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Formation of local protection of the respiratory tract in Holstein calves

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Abstract: The processes of formation of the local protection of the respiratory tract in nasal secretion samples of Holstein calves from birth to 6 months of age that were born from clinically healthy cows were examined. Only clinically healthy animals were involved in the experiment. Nasal secretion samples were collected with the help of a sterile foam tampon with a fixing thread in order to assess local immunity in animals at the age of 1, 5, 10, 30, 60, 90, 120, 150, and 180 days. Total protein, mucin, lysozyme activity, and alkaline phosphatase (ALP) were determined using commercially available methods. The amounts of immunoglobulins were studied using disk electrophoresis in polyacrylamide gel with commercial marker proteins. Studies have shown that the primary potential of local protection of the respiratory tract is formed during intrauterine development. The main factors of primary potential are mucin, lysozyme, alkaline phosphatase, and secretory immunoglobulins of classes A (SIgA) and M (SIgM), which provide safety of adaptation in newborns to the new conditions of existence. There is a decrease in mucin level by 47.3% ($P < 0.001$), in SIgA by a factor of 6.7 ($P < 0.001$), and in SIgM by a factor of 6 ($P < 0.001$) during the first week of life. However, the risk of immunodeficiency is mitigated by the preservation of relatively high levels of lysozyme, ALP, and IgG, creating optimal conditions for the processes of formation of respiratory functions in young animals during the first 2 months of life. After that, SIgA increases by 70% ($P > 0.05$) at the age of 60 days and mucin level increases by 56.7% ($P < 0.001$) between 2 and 6 months.

Key words: Calves, respiratory tract, local immunity, formation

1. Introduction

Intensification of cattle breeding is an objective necessity of the modern stage of the development of agriculture. However, the buildup in the functional load on animals increases their sensitivity to negative environmental factors and the risk of metabolic failures, which often lead to an increase in the incidence of disease, especially among young animals, including respiratory diseases [1,2]. Many authors note a progressive trend towards expanding the etiological spectrum and increasing the relevance of respiratory diseases in calves [3–5], and so new approaches are needed to address this issue. One promising area of prevention of diseases in young animals is reducing the risk of functional overloading of body systems during critical periods of postnatal ontogenesis. Dysontogenesis develops in the context of the effects of impaired antenatal development and/or inadequate environmental conditions [6,7]. The risk of lung tissue contamination and the likelihood of pathology depend on the condition of the respiratory tract barrier structures [8,9]. Therefore, the purpose of our work was to study the processes of formation of local protection of the respiratory tract in calves in the early postnatal period of their ontogenesis.

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2. Materials and methods

2.1. Animals

The study involved 10 clinically healthy Holstein calves from clinically healthy cows that were under constant control before and during calving. The calves were born on their own without complications and without the use of any obstetric aid. The mother licked the calf for 20 min, and then the calf was given 2 L of colostrum from a nursing bottle after 65–75 min and 420–430 min. Later, the diet of the animals corresponded to the recommendations for raising young animals. They were kept in individual cells during the first 60 days, where the air temperature ranged from 18 to 25 °C and relative humidity was 60%–64%. They were transferred to a group cell at the age of 61 days. The study was approved by the ethics committee of the All-Russian Veterinary Research Institute of Pathology, Pharmacology and Therapy.

2.2. Clinical examination

The animals were under constant observation until the calves reached the age of 180 days. Standard clinical examinations included heart rate, respiration rate, and rectal thermometry. Only clinically healthy animals were

used in the experiment. Therefore, when calves were found to have symptoms of pathology, they were excluded from the experiment. Nasal secretion samples were collected with the help of a sterile foam tampon with a fixing thread in order to assess local immunity in animals at the age of 1, 5, 10, 30, 60, 90, 120, 150, and 180 days. The tampon was cylindrical with a diameter of 10–12 mm and a length of 30–40 mm for calves aged up to 30 days, and 12–15 mm and 50–60 mm for older animals respectively. The tampon at fixing was inserted into the nasal passage to a depth of 4–5 cm, and then it was taken out and placed in a sterile container after several cycles of breathing and massage of the soft tissues of the nose.

2.3. Laboratory research

The samples of muconasal secretion were homogenized and examined within 6–8 h of collection. A nephelometric method based on high sensitivity to lysozyme *Micrococcus lysodeikticus* was used to determine the activity of lysozyme [10]. Mucin content was estimated by spectrophotometric method, based on the determination of the difference in protein concentration in the initial material and in supernatant formed after deposition by acid [11]. A biochemical analyzer (Hitachi-902, Japan) was used to investigate the activity of alkaline phosphatase by a method the principle of which is to fix the rate of hydrolysis of p-nitrophenylphosphate (pNPP) by alkaline phosphatase to p-nitrophenol (pNP) in the presence of magnesium ions and diethanolamine as a phosphate acceptor at pH 9.8. The optical density of the pNP formed was measured at 405 nm and was directly proportional to the activity of alkaline phosphatase in the sample (Randox Laboratories Ltd, UK). The amount of total protein was determined by the biuretic method (panel TP L 500 S, ERBA Lachema, Czech Republic), and the amount of γ -globulins by means of disk electrophoresis in polyacrylamide gel with the help of a "Reanal" device (model 69, Hungary). Next 7.5% polyacrylamide gel and tris-glycine electrode buffer (pH 8.3) were used to separate proteins, and bromophenol blue dye was used as an indicator of electrophoretic movement. The electrophoresis was carried out at +4 °C for 45 min at a current of 4 mA per tube. Fractions were identified by a set of recombinant high-purity proteins of known molecular weight in the range from 20 to 1200 kDa (Native Mark Unstained Protein Standard, Bio-Rad Laboratories, USA). The electropherograms were stained with Coomassie G250 and bleached with a mixture of ethanol, acetic acid, and distilled water in 10:1:30 ratio, and then scanned and analyzed using the Gel-Pro Analyzer 6.0 software package (Media Cybernetics, L.P.).

2.4. Statistical analysis

The statistical analyses were performed using SPSS version 22 (IBM Corp, Version 22.0, Armonk, NY, USA, 2013). The independent samples t-test was used for comparison

of measured factors between two groups (analyzed and previous age group). All values were expressed as mean (M) and standard deviation (SD), and $P \leq 0.05$ was considered statistically significant.

3. Results

The maximum level of mucin was found on the first day after birth, but it fell by 47.3% in the next 5 days ($P < 0.001$) (Table 1). The downward trend persisted for 60 days, resulting in a minimum age level of 65% below the baseline ($P < 0.001$). The amount of mucin increased by 56.7% ($P < 0.001$) between 2 and 6 months. However, changes in this indicator were not significant ($P > 0.05$) at the age of 4–6 months.

The maximum level of nasal secretion lysozyme activity was observed in newborns at the age of 1 day, but this indicator decreased by 49% ($P < 0.001$) during the first week of life, and by 5.3-fold ($P < 0.001$) over the next 55 days. As a result, it reached values that did not change significantly in the next 4 months.

The activity of alkaline phosphatase decreased by 31% during the first 5 days of life, but then increased to a maximum of 10 ($P > 0.05$) above the baseline level at 30 days of age. The studied index decreased by 33.6%–40.6% ($P < 0.001$) in the period from 1 to 3–4 months, and reached parameters that did not change significantly thereafter.

The maximum protein content in the nasal secretion was detected on the first day after birth, but during the first week of life this indicator decreased 4.4-fold ($P < 0.001$) to a level that did not change significantly after that ($P > 0.05$). Similar dynamics was observed for gamma-immunoglobulin values.

Three groups of proteins (zones) with molecular weight 150–170, 350, and 900 kDa, respectively, were isolated upon electrophoretic separation of the nasal secretion in γ -globulin fraction (Table 2).

The analysis of electropherograms showed that proteins with a molecular weight of 150–170 kDa in the structure of γ -globulins were 51%–75%, with the exception of the 1st and 90th day when their minimum value was observed, 35.2%. Class G tetrameric immunoglobulin was the bulk of this fraction, which contained two heavy chains (50–53 kDa each) and two light chains (23.5–25.0 kDa), together forming a molecular mass of 155–160 kDa [12]. The maximum amount of IgG in the nasal secretion was noted in the first hours after birth, but then it decreased within 120 days by 62.4% ($P = 0.0011$) to the minimum level for the entire period of observation. The IgG content increased by 40% ($P = 0.088$) in the period from 4 to 6 months.

Protein with a molecular weight of 350 kDa was identified as secretory immunoglobulin A (SIgA), whose

Table 1. Indicators of mucin content, lysozyme activity, and alkaline phosphatase in muconasal secretion of calves of different ages (M ± SD).

Age, day	Mucin, g/L	Lysozyme activity, µg/mL	ALP, U/L
1 (n = 10)	2.037 ± 0.198	1.864 ± 0.070	1313.0 ± 109.67
5 (n = 10)	1.074 ± 0.162*	0.951 ± 0.040*	903.5 ± 5.38*
10 (n = 10)	0.899 ± 0.099	0.620 ± 0.009*	1368.0 ± 110.7*
30 (n = 9)	0.861 ± 0.100	0.402 ± 0.152*	1445.5 ± 39.96
60 (n = 9)	0.713 ± 0.056*	0.178 ± 0.022*	1057.5 ± 22.42*
90 (n = 9)	0.898 ± 0.062	0.183 ± 0.032	859.0 ± 56.0*
120 (n = 9)	1.027 ± 0.100*	0.161 ± 0.058	959.0 ± 39.0*
150 (n = 8)	1.000 ± 0.155	0.170 ± 0.045	943.8 ± 45.04
180 (n = 8)	1.117 ± 0.168	0.167 ± 0.071	933.0 ± 45.06

*- the difference is significant ($P \leq 0.05$) in comparison with the rates for previous age

Table 2. Indicators of total protein and immunity humoral link in muconasal secretion in calves of different ages (M ± SD).

Age, day	Total protein, g/L	γ-Globulins, g/L	IgM, g/L	IgA, g/L	IgG, g/L
1 (n = 10)	31.8 ± 10.03	4.93 ± 1.583	1.45 ± 0.454	1.74 ± 0.543	1.73 ± 0.530
5 (n = 10)	7.22 ± 2.267*	1.87 ± 0.583*	0.24 ± 0.059*	0.26 ± 0.074*	1.37 ± 0.410
10 (n = 10)	7.61 ± 2.386	1.94 ± 0.606	0.25 ± 0.097	0.24 ± 0.070	1.45 ± 0.450
30 (n = 9)	6.78 ± 2.248	1.79 ± 0.612	0.23 ± 0.088	0.20 ± 0.056*	1.33 ± 0.438
60 (n = 9)	6.65 ± 3.980	1.52 ± 0.498	0.26 ± 0.064	0.34 ± 0.096	0.93 ± 0.298
90 (n = 9)	6.03 ± 2.102	1.57 ± 0.508	0.29 ± 0.108	0.36 ± 0.136	0.92 ± 0.320
120 (n = 9)	7.26 ± 2.396	1.27 ± 0.402	0.27 ± 0.074	0.34 ± 0.092	0.65 ± 0.224
150 (n = 8)	6.71 ± 2.429	1.42 ± 0.492	0.29 ± 0.120	0.35 ± 0.099	0.79 ± 0.301
180 (n = 8)	6.71 ± 2.687	1.50 ± 0.522	0.26 ± 0.077	0.33 ± 0.105	0.91 ± 0.314

*- the difference is significant ($P \leq 0.05$) in comparison with the rates for previous age

share of the total number of immunoglobulins was 11%–27%. The exception is the data of days 1 and 90, when the maximum value of 35.3% was observed. The maximum SIgA content was observed in the first 24 h of life of newborns. This rate decreased 6.7-fold during the first week of life ($P < 0.001$) and by another 23% ($P > 0.05$) during the next 25 days. The number of SIgA increased by 70% ($P > 0.05$) at the age of 60 days, and the coefficient of variation increased from 28% to 37.8%. The test indicator did not change significantly in the subsequent period of observation.

The largest protein fraction (900 kDa) was identified as class M immunoglobulin (SIgM), which was 13%–21% in the structure of nasal mucus γ-globulins, although its share increased to 29.5% on days 1 and 90 of life. The

maximum SIgM content was observed on the first day of a newborn's life, but its values decreased in the following 5 days by a factor of 6 ($P < 0.001$) to a level that did not change later on.

4. Discussion

The nasal secretion is formed by the upper and lower respiratory tract glands and so its composition reflects the potential of the barrier, immune, and cleansing functions of the respiratory tract. The mucosal secretion contains a number of nonspecific and humoral protective factors contributing to the clearance and removal of microorganisms. The nonspecific factors include mucin, lysozyme, and others, and humoral factors are represented by immunoglobulins [13].

Mucoproteins (mucins) are a multifunctional component of respiratory tract secretion; they are divided according to the type of external active groups into acidic (sialo- and sulfomucins) and neutral (fucomucins), and by their localization, into secretory and membrane-associated ones [14,15]. Mucins determine the rheological properties of nasal mucus and protect the epithelial membrane from mechanical damage, toxic substances, pH changes, and attachment and translocation of microgerms [16,17]. Such a wide array of mucins' functions explains the biological advisability of their high content in the secretion of the respiratory tract during the adaptation of the newborn to the new conditions of life in an environment with increased concentration of xenobiotics and microorganisms in the air. The need for increased amounts of mucoproteins is reduced at the end of the first 10-day period of life, due to the formation of other mechanisms of local protection. However, it increases again at the age of 2 to 3 months, which is probably due to technological factors, in particular, the increase in microbial air contamination during the group housing of animals.

Lysozyme is also a major factor in nonspecific respiratory protection. It is capable of destroying bacterial cell walls by hydrolyzation of β -1,4 glycoside bonds between N-acetylmuraminic acid C-1 and N-acetylglucosamine C-4 of gram-positive bacteria [18]. Therefore, the relatively high lysozyme activity of the nasal secretion is necessary not only during the period of adaptation of the newborn to the new conditions of life, but also at the initial stage of the early days of the respiratory organs, which lasts for 20–30 days.

The nasal secretion contains alkaline phosphatase, the role of which still needs to be clarified. However, it is known that its activity increases with the development of pathology [19]. Alkaline phosphatase contributes to the neutralization of toxins of gram-negative bacteria by forming a nontoxic monophosphoryl lipopolysaccharide by participating in the dephosphorylation of molecules [20,21]. Thus, alkaline phosphatase is an active component of local antitoxic protection, and its mechanism of action is associated with the need for the presence of an application object (bacteria, toxins). The increased activity of this enzyme in calves remains during the first 2 months of life, probably until the formation of the appropriate level of antitoxic protection of their body and humoral immunity (IgA and IgM).

The secretory immunoglobulins class A and M, which are formed in the plasma cells of the mucous membrane, are among the main components of humoral immunity of the respiratory tract mucous membrane. Unlike serum fractions, they contain a J-chain in their structure, which allows them to bind to a secretory component (glycoprotein). This protects immunoglobulin A and

M molecules from bacterial proteolysis and increases their ability to neutralize soluble antigens and to prevent adhesion of pathogenic microorganisms at the mucosa surface [22]. Our data show that the maximum SIgA content is present at the age of 24 h. Its share in the total number of muconasal secretion immunoglobulins is 35.3%, while in the colostrum of first milk yield it is 5.2% [23], which indicates the synthesis of SIgA in the respiratory tract during the period of intrauterine development, although the activation of its formation from polymeric serum IgA is not excluded. The minimum content of this class of immunoglobulins was noted at the age of 30 days. It is known that selective deficiency of secretory immunoglobulin A is much more common than of its serum fraction and is the earliest symptom of immunodeficiency [24]. Therefore, the risk of primary immunodeficiency developing in calves at 30 days of age can be assumed.

The ratio of immunoglobulins M to G in the first colostrum of the first milk yield is 6.5–6.8 and the ratio of immunoglobulins M to G in the blood serum at the end of the first day of life is 5.0–5.6 [23], but in the muconasal secretion it is 1.2. The increase in the relative amount of SIgM indicates that its pool in the muconasal secretion is formed from the serum but predominantly from the secretory fraction of antenatal origin. Immunoglobulin A is the first line of defense for mucous membranes, providing local immunity [22], and IgM is a main element of the immune response under the initial penetration of the antigen [25]. Therefore, secretory immunoglobulins A and M of antenatal origin form the basic mechanism of humoral protection of the respiratory tract of newborns, and their increased absolute and relative content in the muconasal secretion confirms this point of view. The sharp decrease in SIgA and SIgM indicates that the potential for basic protection is decreasing and is not being replenished by serum immunoglobulins of these classes. However, there is an increase in the number of secretory immunoglobulins at the age of 30 to 60 days, which indicates activation of their synthesis in the respiratory tract mucosa. The risk of immunodeficiency, stipulated by a decrease in SIgA and SIgM levels in the first weeks of calves' lives, could be canceled out by class G immunoglobulin, which provide the neutralization of toxins and bacteria through the mechanism of complement-dependent lysis. Its maximum content in the muconasal secretion was noted at the end of the first day of the calves' life, which is caused by its intake from the blood against the background of active absorption of immunoglobulins from colostrum in the intestines of newborns during the first hours of life. IgG levels are maintained only by means of its diffusion from the blood in older animals, as on the surface of the mucous membrane of the respiratory tract it is quickly exposed to

proteolysis due to the lack of a secretory component. The decrease in the content of secretory immunoglobulins in the mucosal secretion at the age of 30 days and of serumal Ig in 60–90 days is due to the processes of their catabolism, since the half-life of SIgA is 2.5 days, of SIgM is 4 days, and of colostral IgG is 16–32 days [23]. The arising of this relative deficit in the local link of humoral protection creates a risk of actualization of negative microclimate factors and uncontrollable infections, which requires closer attention to the conditions of keeping animals at the age of 30 days, including reducing the risk of air pollution (regrouping, transportation, etc.). Decrease in IgG level in calves at the age of 2–3 months indicates weakening of colostral immunity and a rising threat from vaccine-managed diseases, which makes it necessary to take preventive measures to maintain the appropriate level of specific protection.

In conclusion, the research performed showed that during intrauterine development the primary potential of local protection of the respiratory tract is formed,

the main factors of which are mucin, lysozyme, alkaline phosphatase, and secretory immunoglobulins of classes A and M, providing safety of adaptation of newborns to the new conditions of existence. The indicated potential is reduced during the first week of life, but the risk of immunodeficiency is mitigated by the preservation of relatively high levels of lysozyme, ALP, and IgG, creating optimal conditions for the processes of the formation of respiratory functions in young animals during the first 2 months of life. The age dynamics of the localized immunity of the respiratory tract in young animals depends on the volume of the pool of secretory factors of antenatal origin and on the serum immunoglobulins, admitted into the blood from colostrum, as well as on the conditions the animals are kept in. The revealed regularities of the formation of the barrier function of the respiratory tract in calves in the early postnatal period of their ontogenesis should be taken into account in the development of technologies for obtaining and raising young calves, including the system of vaccine prophylaxis.

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