Porcine Research

The alveoli development and their cellular content during the pigs' ontogenesis

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Abstract. The lungs are one of the few organs that do not perform their specific functions until the birth. Lung's development begins early, and in 45-day fetus lungs already have their shape, subdivided into large main lobes, with branched bronchi, (bronchial tree). However, the differentiation of the respiratory epithelium occurs later, some pneumocytes type II appear only on day 75 of fetal development, and type I pneumocytes appear later. The final formation of the alveoli and their contents occurs at 90 day fetus. Birth and functional activity of the lungs cause an increase in the proliferation of alveolar macrophages (AM). The final formation of the population AM population finished at 1 month of postnatal development of the pig when population increased by more than 15 times compared with newborn.
Key Words: development of the alveols, macrophages, embryonic organs, embryogenesis of the lungs.

Introduction. Study of the pigs' ontogenesis is an important task of the modern medico-biological science. First of all it is connected with the transplantation opportunity of the pig's embryonic organs to humans (Vlahos & Bozinovski 2014). The cellular content of alveoli is also appear to be of crucial importance because the alveolar macrophage (AM) is the basic cell of the respiratory ways' natural immunity, functional state of what predetermines a progress of infectious diseases of different genesis (Eventov-Friedman et al 2005). Recently pigs' AM arouse interest due to their being as an important target-cell for some types of viruses, as well as a convenient model for the study of viral cytopathogenesis in vitro conditions (Carrascosa et al 1982). In spite of that the studies on pig lungs' organogenesis are not sufficient, and moreover, practically there is no investigation of the cellular content, particularly of AM has been conducted. In the purpose to determine the cellular content changes of the pigs' alveoli during ontogenesis we have investigated the alveoli content of the pig fetuses and the newborn piglets. The data obtained were compared with the data of the older pigs (3-4-months aged).

Material and Method. The investigation was encountered on 8 sows of the Large White breed of 11-12 months of ages when attaining 130-140 kg. Thereafter, slaughters of the pregnant sows were performed for study of the embryos and fetuses growth and development. The first slaughter was performed in 15 days after covering, with encountering of the following slaughters on the 25, 45, 55, 65, 75 and the 90 days of pregnancy. An investigation of the newborn piglets aged of 24 hours and of 30 days of birth was also provided. The final stage of the investigation was performed for the pigs of 90 days old. Each time not less than 8 embryos (fetuses) and 3 to 5 piglets were taken into the investigation. Euthanasia of the pigs was encountered according to the protocol Guide for the Care and Use of Laboratory Animals, AVMA Guidelines (Institutional Review Board/Independent Ethics Committee of the Institute of Molecular Biology of NAS, IRB00004079).

When studying the obtained material different methods of anatomical and histological analysis were applied. In the capacity of fixing substances for the investigated samples of embryos' histological investigations the following liquids of Zenker, Phlemming and Buen were used. The samples were embedded in paraffin with the following preparation of the serial histological slides of 5-8 microns of width. The preparations were treated with hematoxylin according to Weigert (Suvarna 2012) and to Karachi (Suvarna 2012) followed by additional staining by eosin, by azan according to Heidenhain and by azan according to Mallory's trichrome (Gray 1954) as well. The main methodic of staining for the morphological and cyto-morphometric measurements provision was comparably scarcely used staining by azan according to Heidenhain. The latter is a modification of the Mallory method (Gray 1954), which enables getting the more precise staining of the connective tissue as compare with the main method. In all the studies performed with the help of the method of staining by azan according to Heidenhain the fixation as per Zenker was applied. For the process of bronchoalveolar lavage (BAL) getting the healthy pigs aged three months old and weighting 25-30 kg was used for the experiment. BAL was performed in a routine way, and for the cells analysis the staining method according to Giemsa was applied. A total counting and analysis of the bronchoalveolar lavage of the cells' population was performed using smears obtained after the lavage process. Counting of the alveolar macrophages was carried out on the slides of the lungs' preparations of embryos and of the newborn piglets. Each time not less than 5 slides of the lungs preparations, and in each slide not less than 30 lines of sight at the small magnification (0.4 mm^2) were scrutinized. The lines of sight were chosen randomly, excepting the capsule of organ. Beyond the BAL, the biopsy material was obtained from the pigs in order to study the bioptate's printings; for the cells analysis the staining method according to Giemsa was applied. Aiming with provision of the cytospectrometric studies the preparations fixed on the glass slide plate simultaneously were stained by the Schiff reactive according to Feulgen (Lillie 1965; Gaub et al 1975). The DNA hydrolysis was performed in the 5 N HCl solution during 1 hour at the temperature t = 22^0C. The quantitative determination of the DNA-Fuxin complex, as well as the surface area of nuclei and nucleoli was performed on the cytospectrometer SPM 05 of the manufacturer's mark (OPTON, Germany) at the wavelength equal to 575 nm together with the depicting visualization (the microscope magnification was 100 x 1.30). Each time the measurements were carried out in 100 nuclei. An average content of DNA in the nucleus and in nucleoli (expressed in the conventionally comparable units) was calculated, and the surface areas of the nuclei and nucleoli were detected and the mean number of nucleoli per nucleus was calculated as well. On the base of obtained material upon the DNA content in nuclei the histograms of their distribution according to the ploidy classes were constructed. For the latter construction the standard diploid amount of DNA in the lymphocytes' nuclei of the healthy pigs' peripheral blood was determined, which appeared to be completely in correspondence with the DNA content in the diploid population at the phases G_0/G_1 of cell cycle. For the cyto-morphometric analysis of sizes of the nuclei, nucleoli, cytoplasm and also of the cells the "ImageJ" standard program of open access was applied, which enables investigating the square area of the cytoplasm, nucleus and, totally, of the nucleoli. For the total protein amount calculation the previously prepared preparations were stained by naphthol yellow. The technique of staining was provided in a routine manner (Gaub et al 1975). Measurement of the optical density of cell protein was performed at the wavelength equal to 434 nm on the cytospectrometer SPM 05 Opton. For the cytospectrometric analysis of the cells the program ImageG was applied. All sizes of the cells were given in mkm^2. All the obtained experimental data were treated by the methods of variation statistics by means of using the package of applied programs "Excel" for the statistical treatment with the help of the program's chapter "Analysis of data" and subchapter "Descriptive Statistics". In each group for all features the mean arithmetical, the standard deviation and the standard error of the mean arithmetical was calculated. The veracity of differences between the mean values was determined with the help of the t-Student statistical criterion. In non-parametric distribution of the features the u-criterion of Wilconson-Manna-Witney was used with the help of SPSS17.0 program (SPSS, INC., Chicago, IL).

Results. The lungs of a 45-days fetus already have the characteristic outlines and are divided into two big lobes, to which the major bronchi ramification corresponds.

At the earlier stage of embryogenesis (the 45-days embryo) in pigs the alveoli anlagen are of a round or vesicular form, and the epithelial cells are of rectangular form (Figure 1A, B, Figure 2A). Thereafter, starting from the 55 days of the development the respiratory bronchioles and the alveolar ways are being formed. However, the respiratory epithelium differentiation is taking place later on. The main part of alveoli is becoming more flatten (Figure 2B).

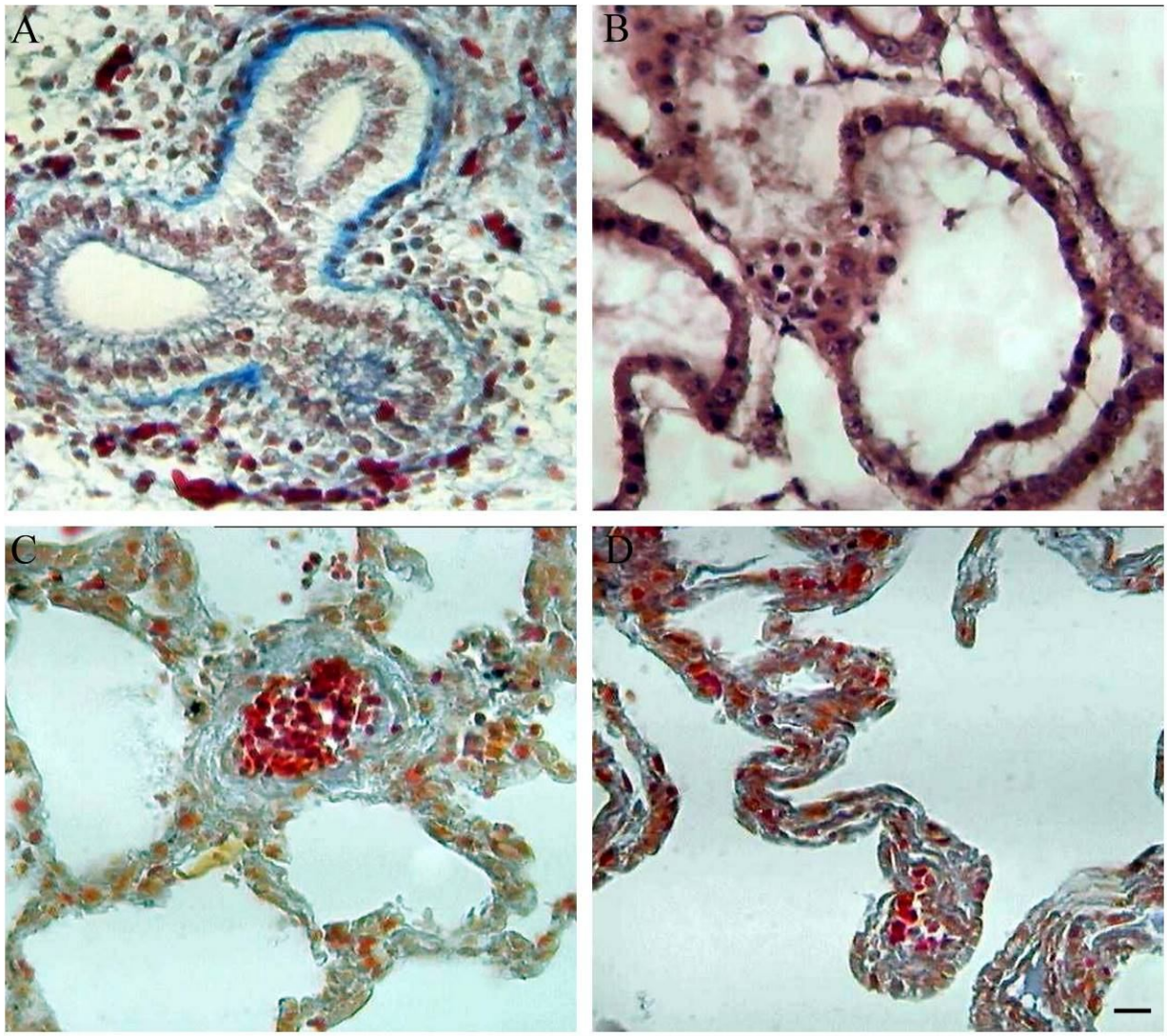

Figure 1. Lungs of the pig embryo and fetus. A – the 45-days pig embryo; the cells of cuboidal epithelium are apparent. Staining used – is by azan according to Haidenhain; B – the 45-days pig embryo. Staining used – is by hematoxilin-eosin. C, D– the newborn pig. Staining used – is by azan according to Heidenhain. Scale – 20 µm.

Separate pneumocytes of the II-nd type are coming to be appeared just by the 75-th day of the fetus development and are characterized by the flattened cytoplasm presence. Pneumocytes of the I-st type are distinct by flatten and prolonged cytoplasm and are coming to be appeared by the 90 days of the fetus development. The final view for the I-st type pneumocytes is coming to being just by the moment of birth (Figure 2 C, D). A significant peculiarity of the 90-days old pig fetus lung is an appearance of the first alveolar ways and alveoli, although they are not in a large quantity yet. The alveoli walls are formed by the flattened epithelium and are surrounded by the connective tissue stroma. The alveoli and alveolar ways are braided by a dens network of capillaries.

The interalveolar connective tissue, anyway, occupies a big area as yet. In the newborn piglet the alveoli are becoming the basic morphological units of lung and occupy an overwhelming part of the lungs' volume (Figure 1 C, D, and Figure 2C, D).

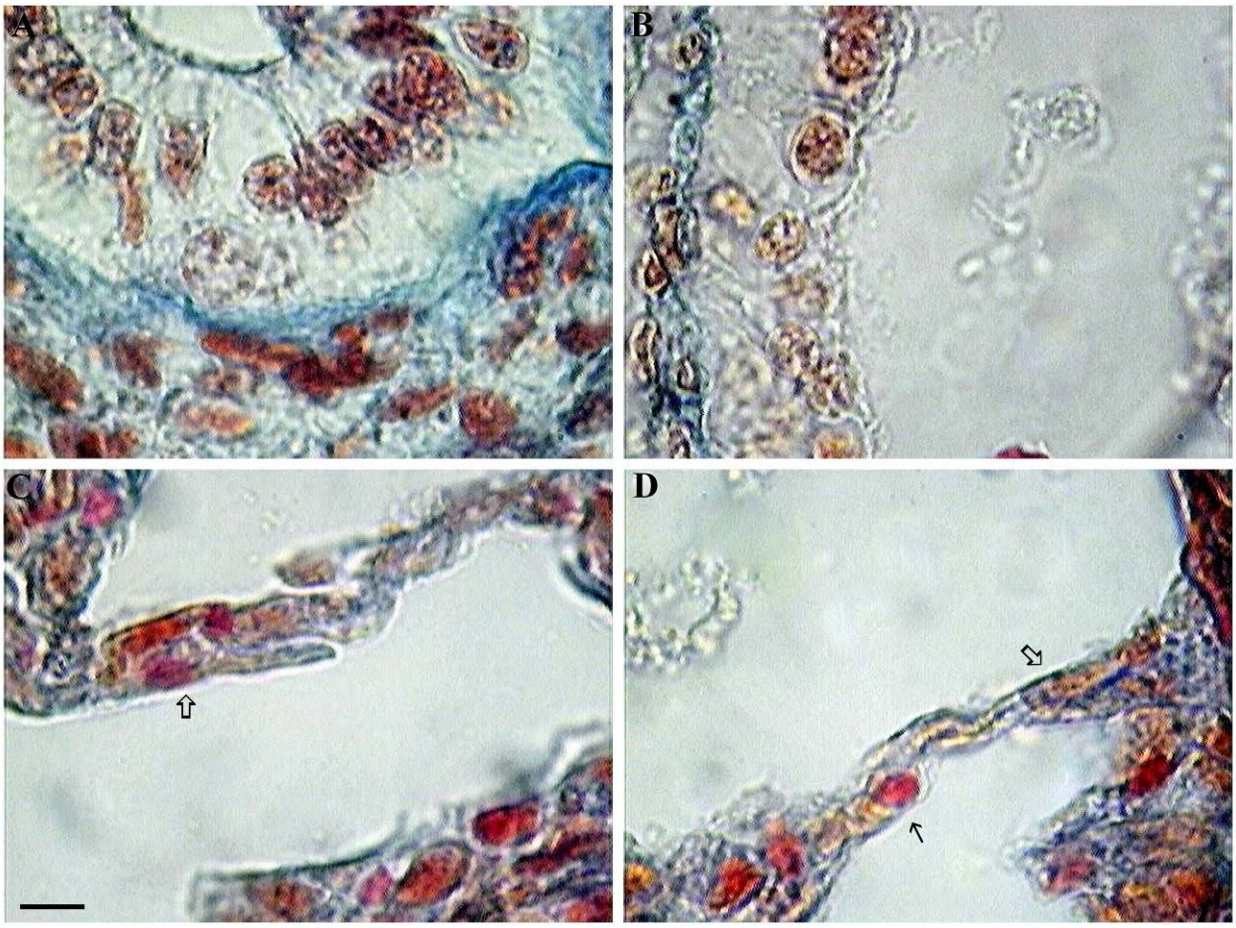

Figure 2. Morphological changes of the epithelial cells of alveoli in the lungs in the pig's embryogenesis. A - the 45-days pig embryo, the cells of cuboidal epithelium are apparent; B– the 75-days pig embryo, the epithelial cells flattening is apparent, but a differentiation into pneumocytes is not observed. C, D– the newborn pig's alveoli. Pneumocytes, practically identical to the corresponding cells of an adult animal are observed. The II-nd type pneumocytes are mentioned by a white arrow, the I-st type pneumocyte is mentioned by a dark arrow. Staining used – is by azan according to Haidenhain. Ocular – 12.5, objective – 25, the scale used – 20 µm.

Appearance of the AM in significant amounts falls on the 65-days period of the fetus development, and the final formation of AM population is observed in the 90-days old fetuses. Under the final formation we assume availability of AM in each part of the lungs' alveoli. Completing of the AM functional morphology conditioning occurs in the beginning period of lungs' pulmonary function provision in the newborn piglets. A role of the alveolar macrophages consists in the phagocytic function accomplishment and also in removal of the dust particles and other substances, which could ingress into the alveoli lumen (Figure 3A, B).

The interstitial macrophages (Figure 4C) in norm are associated with the mucous membrane of airways. During the staining process it becomes obvious, that the large-scale phagosomal vacuoles and phagolysosomes in the interstitial macrophages are not available and the cytoplasm is often somewhat basophilic.

Cells of the ciliar epithelium (Figure 4D) usually have a prolonged cylindrical form with a plenty of cilia; the latter is lining up the respiratory ways, where they provide a transportation of different substances.

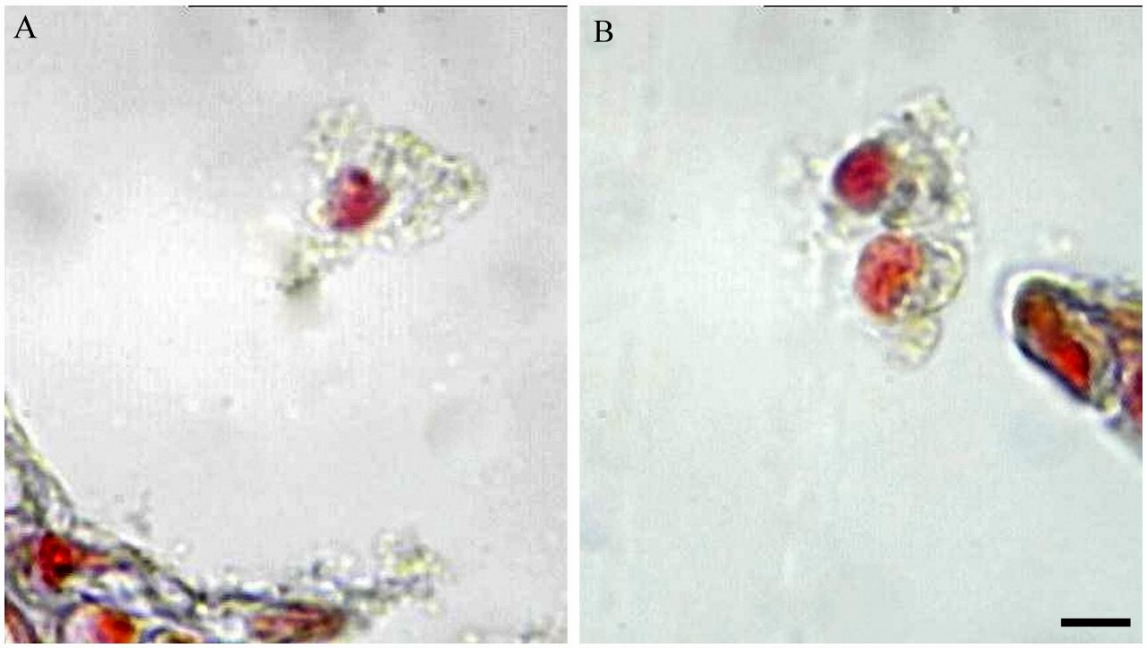

Figure 3. The alveolar macrophages in the newborn pig. A, B– the alveolar macrophages appeared in the alveolar lumen. Staining used – is by azan according to Heidenhain. Scale – 10 µm.

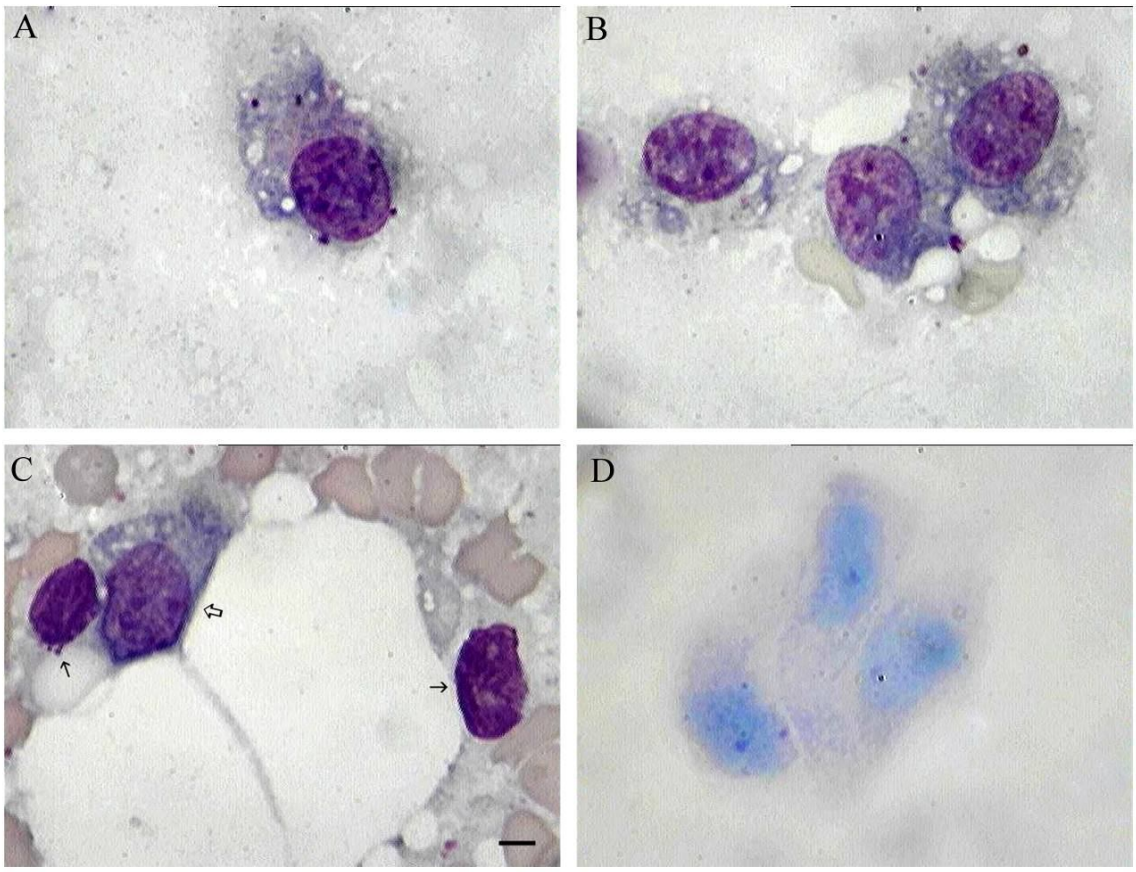

Figure 4. BAL cells (A, B, D) and the bioptate printing (C) of the healthy pig lung (3-months old); A, B - The alveolar macrophages with numerous vacuoles and the particles underwent phagocytosis; C - interstitial macrophage (is mentioned by a white arrow), two lymphocytes (are mentioned by a dark arrows); D – cells of the ciliar epithelium. Staining used – according to Giemsa. Scale – 5 µm.

As it is obvious from the Table 1 the predominating element in the bioptate population of the healthy pigs' lungs were AM, making up 75% from the total cells number.

Table 1

The population analysis of the biopyate cells of healthy pigs lungs (without the erythrocytes consideration) (absolute values and the percentage)

Cells	*Quantity per unit area 0.01 mm²*	*%*
Alveolar macrophages	2-3	75±7.9
Interstitial macrophages	~0.1	3.5±0.8
Lymphocytes	~0.5	10.7±0.9
Neutrophils	<0.1	5.3±0.3
Eosinophils	~0.1	3.5±0.8
Ciliated epithelial cells	<0.05	1.5±0.1
Other cells	<0.01	0.5±0.1

Table 2 contains data on the change in the number of macrophages in the lung biopsy in porcine ontogeny. Obtained data shows that the birth and functioning of the lungs brings to more than tenfold increase of the AM-s number. The AM population organization accomplishment is taking place by the 1-month old period of a piglet, when the population is accelerated more than 15 times comparably with the newborn pig period. At the same time, along with the macrophages maturation process their square area is also increased, what additionally testifies about their maturation.

Table 2

Square area (mkm^2) and the alveolar macrophages number counted onto the square unit (a slide with a square area of 3 mm^2)

Terms	*Quality of alveolar macrophages In sight (3 mm²)*	*Area of alveolar macrophages (μm²)*
45–day embryo	-	-
55-day embryo	0.005±0.001*	216.5±11.3
65-day embryo	0.01±0.01	242.4±14.7
90-day embryo	0.3±0.01	243.1±19.5
Newborn	3.2±0.8**	321.4±18.7
Month-old pig	53.1±9.9**	371.2±31.0
3 month-old pig	85.0±10.2**	397.9±22.7

* - morphological determination at the given stage is rather difficult to perform and the counting is of approximate character; ** - is authentically higher compared with the previous investigations ($p<0.05$ - $p<0.001$)

As it follows from the Figure 5, in norm the main part of AM nuclei does not synthesize DNA, but about 2-4 % of the cells are at the S phase of cell cycle and contain the intermediate values, and about 1% are at the G_2 phase and contain 4c of DNA. At the earlier stages of ontogenesis these types of cells are of lesser amount, and just in the newborns the values are approximately similar to the 3-monthes old piglets.

As it follows from the Figure 6, the authentically found differences in the protein content in AM as compared with the very early AM-s are manifested just in 1-month old piglets and are preserved in the older pigs ($p<0.05$). It is also obvious from the Figure 6, that along with maturation of the pig fetus the protein content in AM is getting increased.

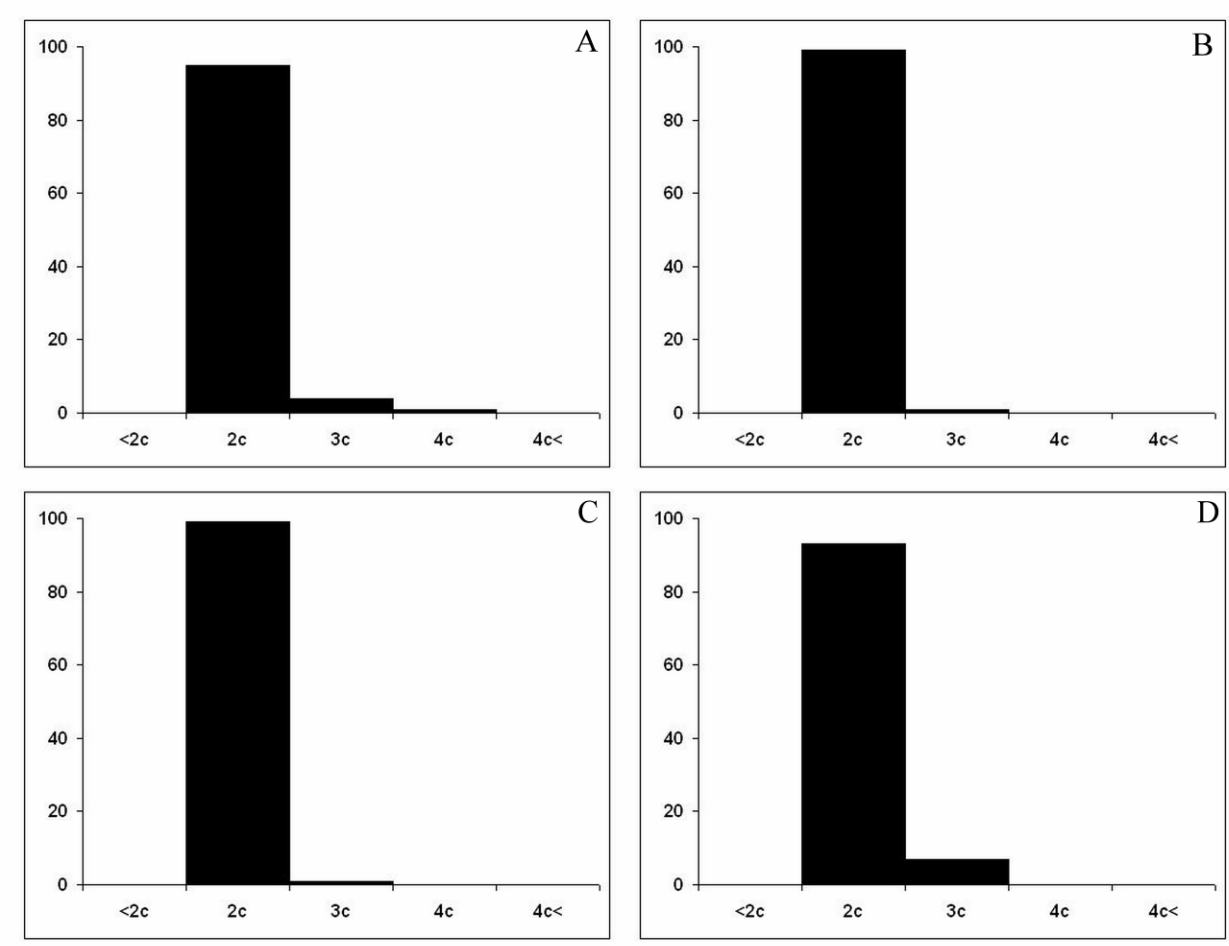

Figure 5. Distribution of the pig's alveolar macrophages nuclei according to the classes of ploidy during the process of ontogenesis. (A – 3-months old piglets; B– the 75-days old fetus of a pig; C – the 90-days old fetus of a pig; D – the newborn piglets. Along the abscess axis – the ploidy units; Along the ordinate axis – the frequency of occurrence (%)).

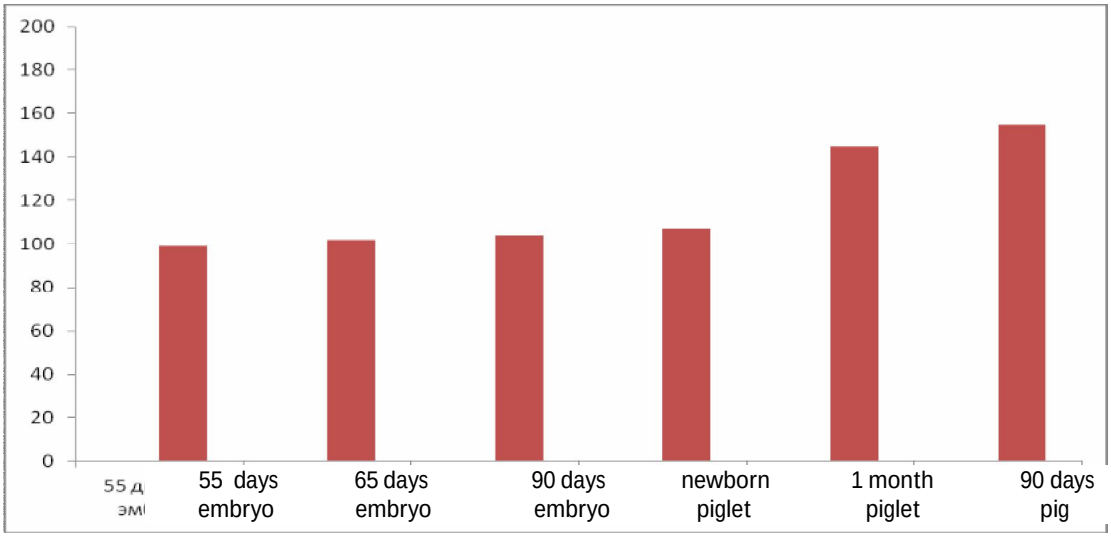

Figure 6. Content of proteins in the alveolar macrophages during the pig ontogenesis process.

Discussion. At present in the lungs' development process there are three main periods or stages detected: the glandular stage, during which the airways are being developed; the canalicular stage, during which the respiratory bronchioles are formed, and the alveolar stage, when the alveolar ways and alveoli are being organized (Carlson 2003). In the pig's fetus development process the glandular stage of the lungs development is initiated very soon, namely, at the beginning of embryonic development. Already in the 25-days old embryos we could find the primary pulmonary sacks arranged dorsally from the heart, which is highly characteristic. The given fact could be explained exclusively from the point of view of phylogenesis, especially when considering the fact that the lungs are the only organs, which do not fulfill their specific function until the birth moment. During the intrauterine life conditions the lungs do not go through significant changes, and just after the birth they obtain a specific alveolar structure. Before birth, the alveoli of the lungs remain in a collapsed state, lined with a cubic or low-prismatic epithelium. The walls of the alveoli are stretched and the epithelium becomes flatened (Baskerville 1976). Our data obtained confirm the conclusions made about the onset of the final stage in the pig lungs' organogenesis accomplishment after the 90-th day from the starting moment of gestation (Widjaja et al 1988). The morphological changes of the epithelial cells of alveoli in pig are correlated with the lungs' development periods. For the glandular period of development a presence the rectangular cells is characteristic, which are arranged in a very tight manner. For the canalicular stage of development a flattening of these cells is characteristic, and the main cells of alveoli, the pneumocytes of the I-st and the II-nd types are coming to appear at the alveolar stage of development, acquiring the accomplished morphological peculiarities by the birth moment.

As it follows from the obtained data the final organization of alveoli and also of their content is taking place in the 90-days old fetus. The basic cells in the intra-alveolar space are represented by the AM-s. It is considered that AM are originated from the interstitial macrophages during a process of their further differentiation (Bowden & Adamson 1976). However, the lungs' interstitial macrophages have a distinct from the AM-s morphology, and, what is of crucial importance, a distinct from them function, as well as they are more heterogenic, what enables differentiating them according to the morphological criteria (Crowel et al 1992). Moreover, the functional activity of the interstitial macrophages plays an important role in inflammation process development, what assumes their differentiation, but probably not final (Zetterberg et al 1998). Nevertheless, despite the fact that even in the embryonic period a part of AM-s is represented by a type of the proliferating cells, the birth and a normal functional activity of the lungs not only does not decrease, but also induces a tendency to increase ($p<0.1$) the proliferation level of these cells. At the same time, the birth and functioning of the lungs brings to more than tenfold increase of AM-s number. The AM population organization accomplishment is taking place by the 1-month old period of a piglet, when the population is accelerated more than 15 times comparably with the newborn pig period. At the same time, along with the macrophages maturation process their square area is also increased, what additionally testifies about their maturation.

In result of the investigation provided our conclusion is yielded, emphasizing on the square area changes and the nuclei distribution according to the classes of ploidy and also the protein content in them. The conclusion we came to is somewhat different from some other authors' conclusion (Zeidler & Kim 1985), testifying about the final formation of AM-s by the end of the first week of postnatal development, and instead considers more prolonged terms – up to 3-4 weeks after the birth. It is possible to get consistent with the conclusions of Banks et al (1999) complying the assumption that namely the respiration is becoming a factor inducing the final maturation of the immune cells (and particularly of AM), and origination of the characteristic cellular population of alveoli, which are revealed in the process of the lungs' content aspiration.

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Received: 01 May 2017. Accepted: 02 June 2017. Published online: 04 June 2017.
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How to cite this article:
Tatoyan M., Abroyan L., Hakobyan L., Avetisyan A., Karalyan Z., Vardanyan A., Karalova E., 2017 The alveoli development and their cellular content during the pigs' ontogenesis. Porc Res 7(1):1-9.