showed the best results for the average live weight on day 35 and the average daily weight gain. In the poultry receiving probiotics that do not have the ability to synthesize lysine, the availability of lysine was higher. The effect of the probiotics studied on cecum composition of pathogenic, opportunistic, transit microorganisms and actinomycetes differes depending on the bacterial preparation and the growing period, which indicates the reserves of increasing effectiveness of probiotic supplements by adjusting the regimens and the doses of their introduction into the diet.

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CHANGES IN LEUKOCYTE AND ERYTHROCYTE BLOOD PROFILE AND PARAMETERS UNDER A COMBINED *Anaplasma marginale* AND BOVINE LEUKEMIA VIRUS INFECTION IN CATTLE

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Abstract

The global spread of infectious diseases and large-scale import of cattle genetic resources lead to necessity of developing screening methods that estimate the danger of co-infection with different pathogens and its impact on animal adaptiveness. In this regard here we analyzed the variability of erythrocyte and leukocyte characteristics in dairy Black-and-White holsteinized cattle naturally infected by *Anaplasma marginale*, the causative agent of bovine anaplasmosis, and bovine leukemia virus (BLV). The results showed that BLV infection of cattle did not facilitate the cross-infection with *A. marginale* in cattle since more than half of *A. marginale*-free animals were BLV-infected, and about one-third cows were characterized by leukocytosis. Except an increased number of leukocytes and lymphocytes due to retroviral infection, *A. marginale*-free animals were characterized only by the absence of statistically significant correlation between the counts of erythrocytes and neutrophils as compared to *A. marginale*-infected cows, which may point at the activation of nonspecific defense mechanisms in *A. marginale*-infected animals. Testing animals for BLV infection by agar gel immunodiffusion (AGID) and polymerase chain reaction (PCR) assays revealed the proviral DNA integration in one AGID-negative cow, whereas in seven out of thirty four AGID-positive cows the proviral DNA was absent. Leukocytosis ($> 20\times10^9$ blood leukocytes per liter) was revealed only in six AGID- and PCR-positive cows. The only common feature of BLV-infected animals with moderate and severe leukocytosis was thrombocytosis, as well as disruption of correlational relationships between the number of agranulocytes and granulocytes in peripheral blood. The detected disruption of the network relationships between different leukocyte populations reflects deep changes in the immune system functioning induced by retroviral infection. We observed a deficiency of neutrophils in cows with leukocytosis, which is in consistency with the data on neutropenia in milk of BLV-infected cows with leucosis (M. Nishiike et al., 2016). Considering the absence of BLV diagnostic tests that are able to reliably exclude the false-positive or false-negative results, it seems that the most effective approach for herd sanitation may consist in a simultaneous quantification of viral load (the number of BLV RNA in peripheral blood cells) and estimation of leukocytosis severity.

Keywords: anaplasmosis, bovine leukemia virus, viral load, leukocytosis, neutropenia, erythrocyte and leukocyte characteristics

A global key problems of modern livestock is the loss from infectious diseases [1, 2]. Substantial change in the situation is not achieved, despite the improvement of vaccines and antibiotics and their wide use. It is assumed that the formation of groups of animals with increased resistance to the most common pathogens can be more promising. However, this requires in-depth understanding of the development of infection and the key links in the interaction between a pathogen and a host organism.

In dairy cattle breeding, the most common cause of economic losses is

due to enzootic bovine leukemia caused by *Bovine leukemia virus*, BLV) [3, 4]. In some countries of South America, for example in Argentina, 90.9 % of bovine cattle herds are BLV-infected [5]. Due to the complex effect on the host's immune system, methods for effective animal vaccination protecting against this retrovirus have not yet been developed [6, 7]. Control of the spread of BLV is complicated by the fact that, as a rule, the host's immune system performs a negative selection against B lymphocytes producing mature viral particles [8]. In order to prevent the spread of BLV, various programs are proposed to detect infected animals based on identification of antibodies to BLV in peripheral blood and (or) proviral DNA in genomes. In addition to immunological and genetic tests, counts of lymphocytes in peripheral blood of animals are used. The combination of all three methods is most effective and allows increasing the reliability of detection of infected cows [1]. However, it should be taken into account that the BLV infection in animals is often accompanied by an increase in the sensitivity to bacterial infectious agents, such as *Mycobacterium bovis* [9], to *Escherichia coli* [10], and by an increase in mastitis [11], which may, in turn, lead to changes in the number of peripheral blood leukocytes.

Anaplasmosis in cattle caused by *Anaplasma marginale* (*Rikskettsiales*: *Anaplasmatacea*) is another reason of significant economic losses in livestock [12-14]. The frequency of infected cattle depends geographically on the region. Thus, in the states of North Africa, for example, in Morocco and Tunisia, it is 25.4 and 29.1 % [15, 16], respectively, while in Central and South Africa this index ranges from 38 to 100 % [17, 18]. In the Russian Federation, according to veterinary reports, anaplasmosis is mostly recorded in the southern regions, Bryansk, Kaluga, Ryazan, Kaliningrad, Saratov, Tyumensk, Vladimir, Nizhny Novgorod, Novosibirsk and Ulyanovsk regions, Altai territory [19]. Thus, 21.4-56.0 % of cattle are infected by *A. marginale* in the south of the Tyumen region [20]. In different climatic regions, lethality at cattle anaplasmosis is also not the same and ranges from 10-30 % [21, 22] to 100 % [23. *A. marginale* refers to erythrocyte parasites. The proportion of infected erythrocytes in the acute stage of anaplasmosis can be 70 % or more [24, 25], and parasitaemia in the blood can reach more than 10^9/ml [26-28]. The main clinical manifestations of anaplasmosis are anemia with a decrease in the amount of erythrocytes up to 1.5×10^6/mm^3 and hemoglobin up to 4-2 g% [21], and jaundice which develop due to the destruction of red blood cells by cells of the reticuloendothelial system [29, 30]. Other symptoms may include fever, weight loss, cardiovascular disorders (arrhythmic pulse) and gastrointestinal disorders (forestomach impaction, constipation); in severe form, abortions, muscle tremors and convulsions are noted [12, 19]. The minimal infecting dose of *A. marginale* leading to the development of clinical signs, is 1.5×10^5 [21], however in *A. marginale* strains this value may vary. The ill animals become lifelong anaplasmic carriers, for which the rickettsemia ration is 10^{2}-10^7 anaplasm per 1 ml of blood [28].

Mechanisms of the immune response to *A. marginale* invasion in cattle have not been fully studied, neveretheless, it is known that γ-interferon secreting CD4+ T-lymphocytes and the production of IgG1 and IgG2, mainly to the immunodominant and hypervariable surface protein MSP2, as well as to the proteins MSP1, MSP3, MSP4 and MSP5 of *A. marginale*, are activated [31-35]. It is suggested that the effect of antibodies is directed at neutralizing *A. marginale* cells prior to their introduction into erythrocytes and/or opsonization followed by phagocytosis by macrophages [22).

In recent years, the neutrophils/lymphocytes ratio, the number of platelets and morphological variability of erythrocytes [36] are widely used as markers of chronic inflammation, including that associated with pre-neoplastic state in

various diseases. These parameters can be determined on an automatic hemoanalyzer, which allows obtaining fairly objective data on hemopoiesis modifications in the development of pathology.

In order to assess the sensitivity of BLV-infected animals to bacterial infections and the accompanying changes in blood morphological parameters, in this study we first tested of black-and-white Holstein cows for the presence of BLV proviral DNA in genomes and *A. marginale* in erythrocytes with regard to counts of cell populations in peripheral blood, i.e. the number of erythrocytes and leukocytes, lymphocytes, monocytes, neutrophils, eosinophils, basophils. In addition, the mean volume and heterogeneity of erythrocytes in diameter (anisocytosis) was compared as indicators reflecting the change in health, for example, when taking antimicrobial drugs, and the development of pathologies, including hormonal abnormalities, bone marrow abnormalities in leukocytosis, and certain malignant diseases. It was found that the infection of animals with BLV does not promote coinfection with anaplasma, moreover, of *A. marginale* free cows, more than a half are infected with BLV and almost $^1/_3$ have leukocytosis.

The aim of the paper was to study the variability of erythrocyte and leukocyte characteristics in specialized dairy cattle, naturally infected with *Anaplasma marginale* and the *Bovine leukemia virus*.

Technique. Blood for the study was taken from the jugular vein of 67 black-and-white Holstein cows aged 2-5 years (ZAO Mozhaiskoye, Moscow Region).

Erythrocyte and leukocyte profiles and erythrocyte characteristics were determined individually for each animal on an automatic hematological analyzer Abacus junior Vet5 (Diatron, Austria, the principle of operation is based on the Coulter method) using 100 μl of EDTA-stabilized fresh whole peripheral blood.

Carriers of BLV proviral DNA were detected using Mancini radial immunodiffusion (RID) and PCR protocol developed by us earlier [37]. Methods for assessing the infection of *A. marginale* and the rickettsemia are described in detail earlier [38, 39].

The data was analyzed in Statistica 6.0 software (StatSoft Inc., USA). Differences were considered significant at $P < 0.05$. The tables show the arithmetic mean (X) and the errors of the arithmetic mean (x).

Results. Of 20 individuals free of BLV proviral DNA in two tests (RID and PCR), 13 ones (65 %) were infected with *A. marginale*, and of 22 BLV proviral DNA carriers *A. marginale* were detected in 11 ones (50 %). That is, in the group of animals infected with BLV, there was no increased sensitivity to *A. marginale*.

When analyzing involvement of different cell populations of peripheral blood in the development of *A. marginale* infection, we assessed their presence in infected *A. marginale* cows and those free from infection (Table 1). According to the manufacturer's protocol, all the parameters studied on the hematological analyzer corresponded to the physiological norm for *Bos taurus*, with the exception of the clearly increased leukocyte counts in the cows not infected by the bacterial pathogen. The fact that in the animals infected by *A. marginale* the number of red blood cells also remained within the normal range could be explained by the persistent stage of the infection, which was indicated by the rickettsemia values of 1.58×10^5 to 2.31×10^6/ml blood characteristic of anaplasmosis [28].

Statistically significant differences ($P < 0.05$) between infected and free from *A. marginale* individuals appeared only in leukocytes and lymphocytes, and was directed towards a decrease in infected animals (see Table 1). Obviously, these differences are due to the fact that BLV-infected animals with a high leukocytosis were infected with a bacterial pathogen (see Table 1). Of 6 individuals with high leukocytosis ($> 20\times10^9$/l), only one cow was infected with *A. marginale*, and 5 cows entered the group of 18 individuals free of anaplasma. Conse-

quently, changes in the profiles of leukocyte populations induced by BLV did not increase the likelihood of infection with *A. marginale*.

1. Erythrocyte and leukocyte profiles of peripheral blood in black-and-white Holstein cows infected with *Anaplasma marginale* and free from infection($X \pm x$, ZAO Mozhayskoe, Moscow Province)

Parameter	Permissible limits for *Bos taurus*	Not infected by *A. marginale*		Infected by *A. marginale* (n = 23)
		total (n = 18)	free of *Bovine leukemia virus* (n = 7)	
Cell population:				
erythrocytes, $\times 10^{12}$/l	5-10	6.53±0.18	6.83±0.18	6.23±0.18
leucocytes, $\times 10^9$/l	4-12	15.21±1.63*	10.18±0.67	10.87±0.98*
lymphocytes, $\times 10^9$/l	2.5-7.5	9.85±1.77*	4.85±0.83	5.62±1.00*
monocytes, $\times 10^9$/l	0-0.84	0.74±0.17	0.52±0.11	0.34±0.08
neutrophils, $\times 10^9$/l	0.6-6.7	4.07±0.69	4.54±0.48	4.41±0.50
eosinophils, $\times 10^9$/l	0.1-1.0	0.51±0.09	0.45±0.05	0.49±0.06
basophils, $\times 10^9$/l	0-0.5	0.0089±0.0011	0.0110±0.0010	0.0104±0.0020
thrombocytes, $\times 10^9$/l	100-800	87.72±35.23	10.29±6.32	86.91±33.94
Morphology of erythrocytes:				
average volume, fl	40-60	45,56±0,89	44,86±0,82	45,00±0,73
variability in diameter, %		19,66±0,33	20,83±0,40	19,63±0,38

* Differences between infected and non-infected individuals are statistically significant at $P < 0.05$.

In the erythrocyte component of uninfected animals, there were statistically significant ($P < 0.05$) correlations between erythrocytes and eosinophils ($r = -0.5$), erythrocytes and platelets ($r = -0.5$), as well as between erythrocyte heterogeneity in diameter and the number of leukocytes ($r = -0.5$), lymphocytes ($r = -0.5$), platelets ($r = -0.6$) and basophils ($r = +0.6$). That is, in uninfected animals, there was correlation between an increase in the number of erythrocytes and a decrease in eosinophils and platelets, the markers of inflammation, and higher morphological variability of erythrocytes correlated with decreased levels of leukocytes and lymphocytes. Several other reliable correlations revealed in infected animals were between the number of erythrocytes and neutrophils ($r = +0.5$), erythrocytes and basophils ($r = +0.5$), between the heterogeneity of erythrocytes in diameter and the number of basophils ($r = +0.4$), platelets ($r = -0.7$) and the mean volume of erythrocytes ($r = -0.6$). Positive correlation of the number of erythrocytes and neutrophils allows us to assume the activation of a nonspecific immune response in *A. marginale* infected cows.

Thus, the generally accepted view that the infection of animals with one pathogen, accompanied by a change in immunoreactivity, contributes to an increase in sensitivity to another pathogen possessed no confirmation for *A. marginale* and BLV infections. In general, this is consistent with the conclusion of several authors that each infectious agent specifically interacts with the host's immune system, and for such pathogens these mechanisms often do not overlap [40].

Infection with the retrovirus BLV, regardless of anaplasma, since its presence, as we showed, did not change the numerical ratio of blood cell populations) (see Table 1) was evaluated in the traditional test for the presence of antibodies to BLV envelope proteins in the peripheral blood (RID) and by integration of the BLV proviral DNA into the host genome. Of 67 cows examined, 33 were seronegative in RID, but one cow contained BLV proviral DNA in the genome. In 7 of 34 RID positive animals no BLV proviral DNA was found. These data are consistent with the findings of previous studies, in which there was also no complete agreement between estimates of animal infection in the RID and the BLV proviral DNA detection [41]. It should be noted that this discrepancy was observed when different viral genes (*env*, *gag*, *pol*) were used to identify BLV proviral DNA integration. The data obtained are consistent with the recent report of M. Nishlike et al. [1]. After examining 774 cows, they showed that in 7 % of the animals with antibodies to BLV in blood the BLV

proviral DNA in the genome, when estimating by viral gene *tax* nucleotide sequences, was not detected.

It is not excluded that this difference can be based on the differences between animals according to the number of B-lymphocytes infected by BLV and the immune response to infection. In this connection, we compared the distribution of peripheral blood cell populations in BLV-infected and non-infected cows (Table 2).

2. Erythrocyte and leukocyte profiles of peripheral blood in black-and-white Holstein cows free from infection and infected by bovine leukemia virus at different development of leukocytosis ($X \pm x$, ZAO Mozhayskoe, Moscow Province)

Parameter	RID^-, BLV^- (n = 21)	RID^+, BLV^+	
		without high leukocytosis (n = 18)	with maximum leukocytosis (n = 6)
Cell population:			
Erythrocytes, $\times 10^{12}$/l	6.59±0.19	6.15±0.17	6.34±0.36
Leucocytes, $\times 10^9$/l	10.00±0.56	11.73±0.88	25.49±1.01
Lymphocytes, $\times 10^9$/l	4.14±0.24	6.12±0.73*	21.70±1.08**
Monocytes, $\times 10^9$/l	0.35±0.06	0.28±0.09*	1.61±0.24*
Neutrophils, $\times 10^9$/l	5.10±0.44	4.63±0.83**	1.66±0.36**
Eosinophils, $\times 10^9$/l	0.43±0.06	0.59±0.09	0.51±0.18
Basophils, $\times 10^9$/l	0.0119±0.0011	0.0088±0.0027	0.0067±0.0021
Thrombocytes, $\times 10^9$/l	12.90±9.91	160.81±47.55**	122.33±78.28**
Neutrophils:Lymphocytes	1.26±0.10	0.88±0.21*	0.08±0.02*
Morphology of erythrocytes:			
average volume, fl	44.19±0.83	46.12±0.86	46.50±1.09
variability in diameter, %	20.84±0.27	18.55±0.34	18.83±0.31

N o t e. RID — radial immunodiffusion, BLV — bovine leukemia virus (proviral DNA detected in the genome by PCR method).
*, ** $P < 0.05$ and $P < 0.01$, respectively, for thresholds of statistical significance of the differences between infected and BLV-free individuals.

3. Distribution of peripheral blood cells in black-and-white Holstein cows with antibodies to bovine leukemia virus in the absence of BLV proviral DNA (n = 7, $X \pm x$, ZAO Mozhayskoe, Moscow Province)

Cell population:	Indeces
Erythrocytes, $\times 10^{12}$/l	6,40±0,19
Leucocytes, $\times 10^9$/l	8,11±0,47
Lymphocytes, $\times 10^9$/l	3,57±0,13
Monocytes, $\times 10^9$/l	0,31±0,10
Neutrophils, $\times 10^9$/l	3,59±0,61
Eosinophils, $\times 10^9$/l	0,64±0,13
Basophils, $\times 10^9$/l	0,0042±0,0020

N o t e. The presence of antibodies was determined by radial immunodiffusion (RID), BLV provirus DNA in the genome was detected by PCR.

In animals with high leukocytosis, there were a statistically significant ($P < 0.05$) increase in the number of lymphocytes, monocytes and platelets, a decrease in neutrophils and, correspondingly, a decrease in the neutrophils and lymphocytes ratio. BLV-infected cows without high leukocytosis significantly differed from uninfected ones only in the number of platelets (see Table 2). In this case, in 7 cows with RID^+, but without the insertion of BLV proviral DNA, the distribution of cellular populations in the peripheral blood corresponded to the physiological norm (Table 3). The peculiarity of this group was an unusually high statistically significant ($P < 0.05$) correlation between the number of leukocytes and neutrophils ($r = 0.871178$).

In the cows free from BLV, as it resulted from RID and lack of proviral DNA, there were numerous positive correlations between the counts of agranulocytes and granulocytes, as well as platelets (Table 4), i.e. between the number of lymphocytes and neutrophils, lymphocytes and basophils, monocytes and eosinophils, neutrophils and basophils, eosinophils and basophils, monocytes and platelets, eosinophils and platelets (see Table 4). That is, in the animals free from infection, the changes in the profiles of the peripheral blood cells of white root were closely interrelated, whereas the BLV infection detected by RID^+ and the insertion of proviral DNA was, in fact, accompanied by the apparent de-

struction of all these positive correlations. In the infected individuals there were positive correlations between the number of lymphocytes and monocytes, and negative correlations between basophils and platelets, lymphocytes and monocytes, lymphocytes and neutrophils. In infected cows with relatively low leukocytosis, only two correlations were statistically significant, a positive one between lymphocytes and monocytes, and a negative one between basophils and platelets, and when high leukocytosis ($> 20\times10^9$/l) was developed, only one statistically significant negative correlation was found between lymphocytes and basophils.

4. Correlation coefficients between the number of agranulocytes, granulocytes and platelets in peripheral blood of black-and-white Holstein cows free from bovine leukemia virus (n = 20, CJSC "Mozhayskoe", Moscow Province)

Cell population	Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils	Platelets
Lymphocytes	1.000000	0.514220*	0.507795*	0.382689	0.591109*	0.064150
Monocytes	0.514220*	1.000000	–0.244767	0.675024*	0.147069	0.488814*
Neutrophils	0.507795*	–0.244767	1.000000	0.151900	0.626046*	–0.285535
Eosinophils	0.382689	0.675024*	0.151900	1.000000	0.464138*	0.558992*
Basophils	0.591109*	0.147069	0.626046*	0.464138*	1.000000	–0.055497
Platelets	0.064150	0.488814*	–0.285535	0.558992*	–0.055497	1.000000

N o t e. Antibodies to the bovine leukemia virus in radial immunodiffusion and proviral DNA in PCR were not detected.

* Correlations are statistically significant at $P < 0.05$.

In general, with regard to the physiological norm for different populations of peripheral blood cells, the marked differences between BLV-infected animals and the control group were manifested in thrombocytosis and at leukocytosis in a sharp drop in the number of neutrophils.

Neutrophils form the first line of cell defense against pathogens, largely providing innate immunity against microorganisms. When the pathogen is phagocytized, neutrophils produce free radicals that destroy it. It was found that in milk of cows infected with BLV, neutrophils are significantly reduced [11], while for individuals with leukocytosis and high load of BLV proviral DNA a reduced expression of γ-interferon (IFN-γ) by peripheral blood monocytes is characteristic. IFN-γ promotes phagocytosis and production of free radicals by neutrophils, which can, in particular, explain the decrease in neutrophil function along with the decrease in their number in BLV infected cows. Earlier publications also noted the fact that blood serum of BLV infected cows suppresses the phagocytic activity of neutrophils [42].

A large amount of data has been accumulated on the pronounced effect of expression of BLV proviral DNA, in particular *tax* gene, on stress reactivity in cells, apoptosis, cell renewal rates, and immune system of infected animals [7, 43, 44]. We have previously shown that it is in animals with high leukocytosis that expression of BLV RNA can be detected by RT-PCR [4]. These data coincide with the reports of many researchers, in particular Japanese ones, who found the greatest amount of BLV RNA in the peripheral blood of cows with the highest leukocytosis [1]. Obviously, the increased expression of BLV proviral DNA accompanied by leukocytosis will inevitably lead to changes in the network relationships between different populations of leukocytes which we observed in our study.

Thus, the prevalence of the combined infection of BLV and *A. marginale* and the changes in peripheral blood cell populations, revealed by us, do not reflect a mutual increase in sensitivity to these pathogens. The profiles of the leukocyte population mostly correspond to the norm, however a feature which distinguishes infected *A. marginale* cows from uninfected, is a statistically significant positive correlation between the number of erythrocytes and neutrophils.

As to BLV, the obtained data indicate that none of the currently available methods of confirming infection with this virus, i.e. detection of antibodies

to BLV in RID, PCR analysis for BLV proviral DNA, excess of the number of leukocytes above physiological norm of $10\text{-}12\times10^9$/l, does not allow to avoid false positive or false negative results. The most distinguishing feature of BLV infection, even at low leukocytosis, is the disappearance of the normally correlated links between the counts of different populations of leukocytes, indicating the destruction of the network relationships between them. Importantly, in animals with high leukocytosis, marked neutrophilopenia is observed, which coincides with the literature data on the change in the neutrophil content in milk at BLV infection and high leukocytosis [11]. Given the need for a simpler method for identifying the most infectious animals in industrial herds, it seems appropriate to simultaneously evaluate the viral load (the amount of BLV RNA in the peripheral blood) and the severity of leukocytosis.

So, infection by bovine leukemia virus (BLV) does not contribute to *Anaplasma marginale* coinfection. Statistically significant correlations ($P < 0.05$) for the erythrocyte component are not the same in uninfected and anaplasma-infected animals, and the positive correlation between the number of erythrocytes and neutrophils suggests the activation of a nonspecific immune response in *A. marginale* infection. The BLV infection leads to the destruction of positive correlations between the sizes of white blood cell populations observed in uninfected individuals. These significant differences result in the development of thrombocytosis, and in individuals with leukocytosis in a sharp drop in the number of neutrophils. When detecting BLV infection in herds, it is most expedient to evaluate leukocytosis in combination with the viral load estimated by the amount of BLV RNA in the peripheral blood.

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DETECTION AND GENOTYPING *Pasteurella multocida* OF FIVE CAPSULAR GROUPS IN REAL TIME POLYMERASE CHAIN REACTION

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Abstract

Respiratory diseases in calves cause significant economic losses in livestock. Bacterium *Pasteurella multocida* plays important role in the etiology of these diseases. It is known that five identified *P. multocida* capsular groups (A, B, D, E and F) differently affect animal epizooty. Identification of bacteria based on the cultural, morphological, biochemical properties is very labor-intensive and time-consuming. Molecular biology techniques, in particular, the polymerase chain reaction (PCR), quickly detect and identify microorganisms directly in samples of biological material, mixed or pure cultures. In this regard, the purpose of our research was to develop multiplex real-time PCR for the detection, genotyping and discrimination of five *P. multocida* capsular groups (A, B, D, E and F) in cattle. The target primers and probes to the highly conserved gene *kmt1* and the genes in the loci of capsule synthesis (*hyaD, fcbD, dcbF, bcbD* and *ecbJ*) specific to the capsular groups have been designed. The sensitivity of DNA detection for different bacterial groups ranged from 1.6×10 to 5.9×10^2 genomic equivalents per reaction, non-specific reactions were not observed. The diagnostic sensitivity of the test was 10^3 CFU/ml for pure cultures and 10^5 CFU/g for biological material. The developed PCR protocol allowed us to type 11 bacterial cultures which were previously characterized serologically and bacteriologically and related to capsular groups A, B, and D. The *kmt1* gene sequencing confirmed the results of PCR analysis. PCR analysis of 260 samples from died calves detected *P. multocida* in lung (63.3 %), the lymph nodes (42.6 %), and spleen (8.8 %). We did not revealed the circulation of *P. multocida* B and E capsular groups among the tested livestock, the majority of the samples contained *P. multocida* group A, in some cases, there was group D, and, in one case, group F.

Keywords: bacteria, *Pasteurella multocida*, real-time PCR, genes, capsular groups

Pasteurella multocida, a gram-negative, immobile, facultative anaerobic coccobacterium, is part of the commensal microflora of the upper respiratory tract of domestic and wild animals. The agent causes septic and respiratory diseases of cattle (with asignificant economic damage to livestock throughout the world [1-2], including in the Russian Federation [3-5]. The bacterium has five capsular groups (A, B, D, E, F) of different epizootological significance. The strains of capsular groups A and D are involved in respiratory diseases of calves and adult animals, B and E cause haemorrhagic septicemia in cattle and buffalo, and F is, though rarely, involved in septic and respiratory diseases of calves [6-8].

Microbiological identification based on the study of cultural and morphological and biochemical properties is a laborious and long-term process. Polymerase chain reaction (PCR) allows rapid detection and identification of microorganisms

directly in samples of biological material, mixed or pure cultures [9].

To identify the bacterium and genotyping its capsule groups, several PCR-based test systems with electrophoretic detection [10-11] of different diagnostic efficacy have been developed [12]. Real-time PCR (PCR-RT) makes it possible to increase the reliability of diagnostic results due to elimination of contamination, as well as to quantify the target DNA in the sample being analyzed. One PCR-RT protocol is currently described for the detection of the sequence of the *est* gene in *P. multocida* serotypes B:2 and E:2 associated with hemorrhagic septicemia of cattle [13]. This technique is based on the use of the intercalating dye SYBR Green which, however, does not allows to differentiate the capsular groups of the bacterium.

Data on the nucleotide sequence of the second region in the locus for synthesis of *P. multocida* capsule allowed identifying unique genes for each capsular group which encode proteins involved in the synthesis of group-specific capsular polysaccharides. The *hyaD* gene is responsible for synthesis of hyaluronic acid and is unique for group A strains, *fcbD* encodes chondroitin synthase in group F, *dcbF* is responsible for synthesis of heparan glycoside in D. Genes *bcbD* and *ecbJ* encode glycosyltransferase in strains of capsular groups B and E, respectively. Also cell wall protein gene *kmt1* highly conserved and unique for the *P. multocida* [14] is identified.

We first designed the primers and TaqMan probes which allow us to identify and genotype serogroups of *P. multocida* in a multiplex PCR with real-time detection with high specificity and sensitivity.

The purpose of our research was to develop a rapid and highly sensitive method for detection of *Pasteurella multocida* and genotyping its five capsular groups in bacterial suspensions and samples from sick animals.

Techniqur. Reference strains of *P. multocida* (1231, 681 and T80), as well as *Mannheimia haemolytica* (strain 16), obtained from the collection of Ya.R. Kovalenko All-Russian Research Institute of Experimental Veterinary (Moscow). Other cultures of *P. multocida* were isolated in the Siberian Federal Scientific Center for Agrobiotechnology RAS in 2013-2014.

A total of 260 samples of lungs, spleen, mediastinal and bronchial lymph nodes served as biomaterials. For study, 10 % suspensions were prepared. Samples were collected from six large dairy complexes in the Tyumen, Novosibirsk and Krasnoyarsk regions from Holstein-Frisian dead calves (aged from 4 days to 3 months) with signs of respiratory infection. Calves were kept in individual houses or cages at temperatures from –5 to –9 °C (cold method). Feeding and husbandry were consistent with physiological and zootechnical standards.

DNA from bacterial suspensions and tissue samples was isolated using the commercial Ribo-prep kit (Central Research Institute of Epidemiology, Russia).

Specific primers and probes were designed in Vector NTI 9.0.0 (InforMax, Inc., USA) using the *kmt1* and *cap* locus gene sequences of *P. multocida* bacteria (capsule groups A, B, D, E, F) deposited in GenBank (NCBI).

The amplification was carried out in real time PCR in a 30 μl reaction mixture containing 5 μl of the DNA template, 1× Taq buffer without Mg^{2+} (OOO Medigene Laboratory, Russia), 3.3 mM $MgCl_2$, 0.2 mM dNTP, 150 nM of each primer, 200 nM of each probe, 1.5 U SmartTaq DNA polymerase (OOO Medigene Laboratory, Russia) and sterile deionized water. The protocol: 5 min at 95 °C; 15 s at 94 °C, 20 s at 53 °C, 20 s at 72 °C (45 cycles) on a CFX96 amplifiers (Bio-Rad, USA); the detection channels was FAM/Green, ROX/Orange, Cy5/Red and R6G/Yellow at a cycling of 53 °C.

Positive control samples (PCS) were obtained by molecular transformation of *Escherichia coli* (Top 10 strain) with plasmids pCR® 2.1 (Invitrogen, USA)

containing specific DNA inserts of amplified bacterial genes. Synthetic fragments of DNA were used as DNA inserts specific for *P. multocida* capsule groups E and F. The plasmid DNA concentration was measured with Quant-iT dsDNA, HS reagent kit (Invitrogen, USA) on a QUBIT fluorimeter (Invitrogen, USA).

The analytical sensitivity of the method was evaluated separately for each determination. The diagnostic sensitivity of the method was established using 10-fold dilutions of *P. multocida* (CP-57, 681, MSC-13) cultures, the concentration of which was previously determined by standard bacteriological methods. The sensitivity of the PCR was evaluated with tissue material. For this, 100 μl of bacterial culture was added to 900 μl of a 10 % suspension of the lung or lymph node, mixed, sedimented, and the upper aqueous phase was examined. The number of bacteria was expressed as CFU per gram of tissue. DNA from the samples was isolated as described above.

To confirm the specificity of the reaction, the amplified gene fragments were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, USA). The products were analyzed by capillary electrophoresis in an automatic sequencer ABI PRISM® 3130xl (Applied Biosystems, USA).

Results. In PCR, using all the primers and probes developed by us (Table 1), all the studied reference strains and *P. multocida* cultures were identified without nonspecific reaction (Table 2).

1. Sequences of primers and fluorescently labeled probes for detection of *Pasteurella multocida* and genotyping of its capsule groups

Target gene	Primer and probe	Sequence (5′→3′)	Position	Reference sequence
		First reaction		
kmt1	P.m.-Kmt1 F	ATAAGAAACGTAACTCAACATGGAAATA	266-292	FJ986389
(*Pasteurella*	P.m.-Kmt1 R	GAGTGGGCTTGTCGGTAGTCTT	456-477	
multocida)	P.m.-Kmt1 Z	(FAM)-AAACCGGCAAATAACAATAAGCTGA-(BHQ1)	322-346	
hyaD	P.m.-A F	TTCGTTAAAAATGACAGCTATGC	9165-9187	AF067175
(capsular	P.m.-A R	ATAATCGTCAGAAGCTCATGCG	9388-9367	
group A)	P.m.-A Z	(R6G)-ATTTCTCAGCATTAACACATGATTGGAT- (BHQ1)	9217-9244	
dcbF	P.m.-D F	ATCGCATCCAGAATAGCAAACTC	3306-3328	AF302465
(капсульная	P.m.-D R	TCCGATGCTTTGGTTGTGC	3661-3643	
capsular	P.m.-D Z	(Cy5)-CCGATTAAACTCAAATCTAGGGACATACTT-		
group D)		(BHQ2)	3350-3379	
		Second reaction		
bcbD	P.m.-B F	GCGTGTATAACCTACATCTTCCCA	12541-12564	AF169324
(capsular	P.m.-B R	CGTCCATCAACACCTTTACTGC	12708-12687	
group B)	P.m.-B Z	(FAM)-TAGGCACAGAATATTCAAAACCCCGT-(BHQ1)	12618-12643	
ecbJ (capsular	P.m.-E F	TGGGCACATGCTCGCTTA	4539-4556	AF302466
group E)	P.m.-E R	CTGCTTGATTTTGTCTTTCTCCTAA	4896-4872	
	P.m.-E Z	(ROX)-ATGTGGCAAAGCGATCAATTCAGA-(BHQ2)	4631-4654	
fcbD (capsular	P.m.-F F	CGGAGAACGCAGAAATCAGAA	2885-2905	AF302467
group F)	P.m.-F R	CAACAACGACTTCAAATGGGTAG	3142-3120	
	P.m.-F Z	(R6G)-CTTGCTCCATTGCCAGATCATGTT-(BHQ1)	2947-2970	

Note. Each sample was examined simultaneously in two reactions. In the first, the bacterium *Pasteurella multocida* was detected and serogroups A and D were genotyped, in the second group serogroups B, E and F were genotyped.

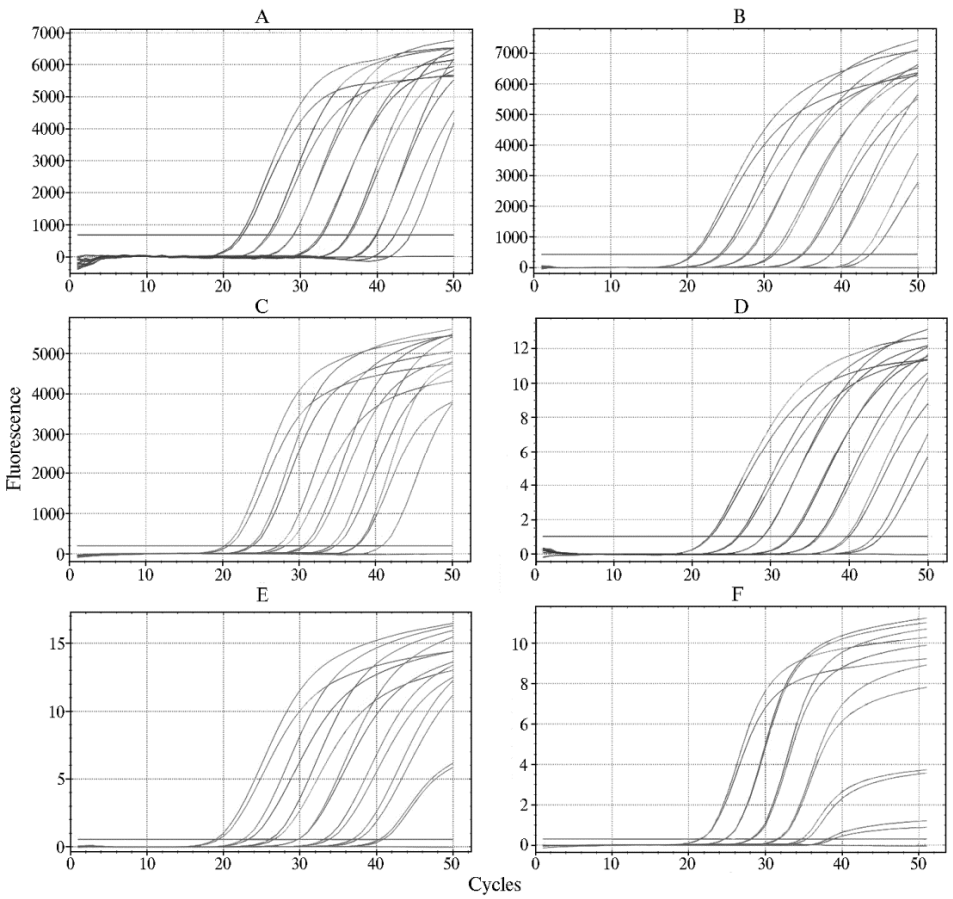
The results of capsular genotyping of bacterial cultures (see Table 2) coincided with the data obtained in previous studies [15]. Analysis of the nucleotide sequences of the *kmt1* gene amplicons in the cultures showed 99-100 % identity with fragments of the *P. multocida* gene form GenBank (KP212385, KP212386, KP212387, KP212388, KP212389, KP212390, KP212391).

The analytical sensitivity of the test was the last PCS dilution in which the PCR result was interpreted as positive. Samples with Ct not exceeding 40 were considered positive. Thus, the analytical sensitivity of the method was from 1.6×10 to 5.9×10^2 genomic equivalents (GE) per reaction (Fig.).

2. Bacterial cultures used in the work, and the results of their genotyping

Type of bacteria	Strain	Origin	PCR test	
			P. multocida	*P. multocida*
Pasteurella multocida	1231	Collection of microorganism cultures	+	A
	681	of the Ya.R. Kovalenko All-Russian	+	B
	T-80	Research Institute of	+	B
Mannheimia haemolytica	16	Experimental Veterinary	–	
Escherichia coli	F-50		–	
P. multocida	T-14	Collection of the Siberian Federal	+	A
	MSC-13	Scientific Center for Agrobiotechnolo-	+	D
	Sib-13	gy RAS	+	A
	Omsk-13		+	A
	UK-59		+	A
	SR-57		+	A
	AGM/2013		+	A
	OB-58		+	A
	T-14		+	A
Mannheimia haemolytica	Sib-13		–	
	Omsk-13		–	
Streptococcus pneumoniae	UK-59		–	
Clostridium perfringens	SR-57		–	
Klebsiella pneumoniae	AGM/2013		–	
Salmonella typhimurium	OB-58		–	
Salmonella paratyphimurium			–	

N o t e. "+" and "–" mean positive and negative result, respectiely.



Evaluation of the analytical sensitivity of the test system using dilutions of positive control samples (PCS) **obtained by molecular transformation of *Escherichia coli* (strain Top 10) with plasmids pCR® 2.1:** A — fluorescence of PCS/P.m.-Kmt1 samples, FAM/Green channel; B — fluorescence of PCS/P.m.-A samples, R6G/Yellow channel; C — fluorescence of the PCS/P.m.-D samples, the Cy5/Red channel; D — fluorescence of PCS/P.m.-B samples, FAM/Green channel; E — fluorescence of PCS/P.m.-E samples, ROX/Orange channel; F — fluorescence of PCS/P.m.-F samples,

R6G/Yellow channel. PCR and recording were performed on a CFX96 thermocycler (Bio-Rad, USA). Experiments were carried out in duplicate. Analytical sensitivity (in genomic equivalents per reaction) was 1.6×10 for PCS/P.m.-Kmt1, 7.2×10 for PCS/P.m.-A, 1.5×10^2 for PSC/P.m.-D, 9.0×10 for PCS/P.m.-B, 8.1×10 for PCS/P.m.-E, and 5.9×10^2 PCS/P.m.-F.

In the test, the primers and probes revealed *P. multocida* with the same efficiency and allowed genotyping capsular groups A, B and D. The diagnostic sensitivity of the test was 10^3 CFU/ml for bacterial suspension, and 10^5 CFU/1 g for the tissue samples.

3. Detection of *Pasteurella multocida* and genotyping of its capsule groups in organs form deid Holstein-Frisian calves

Biomaterial	Number of samples	Detection	Capsular group		
			A	D	F
Lungs	161	102/63.3	89/88.2	12/10.8	1/0.9
Lymph nodes	54	24/42.6	20/86.9	3/13.0	1/4.3
Spleen	45	4/8.8	3/75.0	0	1/25.0
Total	260	130/50.0	112/86.2	15/11.5	3/2.1

N o t e. The number of positive samples and the percentage of samples examined (detection) or those in which *P. multocida* DNA was detected (capsule groups) are Before and after slashes, respectively.

The developed PCR protocol was used to analyze samples of biological material from sick animals. The genome of *P. multocida* was detected in 63.3 % lung samples, 42.6 % lymph nodes, and 8.8 % spleen tissue (Table 3). *P. multocida* genotype A was in 82.6 % of positive samples, genotype D in 11.5 %, and genotype F in three samples (2.1 %). More frequent detection of capsular group A indicates that it played a more important epizootic role in the surveyed farms than the strains of the capsular group D. In one animal, the *P. multocida* bacterium of the capsular group F was genotyped in three samples. The circulation of genotypes B and E among susceptible animals has not been established.

Thus, we developed a method for the detection and genotyping of five capsule groups (A, B, D, E, F) of *Pasteurella multocida* in real-time PCR. The proposed method is high specific and sensitive when testing bacterial cultures and biological material from sick animals. Bacteria of capsular group A were found in the largest number of samples. The developed PCR protocol can be used in veterinary diagnostic laboratories as a simple, accessible and easily reproducible analog of serological typing, which allows to detect the *P. multocida* bacterium and to genotype five of its capsular groups at all stages of bacteriological studies. The use of RT-PCR will shorten the diagnosis and allow to optimize anti-epizootic measures, in particular vaccine selection and the development of a highly effective program for the prophylactic immunization.

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MYCOTOXINS IN THE LEGUMES OF NATURAL FODDER OF THE EUROPEAN RUSSIA

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Abstract

Evaluation of the negative effects of mycotoxins on ruminants and horses is a complex scientific problem and has important economic significance. Mycotoxicoses of animals caused by feeding leguminous grasses have been known for a long time, but their causes remain largely unclear. The purpose of this work was a comparative study of mycotoxin contamination of legumes of the genera *Lathyrus*, *Trifolium*, *Vicia*, *Melilotus*, *Medicago*, *Galega* and *Lupinus* from the natural fodder lands of the European part of Russia. The collection of ground parts of plants was carried out in May-September 2015 in the Moscow, Tver, Leningrad, Pskov, Novgorod, Smolensk, Astrakhan regions, Perm Krai, and the Republic of Karelia. The mycotoxins determined by enzyme immunoassay were T-2 toxin (T-2), diacetoxycirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), 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), PR toxin (PR) and ergot alkaloids (EA). In grasses of the genus *Lathyrus* (peavines), all the analyzed mycotoxins (with the exception of FUM and ROA) were found in more than 80 % of the samples; for clovers (*Trifolium*), the same frequency was found in T-2, OA, MPA, EA, AOL, CPA and EMO, and in species of the genus *Vicia* (vetchs), sweetclovers (*Melilotus*) and alfalfa (*Medicago*) there were only EA, AOL, CPA and EMO. A common feature for the genera *Lathyrus*, *Trifolium* and *Vicia* plants were high, > 1000 μg/kg, accumulation of DAS, AOL and CPA, and for peavines and clovers, PR also. Among the peculiarities of meadow clover (*T. pratense* L.), we found the ultra-high content of EMO, up to 30 000 μg/kg and more. White clover (*T. repens* L.) was characterized by moderate contamination as compared to other species of the genus. Representatives of *Vicia*, bush vetch (*V. sepium* L.) and cow vetch (*V. cracca* L.), showed similarities in the frequency of the majority of mycotoxins, but in cow vetch with a smaller occurrence in comparison to bush vetch, especially visible for FUM, the upper limits of the contents of EA, AOL, ROA, STE, MPA were higher. Meadow sweetclovers and alfalfa were found to be contaminated less than other grasses, while sweetclovers were close to *Vicia* in terms of detection frequency and amounts of OA and PR, and alfalfa was low in MPA and relatively high in EMO accumulation. Among other crops less common in meadows, *Lupinus polyphyllus* Lindl. had the highest mycotoxicological load — of 16 metabolites analyzed, all but FUM and ROA were more than 80 % in frequency. At Caucasian goat's rue (*Galega orientalis* Lam.) and narrow-leaved vetch (*Vicia sativa* L.) mycotoxins were detected less often and in smaller quantities. In this study previously published data on mycotoxicological analysis for meadow clover and white clover (A.A. Burkin and G.P. Kononenko, 2015) have been confirmed, but for other legumes the findings are presented for the first time.

Keywords: meadow grasses, legumes, *Lathyrus*, *Trifolium*, *Vicia*, *Melilotus*, *Medicago*, *Galega*, *Lupinus*, mycotoxins, T-2 toxin (T-2), diacetoxycirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), 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), PR toxin (PR), ergot alkaloids (EA)

Mycotoxicosis of ruminants and horses, in rations of which grain and

grass feed complement each other, have been receiving increasing attention in recent years [1-4]. It is known that fusariose grains and its derivative products are sources of T-2 toxin, zearalenone, deoxynivalenol [5, 6], fumonisins [7]. Besides, there is a threat of contamination of barley and maize with ochratoxin A [8], and the products form sunflower seeds (seedcake, oilcake) are often contaminated ин both ochratoxin A and citrinin. Risks from the use of grass fodders that predominate in the diet or serve as the only feed of ruminants are yet to be evaluated. Recently, there has been confirmation of the extensive combined contamination of mycotoxins for mowing grasses on long-term use fields [10-12], as well as for dry, haylage and ensilage feeds [13-16].

The specific internal mycobiota in mixed spontaneously arising plant communities, the susceptibility of plant stand to fungal diseases, including those caused by toxin-forming fungi, are formed as a result of complex direct and indirect biocenotic relations, the action of ontogenetic and environmental factors. That is why the evaluation of the negative effect of mycotoxins in free grazing is among the extremely complex problems. One of the solutions is a survey of wide samples of a botanically representative homogeneous material with regard to the areas of growth and the phases of vegetation. Recently, in meadow grasses this approach allowed to obtain the first information on the main carriers of toxicological load among wild cereals and to characterize the mycotoxin complex in some leguminous plants [17].

In the present study, for meadow clover and white clover, the results have been confirmed of which we have already reported [17]. Data on the prevalence of mycotoxins in vetches, peas, sweetclovers, alfalfa, lupine and goat's rue were obtained for the first time.

The aim of the work is a comparative study of mycotoxin contamination of leguminous grasses (*Lathyrus*, *Trifolium*, *Vicia*, *Melilotus*, *Medicago*, *Galega* и *Lupinus*) on natural forage lands in the European Russia.

Technique. The study was performed on 842 specimens of *Leguminaceae* family plants of genera *Lathyrus* (n = 111), *Trifolium* (n = 310), *Vicia* (n = 227), and sweetclovers (*Melilotus* spp., n = 76), alfalfa (*Medicago* spp., n = 88), *Galega orientalis* Lam. (n = 18) and Washington lupine (*Lupinus polyphyllus* Lindl., n = 12). For attribution of systematic groups we used identification keys [18, 19]. Species of genera *Lathyrus*, *Trifolium* and *Vicia* are described in the section "Results", among sweetclovers there were *M. albus* Medik. and *M. officinalis* (L.) Pall., though these species could not be identified before the beginning of flowering. To the plants of the genus *Medicago* were referred *M. sativa* L. with significant polymorphism and *M. lupulina* L. Samples was collected from May to September 2015 in Moscow (Balashikha, Dmitrov, Noginsk, Odintsovo, Podolsk regions and forest park in the floodplain of the Setun River, Moscow), Leningrad (Gatchina, Luga, Pushkin, Slantsy, Tosnensky regions), Pskov (Novorzhevsk region), Novgorod (Starorussky region), Smolensk (Gagarinsk region), Tver (Vyshnevolotsk region), Astrakhan (Enotayevsk region), in the Republic of Karelia (Loukhsk region) and Perm (Chaikovsky region), on meadows of different types, cattle stations and pastures, open slopes, on the banks of rivers, streams, lakes (reservoirs), clearing of the woods and outskirts, swaths, openings, willow stands and bushes, roadsides and head lands, near houses, on waste lands, field boundaries and roadside meadows. The aerial parts of plants were cut 3-5 cm from the soil surface, were air-dried in a room and milled.

For extraction, a mixture of acetonitrile and water was used in a volume ratio of 84:16 (10 ml per 1 g of sample). Extracts after 10-fold dilution with phosphate-buffered saline solution (pH 7.4) with Tween 20 were used for indirect competitive enzyme-linked immunosorbent assay. The 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), PR- toxin (PR) were determined using certified immunoenzyme test systems [20]. The lower limits of measurements corresponded to 85 % of antibody binding.

1. Species of genera *Lathyrus*, *Trifolium* and *Vicia* collected in different territories of the European Russia (May-September 2015)

Species	Number of samples
Lathyrus	
L. pratensis L.	93
L. vernus (L.) Bernh.	12
L. palustris L.	3
L. sylvestris L.	3
Trifolium	
T. hybridum L.	56
T. montanum L.	20
T. pratense L.	115
T. repens L.	69
T. medium L.	50
Vicia	
V. sepium L.	87
V. cracca L.	117
V. sylvatica L.	9
V. sativa L.	14

Results. Species of genera *Lathyrus*, *Trifolium* and *Vicia* in the samples are given in Table 1.

Mycotoxicological analysis for five genera of legumes represented by several species reflected an equally high frequency of detection of EA, AOL, CPA and EMO (in 80 % and more samples) (Table 2). All other mycotoxins were found less often in peas (*Vicia*), sweetclovers (*Melilotus*) and alfalfa species (*Medicago*), though T-2, OA and MPA were also common in clovers (*Trifolium*), and all tested mycotoxins, except of FUM and ROA, was frequent in vetches (genus *Lathyrus*) (see Table 2). Moreover, peas, sweetcloves and alfalfa were noticeably inferior to vetches and clovers in the frequency of contamination with fusariotoxins T-2, DON, ZEN, FUM, and also AB_1, OA, PR.

2. Occurrence (%) and accumulation of mycotoxins (μg/kg) in legumes of different genera (European Russia, May-September 2015)

Mycotoxin	*Lathyrus* spp. (n = 111)	*Trifolium* spp. (n = 310)	*Vicia* spp. (n = 227)	*Melilotus* spp. (n = 76)	*Medicago* spp. (n = 88)
T-2	89 (2-10-41)	86 (2-10-205)	45 (2-17-445)	63 (2-6-110)	59 (2-6-41)
DAS	86 (170-995-3020)	55 (50-325-4680)	20 (79-365-1035)	13 (100-260-425)	28 (79-265-630)
DON	81 (76-340-2065)	41 (55-190-520)	22 (74-150-375)	11 (74-125-180)	20 (79-140-250)
ZEN	88 (24-59-200)	55 (20-45-380)	22 (21-43-100)	30 (15-32-56)	35 (20-37-67)
FUM	61 (52-307-775)	22 (66-170-1780)	12 (76-185-420)	8 (74-95-120)	16 (87-190-265)
EA	98 (2-48-795)	93 (2-16-280)	85 (2-16-600)	97 (2-13-89)	89 (1-7-26)
AOL	98 (28-830-6310)	99 (30-300-1905)	96 (14-98-1515)	100 (20-105-1070)	100 (21-110-795)
ROA	77 (3-63-675)	30 (3-25-240)	22 (4-24-105)	11 (4-16-38)	20 (4-14-26)
AB_1	87 (2-15-89)	56 (2-5-79)	27 (2-5-19)	22 (2-3-7)	25 (2-4-6)
STE	92 (15-82-500)	65 (6-29-315)	48 (8-68-1320)	61 (8-20-45)	32 (10-24-56)
CPA	99 (79-885-5130)	99 (52-675-3550)	98 (63-425-2040)	99 (62-435-1335)	98 (76-330-935)
EMO	99 (21-205-775)	99 (20-2300-35500)	82 (10-49-775)	78 (16-51-335)	86 (13-170-2950)
OA	95 (6-73-200)	87 (3-25-245)	48 (4-9-28)	32 (5-8-13)	32 (4-11-76)
CIT	91 (31-145-500)	69 (8-63-820)	34 (25-76-315)	53 (19-49-185)	30 (20-52-200)
MPA	86 (14-88-830)	88 (12-45-530)	41 (11-40-280)	68 (13-39-250)	32 (10-25-47)
PR	85 (190-835-2755)	71 (23-510-1700)	26 (30-355-685)	28 (104-235-515)	31 (105-325-765)

Note. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, ROA — 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 examined. The minimum-average-maximum content of mycotoxin is given in parentheses.

Common to the genera *Lathyrus*, *Trifolium* and *Vicia* plants was a high accumulation of DAS (> 1000 μg/kg) and ZEN, EA, ROA, STE (> 100 μg/kg), while *Vicia* had amounts of STE > 1000 μg/kg. In addition, vetches and clovers were distinguished by a significant PR (> 1000 μg/kg) and OA content (> 100 μg/kg), the highest levels of occurrence of ROA, STE and accumulation of DON, EA, AOL, CPA were noted in vetches, and the ultrahigh contamination of EMO (> 30,000 μg/kg) was characteristic of clovers. Meadow sweetclovers and alfalfa were found to be less contaminated with mycotoxins with the maximum ac-

cumulation of DAS, ZEN, FUM, EA, PR, AB_1, STE was an order of magnitude lower, and there was a similarity to peas according to the frequency of detection and the amounts of OA and PR. In alfalfa, a low level MPA and a relatively high EMO (up to 2950 μg/kg) were noted.

Within each of the genera, groups with a narrow range (by an order of magnitude or within the order of magnitude) and a wide (two to three orders of magnitude) range of variation in the accumulation of mycotoxins were distinctly distinguished. The variability in the amounts of toxins could be due to both specific plant characteristics and seasonal fluctuations. Most often wide variation was noted in clovers (for 12 toxins), vetches and peas (for 7-8 toxins). These legumes were the most diverse in species composition (5 species of *Trifolium* and 4 species of *Lathyrus* and *Vicia*) (see Table 1). On the contrary, only a few metabolites (T-2, AOL, CPA in sweetclover, and EMO in alfalfa) varied considerably in quantity among the white sweetclover and yellow melilot, as well as wild medic with an admixture of clover (18 samples out of 88 ones). Perhaps, these species differed little in mycotoxin contamination due to the close composition of micromycetes and their similar response to external factors.

The interspecies differences in *Lathyrus* and *Trifolium* genera plants can be seen from Table 3.

3. Occurrence (%) and accumulation of mycotoxins (μg/kg) in legumes of genera *Lathyrus* and *Trifolium* (European Russia, May-September 2015)

Mycotoxin	Meadow vetch (n = 93)	Spring vetch (n = 12)	Meadow clover (n = 115)	Zigzag clover (n = 50)	White clover (n = 69)
T-2	96 (2-10-41)	75 (4-5-7)	98 (3-11-205)	86 (3-13-160)	78 (2-6-41)
DAS	97 (240-1035-3020)	33 (170-240-315)	71 (50-370-4675)	56 (63-225-500)	19 (50-170-315)
DON	89 (76-360-2065)	58 (105-125-195)	59 (55-190-520)	48 (120-225-390)	9 (84-135-200)
ZEN	99 (24-60-200)	42 (26-34-42)	62 (20-50-380)	72 (28-46-79)	59 (20-32-47)
FUM	72 (52-230-775)	8 (125	38 (77-195-1780)	20 (84-110-165)	7 (84-98-125)
EA	98 (2-56-795)	100 (2-13-48)	97 (2-22-280)	94 (2-20-115)	88 (2-9-60)
AOL	100 (28-940-6310)	100 (48-165-255)	100 (100-395-1905)	100 (33-440-1585)	99 (30-100-315)
ROA	87 (3-66-675)	33 (3-11-32)	43 (3-26-215)	36 (5-33-240)	19 (4-12-54)
AB_1	98 (3-16-89)	42 (2-6-12)	86 (2-6-79)	62 (2-5-14)	22 (2-3-4)
STE	99 (15-87-500)	75 (16-45-100)	84 (10-30-195)	76 (8-30-91)	41 (8-21-37)
CPA	100 (115-975-5130)	100 (205-445-1150)	100 (150-910-2500)	100 (140-1025-3550)	99 (74-350-935)
EMO	99 (25-235-775)	100 (39-66-125)	100 (125-5535-35500)	94 (39-330-4265)	100 (20-345-3090)
OA	100 (9-82-200)	75 (6-8-12)	100 (7-36-245)	90 (6-18-39)	58 (3-8-16)
CIT	96 (31-150-500)	92 (31-130-325)	78 (23-75-820)	82 (25-68-255)	61 (22-46-125)
MPA	95 (14-93-830)	67 (16-30-52)	97 (15-57-530)	82 (12-39-130)	86 (15-36-85)
PR	96 (190-860-2755)	33 (300-455-590)	99 (145-570-1700)	84 (190-560-1215)	14 (23-290-595)ë

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, ROA — 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 examined. The minimum-average-maximum content of mycotoxin is given in parentheses.

Meadow vetch predominating among the samples (93 of 111 ones) showed the specialties of contamination, characteristic of the genus (see Table 2). All mycotoxins, other than FUM, were present in more than 80 % of the samples, and there was a significant accumulation (> 1000 μg/kg) of DAS, DON, AOL, CPA, PR and > 100 μg/kg for the rest toxins, except for AB_1 and T-2. Given such a richness of mycotoxins, grass mixtures with meadow vetch should be used with caution. In addition, this species is characterized by a wide quantitative variability in DAS, DON, EA, AOL, ROA, which may be a consequence of hypersensitivity of mycobiota to growth conditions and plant growth phases. Spring vetch which was rare among the samples (12 out of 111 ones) was noticeably inferior to the meadow vetch in contamination (Table 3). Only EA, AOL, CPA, EMO were found regularly, the ranges of none of the mycotoxins went beyond the limits found in the meadow vetch, the maximum accumulation > 1000 μg/kg was retained only in the CPA, and the amounts of ZEN, EA,

ROA, OA and MPA were an order of magnitude lower, not reaching 100 μg/kg. When comparing these species, one can not ignore the fact that they have different areas, i.e. the meadow vetch usually grows in meadows, and is rarely found in the outskirts, clearings of the woods or in the clarified areas of the forest, whereas the spring vetch is common in forests and openings.

For meadow clover, the results coincided with those described earlier [17]. In the preliminary study in 2014, conducted on a collection of 35 samples in the Moscow, Tver regions and the Republic of Karelia, mycotoxins were represented by 12-15 components in all habitats and in different periods of vegetation (July, August and September), and the EMO content was kept ultrahigh (up to 27,540 μg/kg). In this large-scale study, accumulation of > 1000 μg/kg was found for a greater number of toxins, i.e. for DAS, FUM, AOL and PR in addition of CPA. In meadow clover in comparison to the meadow vetch, T-2, DAS, FUM and EMO also were among the mycotoxins with a wide varying accumulation. Apparently, a greater number of toxin-forming micromycetes affecting this culture are sensitive to habitat. Both species with the greatest variety of putative sources of toxic metabolites deserve special attention of researchers as objects for the assignment and identification of epiphytic fungi.

Two species of the genus *Trifolium*, the zigzag clover and the white clover, were represented in a smaller number of samples, but with comparable sample sizes. In the zigzag clover, the same mycotoxins and in the same amounts as in meadow clover were regularly detected, but, as a rule, with smaller range of variation. The differences concerned only the indicators of the greatest accumulation of DAS, ZEN, FUM and OA, as well as EMO, for which the range amonts shifted by an order of magnitude toward lower values (see Table 3). For the white clover, a lesser degree of contamination was revealed compared to two other species of this genus. The upper limits of DAS, ZEN FUM, EMO, OA levels coincided with the zigzag clover (unlike meadow), but with a much more rare detection of DAS, DON, FUM, ROA, AB_1, STE, PR and reduced accumulation of EA, ROA, as well as AB_1, STE, CPA. Moreover, the MPA level did not exceed 100 μg/kg. Thus, a large-scale mycotoxicological study confirmed the earlier conclusion [17] about clear advantages of white clover, the main component of pastures throughout the European Russia and in Siberia, over meadow clover.

4. Occurrence (%) and accumulation of mycotoxins (μg/kg) in lupine, goat's rue and three species of *Vicia* (European Russia, May-September 2015)

Mycotoxin	Bush vetch (n = 87)	Tufted vetch (n = 117)	Common vetch (n = 14)	Washington lupine (n = 12)	Goat's rue (n = 18)
T-2	51 (2-17-445)	40 (2-9-62)	71 (2-56-280)	100 (3-8-12)	56 (2-3-5)
DAS	26 (160-340-850)	15 (79-375-955)	14 (130-135-140)	83 (195-255-405)	11 (215-275-330)
DON	36 (78-145-375)	12 (87-175-315)	36 (74-130-190)	92 (105-205-365)	11 (110-115-120)
ZEN	29 (21-44-78)	17 (25-44-100)	7 (30)	100 (31-51-83)	22 (31-48-63)
FUM	23 (76-200-420)	3 (83-105-130)	Не выявлен	33 (79-130-230)	17 (110-175-275)
EA	89 (2-10-37)	80 (2-17-600)	100 (3-44-345)	100 (5-33-75)	89 (2-20-79)
AOL	100 (14-115-860)	94 (17-85-1515)	100 (50-95-245)	100 (140-425-795)	100 (30-195-1410)
ROA	26 (4-22-76)	21 (4-20-105)	Не выявлен	50 (6-16-27)	6 (3)
AB_1	32 (2-5-19)	19 (2-4-12)	50 (2-3-5)	100 (3-8-23)	6 (2)
STE	52 (9-45-325)	47 (8-92-1320)	29 (12-20-35)	100 (16-38-71)	28 (12-16-23)
CPA	100 (77-445-1585)	96 (63-420-2040)	100 (91-485-1660)	100 (315-630-1260)	89 (81-355-610)
EMO	83 (10-57-775)	85 (16-44-250)	79 (17-34-74)	100 (165-415-740)	72 (18-49-125)
OA	71 (4-9-28)	32 (4-8-24)	71 (8-10-13)	92 (8-15-24)	56 (5-9-13)
CIT	39 (25-100-315)	29 (25-61-165)	43 (27-40-68)	83 (33-82-210)	22 (32-37-42)
MPA	54 (13-35-82)	33 (11-50-280)	29 (17-20-25)	67 (16-33-66)	44 (12-23-32)
PR	31 (31-350-685)	24 (30-350-655)	7 (250)	100 (220-355-595)	17 (135-215-315)

Note. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergot alkaloids, AOL — alternariol, ROA — 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 examined. The minimum-average-maximum content of mycotoxin is given in parentheses.

Members of genus *Vicia*, the bush vetch and tufted vetch, had similarities in the frequency of detection of the majority of mycotoxins, but in tufted vetch there was a tendency to a decrease in this parameter, especially noticeable for FUM (Table 4). However, in this species the upper limits of the EA, AOL, ROA, STE, MPA contents were higher. The possibility of intensive accumulation of STE (up to 2320 g/kg), noted earlier for *Vicia* spp. [17], was also characteristic of tufted vetch (see Table 4). By the levels of fusariotoxins DAS, DON, ZEN and a number of other toxins, the species did not differ, the maximum content of DAS (1035 μg/kg) in the *Vicia* spp. (see Table 2) was found for wood vetch. In tufted vetch, a wide fluctuations in the amounts of mycotoxins were much more frequent than in bush vetch. Here, too, we can assume the effect of higher environmental plasticity of micromycetes, but one can not exclude the consequences of the fact that clear identification of closely related species and varieties is often hampered by morphological differences which are hard to detect.

Cultivated leguminous plants are often found in grass stands of natural forage lands. In the areas of sample collection, a single growth or clumps of Washington lupine, goat's rue and common vetch were observed. Among them, Washington lupine was the most abundant in mycotoxicological load. Of the 16 mycotoxins tested, 13 (all but FUM, ROA and MPA) were detected at a frequency of more than 80 %. Fusariotoxins T-2, DAS, DON and ZEN were almost common, although in small amounts. Significant susceptibility to different species of *Fusarium* was previously established for annual fodder lupins, *L. luteus* L., *L. albus* L. and *L. angustifolius* L. [21], but the formation of metabolites is uncharacteristic of the main pathogens *F. avenaceum* (Fr.) Sacc. and *F. oxysporum* (Schlecht) Snyd et Hans. Perhaps, their appearance in the plant is provided by accompanying species. Frequent contamination of Washington lupine with a wide range of mycotoxins should be considered when growing this crop for sideration and especially for subsequent silage.

Goat's rue is considered promising because of its high yield, it is attractive for grazing, harvesting hay, haylage, silage. We have shown that it was characterized by a moderate contamination with mycotoxins by prevalence and content (see Table 4). In goat's rue, as well as in peas, sweetcloves and alfalfa, only EA, AOL, CPA and EMO were regularly identified, but the goat's rue plants differed from peas by a smaller accumulation of DON, CIT, ROA, and STE, and was close to sweetclovers in EMO levels, and to alfalfa in low level of MPA (see Table 2).

Common vetch on the whole was characterized by weak contamination, it combined certain features of other species of this genus, tufted vetch (practical absence of FUM) and bush vetch (occurrence of OA), but there were distinctive signs (absence of ROA and smaller contents of EMO, STE and CIT).

Comparison of susceptibility to contamination with mycotoxins for species within the genus (see Table 3, 4) and between genera of leguminous plants (see Table 2) showed that the differences were comparable and could be very significant. We have previously marked this for lichens [20, 22], which, forming the basis of vegetation cover for deer pastures, serve as a food for many other animals in the wild.

Sources and mechanisms for the formation of the mycotoxicological status of plants are still unclear, but lately an increasing role in this has been attributed to endophytic fungi [23]. Thus, the cause of widely known intoxication of ruminants and horses (salivation, locoism, "pea disease") accompanying grazing in legumes, the red clover (*Trifolium pratense* L.), *Astragalus* spp., *Oxytropis* spp. in the USA and *Swainsona canencens* (Benth.) F. Muell. in Australia, are indolizidine alkaloids of fungi inhabiting tissues of these plants [24]. The presence of EA in pasture cereals is associated with the endophytes of the genus *Ne-*

otyphodium [25], the hypericin-emodin metabolism in Saint-John's wort (*Hypericum perforatum* L. common for the meadow is provided by endophytic fungus *Thielavia subthermophila* Mouch. [26], and search for fungi responsible for EMO biosynthesis in plants is expanding [27]. Therefore, a reasonable approach to the formation of grass stands with productive longevity, is based on diversity of the cenotic interactions and environmental factors [28, 29].

Thus, the predisposition to the accumulation of fungal metabolites toxic to animals should be taken into account in the economic use of leguminous crops along with their yield, nutritional value, and resistance to drought, salinity, temperature changes, pest damage and diseases.

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DIETARY PROBIOTIC *Lactobacillus plantarum* L-211 FOR FARM ANIMALS. II. THE ADDITIVE FOR PIGLETS

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Abstract

The lack of lysine in the diet of pigs negatively affects appetite, weight gain, metabolism and immunity of animals. Most feeds for pigs are deficient in lysine. Synthetic amino acids, as feed additives, make feeds significantly more expensive. In this regards, the biologcs based on microbial producers able to synthetize lysine in the gastrointestinal tract of animals are promising. However, common producers of lysine, *Brevibacterium lactofermentum*, *Escherichia coli* and the genus *Corynebacterium*, are conditionally pathogenic as a causal agents of opportunistic infections. In the present study, we first examined the changes in intestinal bacterial community and the productive performance in Large White pigs («Novgorod bacon», Novgorod Province) under the influence a lysine producing strain *Lactobacillus plantarum* L-211 (JSC «Bioreactor», Moscow). Taxonomic composition of microorganisms was determined by T-RFLP (terminal restriction fragment length polymorphism) analysis. For surveillance we used two groups of pigs from 28- to 84-day age, fed with the basic diet (n = 715, group 1, control) and the same diet supplemented with *L. plantarum* L-211 at the dose not less than 10^9 CFU per animal added to water (n = 657, group 2). Microbial community in the pigs' large intestine was taxonomically divers and included a number of unidentified phylotypes. Here, the predominating bacteria were representatives of the phylum *Firmicutes*, including mainly *Clostridia* possessing cellulolytic and amylolytic properties, as well as the members of order Negativicutes able to ferment acids. The phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were less abundant. The counts of genus *Lactobacillus* was lower than previously assumed, moreover, there was a complete absence of enterococci and bifidobacteria, which are usually attributed to the autochthonous microbiota of the large intestine of animals and birds. Lysine synthesizing strain *L. plantarum* L-211 had a high probiotic effect resulting in a significant increase in the counts of genera *Lactobacillus* (2.94-fold, $P < 0.01$) and *Bacillus* (3.29-fold, $P < 0.01$), of phylum *Bacteroidetes* (5.29-fold, $P < 0.01$), and class *Clostridia* (2.05-fold, $P < 0.01$), whereas the proportions of pathogens from *Staphylococcus* genus and *Campylobacteriaceae* family were below the T-RFLP sensitivity, and the family *Pasteurellaceae* decreased in number 1.41-fold ($P < 0.05$). Both the survival and the average daily weight gain ($P < 0.05$) in pigs, as influenced by the probiotic strain *L. plantarum* L-211, were higher. *L. plantarum* L-211 also improved feed conversion efficiency as compared to the control pigs.

Keywords: lysine, intestinal microflora, pigs, bacterial community, T-RFLP, probiotic, *Lactobacillus plantarum*, productivity, pigs' survival, feed conversion

In baby pigs, digestive system is formed and live weight is intensively incresed which is important for obtaining highly productive livestock [1, 2]. In this, full amino acid composition of the diet, including essential amino acids, plays a special role. The lack of lysine is associated with a decrease in appetite and productivity of animals, weight loss, impaired calcification of bones, general

exhaustion and anemia [3, 4]. In addition, immunity suppression occurs and susceptibility to infectious diseases increases [5]. Due to the lack of lysine in vegetable fodder mixtures, their use in feeding pigs is ineffective. The main raw materials in production of domestic mixed fodders for pigs are the components deficient in the lysine (grain, by-products of their processing, sunflower meal), therefore, as a rule, it is not possible to provide enough lysine level without the use of synthetic amino acids, which considerably increases the fodder cost [6, 7].

The development of probiotics synthesizing lysine in the gastrointestinal tract of animals [8, 9] performed in Russia and abroad, are considered as the most promising approach in the formation of the optimal intestinal microflora. In this, *Brevibacterium lactofermentum*, *Escherichia coli* and representatives of the genus *Corynebacterium* [10-12] were mainly studied as lysine producers, but the listed microorganisms are conditionally pathogenic and capable of causing opportunistic infections, so their use as probiotics is undesirable. Some strains of the genus *Lactobacillus* are also able to synthesize lysine [8, 9, 13] and positively affect growth, carcass quality, animal immunity [14, 15]. The positive effect of dietary lactobacilli in pigs is also associated with the synthesis of organic acids and bacteriocins that inhibit the growth and development of various pathogens, i.e. *Salmonella*, *Proteus*, *Staphylococcus*, *E. coli*, *Pseudomonads*, *Streptococci* [15, 16].

In recent years, successful use of molecular genetic approaches to study the microbial community of the digestive tract of pigs has been reported, including differences in the composition of microbiocenosis of healthy pigs and animals with intestinal disorders [17, 18]. The T-RFLP (terminal restriction fragment length polymorphism) and NGS (next generation sequencing) methods allow a detailed description of the microbial community, revealing not only taxonomic dominants, but also minor components, including non-cultivated microorganisms, whose proportion in different ecosystems can reach 90 % [19, 20[. However, studies of the intestinal microbiom of pigs carried out to date are extremely few [21, 22], and data on the complex analysis of the bacterial community, as influenced by probiotic lactobacilli, are absent. The strain *Lactobacillus plantarum* L-211 is described as a lysine producer with a fairly high yield in culture [8, 9].

For the first time, using the T-RFLP method, we determined the composition of the bacterial community in the thick intestine of the baby pigs when the preparation of the lysine-producing strain of lactobacilli was supplemented with water and revealed its high probiotic activity which was accompanied by positive changes in the zootechnical indices, i.e. an increased survivability and daily live weight gain.

The purpose of this work was to study the effect of dietary lysine-producing strain *Lactobacillus plantarum* L-211 on the bacterial community of the intestine and the productive indices in baby pigs during early growth.

Technique. Farm experiment was carried out on two groups of baby Large White pigs from day 28 to day 84 of life (OOO Novgorodsky Bacon, Novgorod region). The feeding and housing pigs from groups I (control, $n = 715$) and II ($n = 657$) were in compliance with all technological parameters and equivalent feed compositions (OOO Novgorodsky Bacon, Novgorod region) for lysine corresponding to the norms for Large White breed. Baby pigs of group II were additionally given dietary *Lactobacillus plantarum* L-211 (OOO Bioreactor, Moscow) in a dose of at least 10^9 CFU per animal. Survivability of livestock, the live weight of baby pigs at the age of 28 and 84 days in individual weighing, its daily gain, feed consumption and feed costs per 1 kg of live weight were recorded.

Contents of the large intestine from three slaughtered pigs of each group were collected on day 84 with strict sterility for molecular genetic studies. T-RFLP analysis of bacterial community was performed according to description

[23]. Total DNA was isolated from the samples using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to manufacturer's recommendations. PCR was performed on a Verity DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers 63F CAGGCCTAACACATGCAAGTC labeled at the 5'-end (WellFed D4 fluorophore, Beckman Coulter, Inc., USA) and 1492R TACGGHTACCTTGTTACGACTT. The fluorescently labeled amplicons of 16S rRNA gene was purified as described [24], the restriction (30-50 ng DNA) with endonucleases HaeIII, HhaI and MspI was performed following the manufacturer's recommendation (Fermentas, Inc., Lithuania). Restricts were analyzed using CEQ™ 8000 (Beckman Coulter Inc., USA) according to the manufacturer's protocol. The taxonomic attribution was performed in the Fragment Sorter program (http://www.oardc.ohiostate.edu/trflpfragsort/index.php).

The data were processed by an analysis of variance. Differences with the control were considered significant at $P < 0.05$; $P < 0.01$ and $P < 0.001$.

Results. T-RFLP analysis of the microbial community of thick intestine where the most important digestion of carbohydrates, including fiber, occurs with participation of intestinal microbiota and formation of volatile fatty acids (VFA) and other metabolites [25], revealed a number of taxonomic groups (Table 1).

1. The ratio of bacterial taxa (%) in the thick intestine in 84-day old the Large White pigs receiving probiotic *Lactobacillus plantarum* L-211 ($X \pm x$, OOO Novgorodsky bacon, Novgorod region)

Taxon	Group I (control, $n = 3$)	Group II ($n = 3$)
Phylum *Bacteroidetes*	0.17±0.01	0.90±0.04**
Phylum *Firmicutes*	35.46±1.69	65.38±2.95**
class *Clostridia*	5.35±0.21	10.96±0.43**
family *Lachnospiraceae*	1.48±0.06	0.69±0.03**
family *Eubacteriaceae*	1.07±0.04	8.18±0.03***
family *Ruminococcaceae*	0.84±0.03	0.47±0.02**
family *Clostridiaceae*	1.89±0.09	1.62±0.07
genus *Peptostreptococcus*	0.07±0.01	Brd
genus *Lactobacillus*	7.83±0.33	23.07±1.13**
genus *Bacillus*	2.93±0.13	9.65±0.61**
genus *Staphylococcus*	0.25±0.01	Brd
order *Negativicutes*	19.10±0.97	21.70±1.03
Phylum *Actinobacteria*	0.16±0.01	Brd
Phylum *Proteobacteria*	2.04±0.03	2.64±0.06**
family *Enterobacteriaceae*	0.87±0.23	1.95±0.15*
family *Campylobacteriaceae*	0.21±0.01	Brd
family *Pseudomonadaceae*	0.96±0.04	0.21±0.01***
genus *Acinetobacter*	Brd	0.48±0.02
family *Pasteurellaceae*	2.60±0.14	1.84±0.08*
Phylum *Fusobacteria*	0.07±0.01	0.14±0.01*
Unclassified sequences	59.49±2.98	29.10±1.39**

N o t e. Description of the groups is given in the *Technique* section. Brd — below the limit of reliable determination by the method of T-RFLP (terminal restriction fragment length polymorphism).
*, **, *** Differences with control are statistically significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Identified bacteria belonged to five phylums, of which the members of the phylum *Firmicutes*, including *Clostridia* and the order of *Negativicutes*, predominated. Traditionally, the bacteria of the families *Lachnospiraceae*, *Clostridiaceae*, *Ruminococcaceae*, *Eubacteriaceae* belonging to class *Clostridia*, are considered as the main producers of enzymes (cellulase, hemicellulase, amylase, etc.) necessary to metabolize carbohydrates from plant fodder. As a rule, the VFAs formed during this process are used by representatives of the order of *Negativicutes,* including bacteria *Megasphaera*, *Selenomonas*, and others. Note that these processes were previously described primarily for ruminants [25]. The phyla of *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were less abundant.

Some gut microorganisms found were opportunistic and pathogenic. Bacteria of the genus *Fusobacterium*, revealed in both groups, have traditionally been regarded as causative agents of cattle necrobacteriosis [26], and their presence in the intestine, various organs, on the skin in animals and humans has now been confirmed using molecular genetic methods [27]. Representatives of the families *Enterobacteriaceae* and *Campylobacteriaceae* detected in the intestine in small quantities are typical pathogens of the dysbiosis in animals. Of interest is the fact that bacteria of the family *Pasteurellaceae*, the pathogens of respiratory tract in animals and poultry, have been detected in the intestines of baby pigs

[16]. At that, bacteria of the genus *Staphylococcus* common in intestinal contents of animals were practically absent in baby pigs.

A part of the bacterial community could not be taxonomically identified which agrees with foreign and domestic studies of the microbiom of the digestive tract in various species of animals and poultry [23, 28]. As to the identified bacteria, the results obtained using the T-RFLP method also did not contradict the known concepts [16, 21, 25] with some exceptions. Thus, the bacteria of the genus *Lactobacillus*, which are commonly considered to be the dominant inhabitants of pig intestines, we found in small amounts. In addition, enterococci and bifidobacteria, previously described as representatives of the autochthonous microbiota of the large intestine in animals and birds, were completely absent in the intestines of these baby pigs [16, 21].

Dietary probiotic *L. plantarum* L-211 which produces an average of 148.4±4.45 mg/l lysine in minimal media culture [8, 9], when given with water to pigs once a week at a dose of at least 10^9 CFU per animal, changed microbiota of the large intestine. The percentage of genus *Lactobacillus* representatives increased 3 times ($P < 0.01$), which was probably due to the good persistence and growth of the introduced lactobacilli in intestinal contents. The ability of a number of lactobacilli to adhere to the intestinal walls is known which allows them to colonize the digestive tract [29] and occupy free ecological niches in its microbiome, exerting a probiotic effect. In addition, *L. plantum* strain L-211 contributed to a 3-fold increase in the number of bacteria of genus *Bacillus* ($P < 0.01$) which also, due to the synthesis of organic acids and bacteriocins, are capable of competitive exclusion of pathogens [16]. *L. plantarum* L-211 also affected the multiplication of bacteria from the phyla *Bacteroidetes* and *Clostridia*, producing cellulolytic and amylolytic enzymes, and caused 5-fold ($P < 0.01$) and 2-fold ($P < 0, 01$) increase, respectively, in their number in the large intestine compared to control. The proportion of bacteria from the order *Negativicutes* under the influence of probiotic lactobacilli did not change significantly.

2. Zootechnical indices in 84-day-old Large White pigs receiving probiotic *Lactobacillus plantarum* L-211 ($X±x$, OOO Novgorodsky bacon, Novgorod region)

Parameter	Group I (control, $n = 3$)	Group II ($n = 3$)
Total weight, kg	8567	6753
Death loss:		
by number, heads	13	8
by weight, kg	203	116
Sanitary butchering:		
by number, heads	9	11
by weight, kg	181	299
Transmitted for fattening:		
by number, heads	693	638
by weight, kg	21550	19875
Weight, kg per head ($X±x$)	31.10±1.48	31.15±1.39
Preservation, %	98.18	98.78
Gross increment, kg	13367	13537
Age at transfer, day	83	84
Feed-days, total	28675	27288
Average daily weight gain, g ($X±x$),	466.20±11.29	496.10±10.14*
Fodder consumption, kg:		
total	26700	26400
mixed fodder CK-4	17890	18300
mixed fodder CK-5	8810	8100
Conversion of feed ($X±x$), kg	1.997±0.040	1.950±0.050
Consumption of mixed fodder for the feed-day, kg	0.931	0.967

N o t e. Description of the groups is given in *Technique* section.
* Differences with control are statistically significant at $P < 0.05$.

Importantly, *L. plantarum* L-211 positively affected opportunistic and pathogenic bacteria which percentage decreased. The counts of *Staphylococcus* and *Campylobacteriaceae* bacteria decreased to the levels not detectable by T-RFLP, and the *Pasteurellaceae* was 1.41-fold less abundant ($P < 0.05$). Also, the counts of *Pseudomonadaceae* bacteria, transit microorganisms entering intestine with food, decreased 4.57 times ($P < 0.001$). Unidentified bacteria decreased 2.05 times ($P < 0.01$) compared to control.

Zootechnical indicators (Table 2) confirmed an increased viability, the daily weight gain ($P < 0.05$), and the efficiency of feed conversion due to *L. plantarum* L-211.

Thus, *Lactobacillus plan-*

tarum L-211 synthesizing lysine has a high probiotic activity with a positive effect on the composition of the bacterial community of the large intestine of baby pigs. When feeding the probiotic, the normoflora (genera *Lactobacillus* and *Bacillus*) significantly increased, and the number of microorganisms traditionally associated with intestinal dysbiosis in humans and animals (the family *Campylobacteriaceae*, *Pasteurellaceae*, genus *Staphylococcus*) decreased. The change in the microbial community positively affected zootechnical indicators of pigs which survivability and the daily weight gain ($P < 0.05$) increased at better feed conversion. Therefore, this probiotic used during early growing can provide for highly productive livestock and reduces the costs of compound feed per unit of production.

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