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Ontogenesis of the pig erythroid cells

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Abstract. In the 15-days aged embryos the main part (70%) of primitive erythroid cells are diploid and capable to proliferate, what is testified by the hyperdiploid and tetraploid populations' presence (an approximate correspondence to the S and G2 phases of mitosis). A difference between the hemoglobin content in primitive erythroid cells and the primitive erythrocytes of the 15- and 25-days aged pig embryos is not significant. Anyway, these cells contain much more hemoglobin ($p<0.01$) comparably to the yolk sac erythroid cells' non-differentiated precursors. We did not reveal any difference in the hemoglobin content in di- and tetraploid cells of the 15-days aged pig embryo. In the investigation it was revealed, that the 25-days embryo has simultaneous functioning of two centers of hemopoiesis – the yolk sac and the liver, as well as the primitive erythroid cells long time persistence (up to 55 days of embryonal development) in the pig's fetus blood.
Key Words: ontogenesis, erythropioesis, precursors of erythroid cells, yolk sac, embryonal development.

Introduction. It is well-known, that the embryo erythropoiesis occurs in four successively altering locations: the yolk sac, liver, spleen, the red bone marrow. Actually, the embryo hemopoiesis in the yolk sac is the primary erythropoiesis, which is attributed by the preserved nucleus in all the stages of erythrocytes' maturation and by the fetal type hemoglobin (HbE) synthesis (Wong et al 1986). The first cells are being formed out of the embryo's body, in the sites of mesodermal cells fewer accumulation, called the yolk sac blood islands. The yolk sac blood islands are the first sites of hematopoiesis and vascular development in the vertebrates' embryo (Ferkowicz & Yoder 2005).

The next stage of the process is hematopoiesis in the liver, occurring along vessels in the extra-vascular way. The stem hematopoietic cells first are being differentiated into the blasts and thereafter into the secondary erythrocytes. The central macrophage containing erythroblastic islands' primary formation is taking place in the liver (Sasaki & Iwatsuki 1997). Then the liver becomes the main site of hemopoiesis and stands active till the birth first weeks, but with the activity's gradual attenuation.

Erythropoiesis process in the embryo's liver is represented by the nuclear erythroid precursors of different grade maturation, containing the fetal hemoglobin (HbF), which is due-to its higher affinity to oxygen provides an effective absorption of the latter from the mother's blood (De Simone & Mueller 1979).

The spleen in mammals, usually, is being formed earlier than the hemopoiesis processes rise in it. Probably, because of a big amount of blood accumulation the embryo's spleen becomes the center of hemopoiesis before the birth moment followed by the splenic erythropoiesis gradual cessation. In general, the spleen erythropoietic activity of the embryo and the fetus relatively is not of great significance (Fruhmann 1968; Rifkind et al 1974).

The next stage and localization of erythropoiesis is in the red bone marrow. A formation of the cells, morphologically alike the liver hepatoblasts and the yolk sac cells starts from the bone marrow early mesenchimal cells. Analogically to the latter they give birth to megakaryocytes, the erythroid and the myeloid cells, including neutrophils,

basophils and eosinophils. The embryo's bone marrow significantly differs from the hemopoiesis earlier centers by the myeloid cells formation prevalence as compared to erythropoiesis in it. The early myeloid cells formation process starts in the bone marrow cavity's central part (usually the processes of hemopoiesis begin in the clavicle and then propagate over the rest bones) and further entrain the bone marrow's whole cavity. Erythropoiesis in the embryo's bone marrow develops later and mainly occurs together with the myelopoiesis process, so amongst the majority of the myeloid line maturing cells the erythropoiesis small foci are becoming apparent (King & Ackerman 1967; Carbonell et al 1982).

The embryo's erythroid population cellular content in the late embryonal and early fetal periods (the 24^{th}, 30^{th} and 40^{th} days of gestation) was investigated by Pearson et al (1998). Miller et al (1961) have investigated the pig erythrocytes in the postnatal period. In the given research by means of the methods of cytomorphology, cytomorphometry and cytospectrophotometry the cellular content of embryo's erythroid population was studied, started with the yolk hemopoiesis till the postnatal period with intervals of 10 days, also including the one-day, one-month and three-month aged piglets. Herein, the size indices change dynamics, a content of total protein, hemoglobin, DNA and RNA in all the mentioned cells was studied for the same period of the pigs' embryo development.

Materials and Method

Animals. A total of 16 sows, Large White breed, aged in 11 months were used in the given experiment. The pregnant sows being grown up till 130-140 kg in 11-12 months of age underwent slaughter to study the embryos and fetuses development and growth.

A slaughter was done on the 15^{th}, 25^{th}, 35^{th}, 45^{th}, 55^{th}, 65^{th}, 75^{th} and 90^{th} days of the sows' pregnancy. For each mentioned day two sows were slaughtered. Also the piglets of 24 hours, 30 days and 90 days of birth were studied. For each time not less than 8 embryos (fetuses) and 3-5 piglets were studied. Euthanasia of the pigs was performed according to the Guide for Care and Use of Laboratory Animals, AVMA Guidelines (International Review Board/Independent Ethics Committee of the Molecular Biology of NAS, IRB00004079).

Laboratory investigation. As fixations for the histological samples of embryos the Zenker's, Flemming's, Buen's liquids and 96% ethanol solution were used. The samples were flooded in paraffin, followed by preparations of the series of histological slides of 5-8 μm thick. The preparations were stained by Hematoxylin solution according to Weigert and then by eosin (Lillie 1965) and azan according to Mallory (Gray 1954).

Morphology of the erythroid cells. For cells analysis, slides were fixed in pure methanol and stained by Giemsa modified solution (azure B/azure II, eosin and methylene blue) according to the manufacturer's protocol (Sigma-Aldrich). Cells were examined under the light microscope at 1250 x magnification in a random sequence. At least 200 cells in each sample were classified. Morphological determination of the stages of primitive and early liver erythropoietic development was performed as per Baron et al (2012). The erythroid cells' classification was done according to Douglas & Weiss (2010).

Morphometric analysis of the liver. The embryo's liver hematogenic tissue size was determined on the preparations slides with the help of programming support ImageJ. In each case not less than 4 liver slides were used, and in each slide not less than 30 fields of view (0.4mm) were observed. Fields of view have been chosen randomly, excluding the liver capsule.

Protein staining with nafthole yellow and the protein cytophotometry. For the total protein counting the preparations were stained with nafthole yellow by routine method (Gaub et al 1975). The protein optical density measurement was performed in the wave length of 434nm on the cytospectrophotometer SMP 05 Opton. For the cells sizes cytometric analysis the programming support ImageG was used.

DNA protein staining. All preparations were treated with the combined Feulgen-Naphthol Yellow staining (FNYS) procedure (Gaub et al 1975). This method permits simultaneous microspectrophotometric analyses of DNA and protein in single cells and the protein value is closely correlated to the amount of dry mass of the cell.

Image scanning cytometry. For image scanning cytometry and DNA measurement, blood slides were fixed in 96% ethanol for 30 minutes and stained in fresh Schiff's reagent (DNA hydrolysis in 5 N hydrochloric acid for 60 minute at 22°C) by the method of Feulgen (Lillie 1965). In order to measure DNA content (in conventional units) by image scanning cytometry, computer-equipped microscope-cytometer SMP 05 (OPTON) was used at 575 nm wavelength and at 1250 × magnification. Before the scanning process, each nucleus was contoured, and cytometry of nuclear DNA content of all studied types of cells were carried out at 1 to 7 dpi.

Ploidy of cells. DNA content was expressed on a "c" scale, in which 1 c is the haploid amount of nuclear DNA occurred in normal (non-pathologic) diploid populations in G0/G1. The DNA content of unstimulated swine lymphocytes was used as a diploid standard for measurements. DNA measurements identify nuclei as aneuploid if they deviate more than 10% from 2 c, 4 c, 8 c, or 16 c; i.e. if they are outside of 2 c ± 0.2, 4 c ± 0.4, 8 c ± 0.8, or 16 c ± 1.6 values. The total number of cells in euploid areas of the DNA histogram rescaled by the mean corrective factor (1.8 c - 2.2 c, 3.6 c - 4.4 c, 7.2 c - 8.8 c, and 14.4 c - 17.6 c) was also calculated. The variability of DNA content in unstimulated lymphocytes did not exceed 10%.

Hemoglobin quantification. Hemoglobin amount in cells was determined spectrophotometrically on unstained preparations. Wave length scans of the diluted rat blood consistently showed the greatest absorbance at 414 nm, which is the Soret peak from hemoglobin (Tanaka et al 1989).

Statistical analysis. The significance was evaluated by two-tailed Student's t-test, and Mann-Witney u-test.

Results and Discussion. Our data show that porcine early yolk sac derived haemopoietic cells in the foci were large and generally oval in shape. Their nuclei appeared to be oval, 5-7 μm wide.

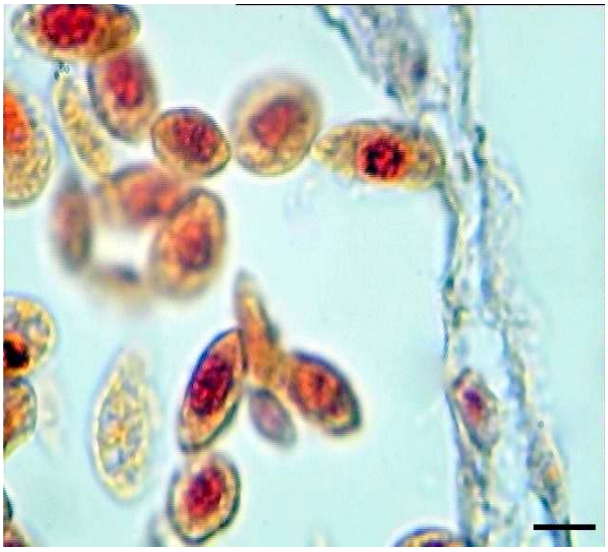

Fig. 1. Primitive yolk sac derived haemopoietic cells (red colored) 15 day swine embryo. Present dividing cells. Scale bar is 10 μm.

Morphologically they were much likely to those of mice (Sasaki & Matsumara 1986). In the following differentiation a rounding of the hemopoietic cells and formation of the primitive erythropoietic cells occur.

The primitive cells of blood are being formed out of the embryo body in the mesenterial cells accumulation small sites, named blood islands (yolk sac blood islands). Yolk sac blood islands are the first sites of hemopoiesis and vascular development in the vertebrates' embryo (Ferkowicz & Yoder 2005). The blood island is an early embryonal formation composed of the mesenteric cells, lining the yolk sac. Representation of a typical blood island of the 15-days pig embryo with different hemopoietic elements

surrounded by endothelium and arranged between the visceral entoderma and the internal mesothelium of mesodermal origin is presented in Fig.2 (Ferkowicz & Yoder 2005). According to our data hemopoiesis in the blood islands is dominating in pigs (rather the only type of hemopoiesis) on the 15th day of the pig embryo's development, remains significant (not lesser than 40%) on the 25th day and practically is disappeared on the 35th day.

Morphology of early embryonal erythroid cells of pig both of the yolk sac origin and of the liver origin is presented in Fig.3. It should be mentioned that the 25-days aged pig embryo is attributed by simultaneous presence of both primitive erythropoietic cells and early liver erythropoietic ones as well.

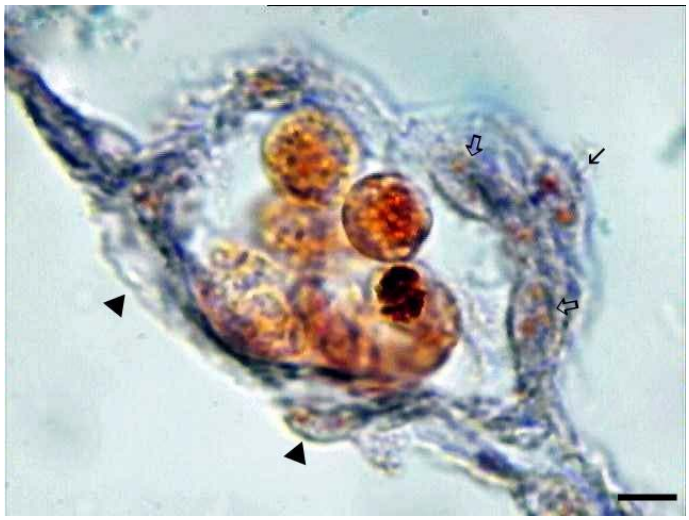

Fig. 2. Primitive yolk sac blood islands in 15 day swine embryo. Staining: trichrome by Mallory. Primitive erythroid cells (red coloured), endothelial and mesothelial cells (blue colored cytoplasm and red nuclei). Yolk sac includes all the erythroid elements and the surrounding endothelium (transparent arrows), situated between the outer visceral endoderm (black arrows) and the inner mesodermally derived mesothelium (triangles). Scale bar is 10 μm.

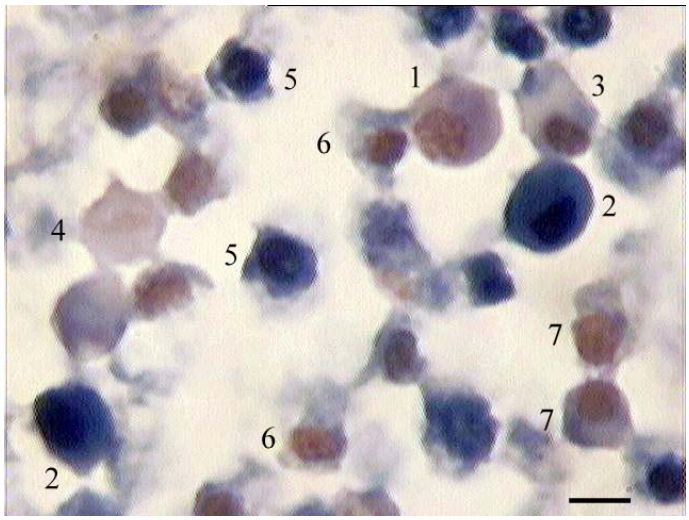

Fig.3. Early erythroid cells of the 25 day swine embryo. Paraffin embedded tissue Section of the 25 day embryo vessel. Stages of primitive and liver early erythropoietic development. 1.First stage primitive erythroid cells (yolk sac origin); 2.Second stage primitive erythroid cells (yolk sac origin); 3.Third stage primitive erythroid cells (yolk sac origin); 4.Primitive erythrocyte (yolk sac origin); 5.Basophilic erythroblast (liver origin); 6.Polychromatophilic erythroblast (liver origin); 7.Orthochromatophilic erythroblast (liver origin). Staining by Giemsa. Scale bar is 10 μm.

Population analysis of the pig erythroid cells in its development embryonal stage is presented in Table 1.

Table 1

Population content of the pig embryo's, fetus's, newborn's and the 30-days aged piglet's peripheral blood (in %)

Cells	Terms of embryonic, fetal and postnatal development (days)									
(%)	15	25	35	45	55	65	75	90	newborn	30
primitive erythroid cells	98.0±2.1	42.0± 3.5	0.1± 0.01	0.01± 0.0001	0.001± 0.0001	-	-	-	-	-
Primitive erythrocyte	2.0± 0.3	0.01± 0.001	0.001± 0.0001	-	-	-	-	-	-	-
Basophilic erythroblast	-	6.0± 0.8	1.5± 0.2	0.2± 0.01	0.1± 0.01	0.1± 0.02	0.01± 0.001	-	-	-
Polychromatophilic erythroblast	-			0.4± 0.01	0.3± 0.07	0.2± 0.02	0.1± 0.03	0.1± 0.02	0.01± 0.001	-
Orthochromatophilic erythroblast	-	51.0** ± 3.4	82.6** ± 4.7	6.2± 1.3	5.5± 1.0	1.2± 0.1	0.1± 0.01	0.1± 0.01	0.01± 0.001	0.001*± 0.0001
Reticulocyte	-	-	11.5±1.3	27± 3.3	22.8± 4.5	11.3± 2.8	4.9± 1.1	2.6± 0.7	1.1± 0.4	1.7± 0.3
Erythrocyte	-	0.9± 0.05	4.3±0.7	66.2± 8.1	71.1± 10.1	87.2± 7.9	94.9± 9.3	97.2± 4.6	98.88± 1.3	98.3± 1.8

* the presence of orthochromatophilic erythroblast 30 day piglets noted only in some animals;
** polychromatophilic and orthochromatophilic erythroblast are hardly differentiated at this stage.

From the table below it is obvious, that erythropoiesis in primitive yolk sac successively turns into another type of erythropoiesis in erythroblastic islands (EI). This substitution takes place in the 25-days aged embryo and practically ends in 35-days aged one, being accompanied with a complete disappearing of the yolk sac cells population.

Table 2

A dynamics of the erythroid cells nuclear area and cytoplasm changes in the pig vessels during ontogenesis (μm^2)

Age of the embryo (days)	Types of the cells	Localization	Area of cell nucleus	Area of cell
15	Primitive erythroid cells	Yolk sac	21.1±3.1	72.7±12.9
15	Primitive erythrocyte	Yolk sac	-	58.9±4.2*
25	Erythrocyte	Liver	-	52.1±5.3**
35	Erythrocyte	Liver	-	45.2±3.5†
45	Erythrocyte	Liver, spleen	-	45.7±4.1†
55	Erythrocyte	Liver, spleen	-	47.2±5.1†
65	Erythrocyte	Liver, spleen	-	46.1±2.8†
75	Erythrocyte	Liver, spleen	-	46.2±4.4†
90	Erythrocyte	Liver, bone marrow	-	45.3±3.3†
Newborn	Erythrocyte	Liver, bone marrow	-	44.8±4.0†
3 months	Erythrocyte	Bone marrow	-	35.4±1.6

* significantly higher, comparably to the postnatal erythrocytes ($p < 0.05$-0.01);
** tendency comparably to the postnatal erythrocytes ($p < 0.01$);
† the total tendency to decrease comparably to the primitive erythrocytes, the number of inversions per u-criterion is equal to 2 ($p < 0.01$).

We also studied the primitive erythroid cells by the nuclei distribution on the classes of DNA ploidy and presented the results in Fig.4.

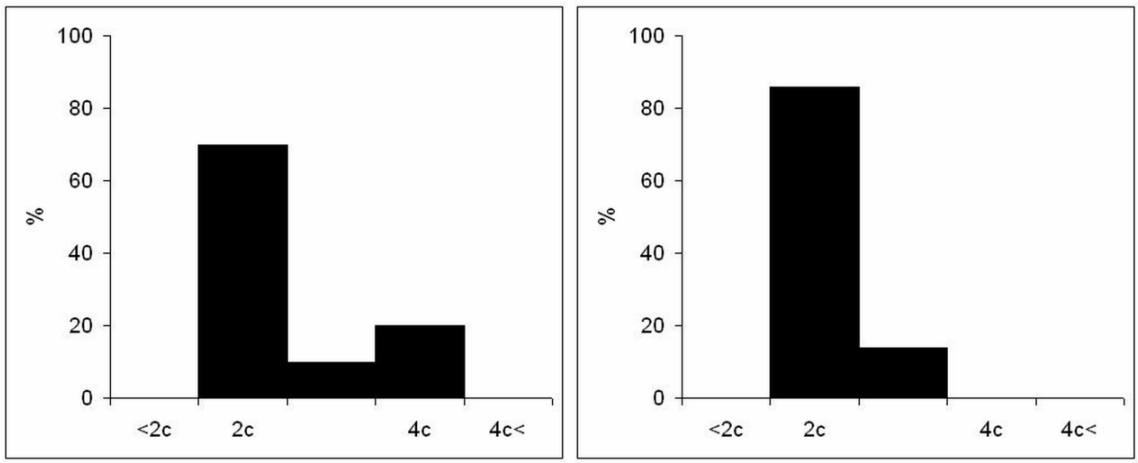

Fig. 4. Distribution of the primitive erythroid cells by the DNA ploidy in 15 and 25 day swine embryo. A - primitive erythroid cells in 15 day swine embryo; B - primitive erythroid cells in 25 day swine embryo.

As it is seen from the Fig.4, the main part (about 70%) of the primitive erythroid cells of the 15-days aged embryo are diploid and capable to proliferate, what is testified by hyperdiploid and tetraploid populations' presence (an approximate correspondence to the S and G2 phases of mitotic cycle). The main cell population on the 25th day of embryonal development is represented by diploid and hyperploids cells. The tetraploid cells disappear from general population of cells.

Table 3

Hemoglobin content in the pig's erythroid cells during its ontogenesis (pg)

Age of the embryo (days)	Types of the cells	Localization	Content of hemoglobin (pg)
15	Primitive erythroid cells	yolk sac	40.2±7.4*
15	Primitive erythrocyte	yolk sac	33.7±9.0*
25	Primitive erythroid cells	yolk sac	33.4±5.1*
25	Primitive erythrocyte	yolk sac	41.4±5.2*
25	Early embryonic erythroblast	Liver	14.9±4.1
25	Late embryonic erythroblast	Liver	15.5±3.2
25	Erythrocyte	Liver	20.3±0.5**
35	Erythrocyte	Liver	21.1±1.2**
45	Erythrocyte	liver, spleen	19.9±1.1
55	Erythrocyte	liver, spleen	19.2±1.4
65	Erythrocyte	liver, spleen	18.7±1.0
75	Erythrocyte	liver, spleen	19.1±0.9
90	Erythrocyte	liver, bone marrow	18.9±1.5
Newborn	Erythrocyte	liver, bone marrow	18.6±0.8
3 months	Erythrocyte	Bone marrow	18.2±2.1

* significantly higher, comparably to the postnatal erythrocytes ($p < 0.05$-0.01);
** comparing to the mature erythrocytes, the number of inversions as per u-criterion is equal to 2-3 ($p < 0.01$).

The data on hemoglobin amount in the erythroid cells of the pig's yolk sac in the 15- and 25-days aged embryos are presented in Fig.5. As it is seen from the Fig.5, a difference between the hemoglobin content in primitive erythroid cells and the primitive erythrocytes of the 15- and 25-days aged pig embryos is not significantly expressed.

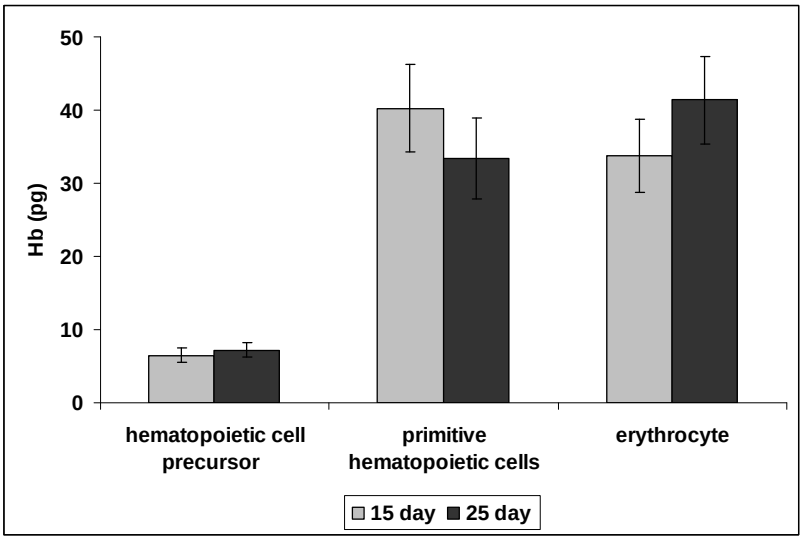

Fig.5. Hemoglobin content (pg) in the pig's yolk sac erythroid cells.

Anyway, these cells contain much more hemoglobin ($p < 0.01$) comparably to the yolk sac erythroid cells' non-differentiated precursors. We did not reveal any difference in the hemoglobin content in diploid and tetraploid cells of the 15-days aged pig embryo.

As it is yielded from the Fig.6, a difference between the total protein content in the non-differentiated precursors of the yolk sac erythroid cell, the primitive erythroid cells and the primitive erythrocytes is not not significantly expressed.

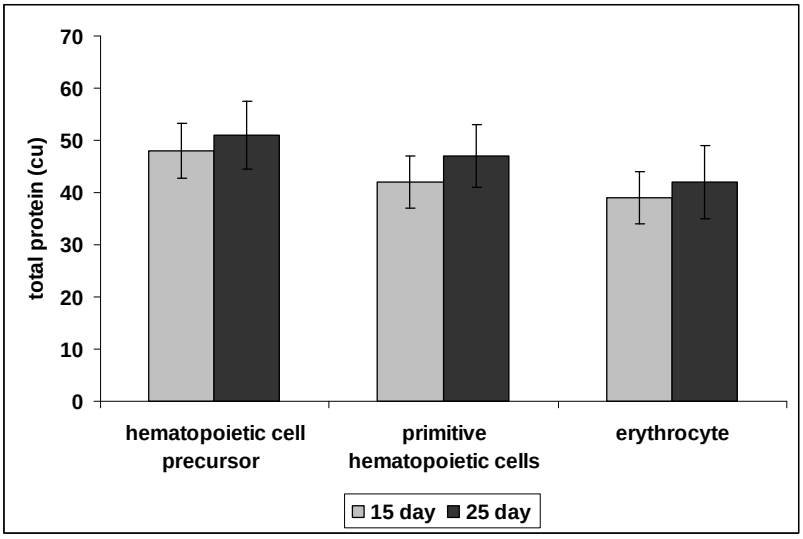

Fig.6. Total protein content (c.u.) in the pig's yolk sac erythroid cells.

From the Fig.7 it comes obvious, that the RNA content is reduced along with the deepening of differentiation in the pig's yolk sac erythroid cells. The given fact is well elucidated in scientific literature (Van Hove et al 1990). Content of RNA in the non-differentiated precursors of the yolk sac erythroid cell was more than in the primitive erythroid cells and in the primitive erythrocytes (both on the 15^{th} day and the 25^{th} day of embryo development) ($p<0.01$); but it was more in the primitive erythroid cells compared to the primitive erythrocytes ($p<0.01$) both on the 15^{th} day and the 25^{th} day of embryo development.

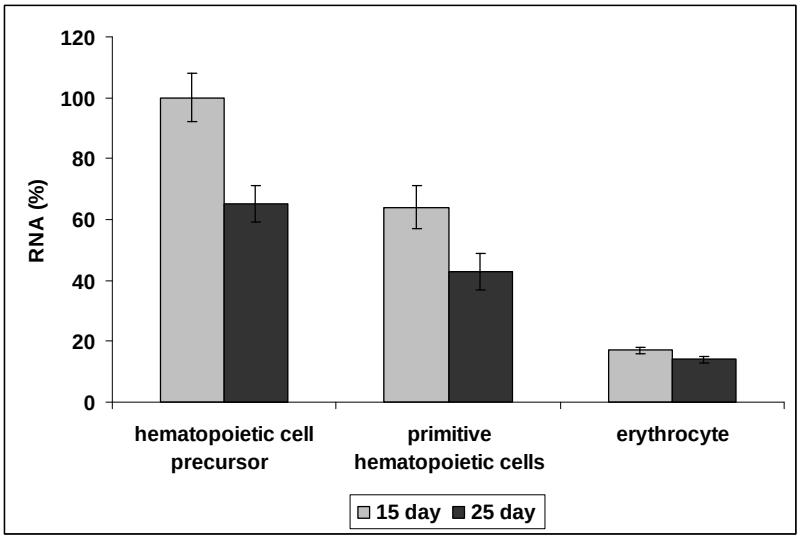

Fig.7. RNA content (c.u.) in the pig's yolk sac erythroid cells. * For 100% of RNA its content in the hemopoietic precursors cells of the pig's 15-day embryo was accepted.

As it is yielded from the Fig.6 and Fig.7, in the hemopoietic cells precursors the total protein content, in general, was not lesser than it was in mature erythroid cells of the pig's yolk sac. But the hemoglobin content in the early cells was significantly lesser (more than 3.5-4 times). Probably, it could be explained by incomplete differentiation level of the given cells. Along with deepening of differentiation the hemoglobin intensified synthesis is started, so the main protein both in the primitive erythroid cells and the primitive erythrocytes is represented.

There is a rather less number of investigations on primitive erythropoiesis in pigs considering just some discrete researches on the given topic. Thus, the yolk sac in pig's embryo was studied by Liwska & Grabinski-Baranowski (1994). In the research they indicated that the hemopoietic functions transfer from the yolk sac to the liver is occurred by the 27^{th} day of embryo development and the hematopoiesis' functioning till the 51^{th} day of pig's embryo development. Our data indicate on the earlier terms (the 25^{th} day for simultaneous functioning of two centers of hematopoiesis – the yolk sac and the liver) and almost a complete cease of it in the yolk sac after the 35^{th} day of embryo development. Probably, the data difference (with the mentioned authors' data got) is a consequence of different pig breeds use in experiment, what predetermines the erythropoiesis terms (Pearson et al 1998).

So, considering the literature data, it is possible to testify to a variability of the erythropoiesis terms in different pig breeds. It also should be noted, that the primitive erythroid cells insignificant presence in the pig embryo's vessels after the 35^{th} day of development does not surely indicate on the erythtopoiesis in yolk sac. It is known, that the primary mesenchimal cells observed all over the organs of embryo and/or fetus (Emura et al 1983) and in the body cavities, especially in anterior primary pericardial region, play an auxiliary role in early embryonal hemopoiesis.

In the taken early terms of gestation (the 15^{th}, 25^{th}, 35^{th} days, etc) we have revealed the un-nuclear cells population arising during the yolk sac erythropoiesis process at the early stage of ontogenesis.

As it could be seen from the Tab.2., the sizes of the similar erythrocytes (which are being formed in the yolk sac) significantly exceed not only that of the mature ones (of the bone marrow origin), but also of those being formed in early (embryonal) erythroblastic islands with central macrophage in it.

As it is known, lost of nuclei is a unique behavior during definitive type of erythroid maturation in mammals (Bills et al 1992), and the extruded nuclei are immediately taken up by macrophages of EI. But in the research of Kingsley et al (2004) the primitive erythroid cells' possible enucleating found in mice was shown. Presence of the erythroblastic islands with central macrophage, especially in the 15-days aged

embryo seems to be less likely. So, a mechanism of the primitive erythroid cells enucleating is still to be investigated.

The data got on the hemoglobin content per cell in the pig's embryogenesis testifies to the hemoglobin content per cell significant decrease along with the gestation period increase. These data correlate with those got by O'Connor (1952) in the birds' embryonal erythropoiesis research. It should be noted, that the primitive hematopoietic cells contain approximately two times more hemoglobin comparably to the mature erythrocytes, and their square area exceeds that of mature ones more than two times, and consequently, their volume also exceeds the mature ones' volume more than three times (if not considering the present nucleus). The primitive un-nucleated erythrocytes contain hemoglobin over two times more than the mature ones, approximately in two times more volume. So, the hemoglobin saturation grade in primitive hematopoietic cells, primitive erythrocytes and the mature (postnatal) ones is nearly the same.

Resuming the pig's embryonal erythropoiesis research we have summed up the hematopoietic tissues' temporal localization with their moiety in total erythropoiesis of pig during its intrauterine development.

A comparative analysis of the hematopoietic tissues' temporal localization during the pig's intrauterine development (Fig.8) revealed that unlike the human embryo the liver participation in the pig's embryo erythropoiesis occurs already starting with the 20th day of gestation.

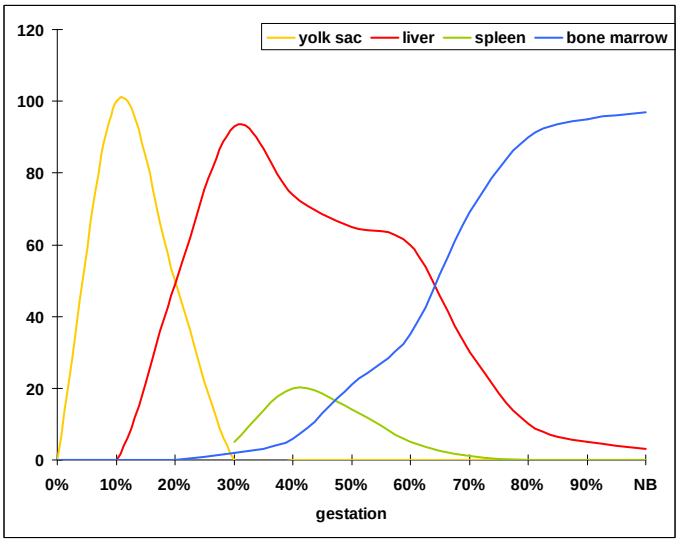

Fig.8. The hematopoietic tissues' temporal localization during the pig's intrauterine development. On the abscissa axis – a moiety of organ's participation in hemopoiesis (%); on the ordinate axis – a term of the intrauterine development, expressed in percents from the pregnancy duration (in pigs the total pregnancy period lasts 115 days; NB - newborn).

At the hepatic hemopoiesis' initial stage the primitive erythroid cells formation is similar to those in the yolk sac (Isern et al 2011). These cells form hemopoiesis loci similar to the yolk sac blood islands very soon (Ferkowicz & Yoder 2005). But already at the early stages the nuclear cells of much lesser size than the primitive erythroid cells are observed in the hepatic hemopoiesis loci. At the same time the erythrocytes of lesser size than the cells formed in the blood islands of yolk sac are appeared. This fact testifies to the eryhtropoiesis different types' presence in the mentioned period of pig embryonal development. It is also necessary to mention, that we revealed a few amount of the primitive erythroid cells even after the yolk sac reduction on the 35-55th days of embryonal development. According to the contemporary conception of embryonal erythropoiesis in mammals (Sequera Lopez et al 2003) the early hemopoietic cells formation is feasible in all the tissues. The fact is that, hemopoiesis during the embryonal development occurs first in yolk sac, and then in the aorto-gonado-mesonephric region, the fetus's liver, spleen, and eventually in the bone marrow. Probably, a long lasting

persistence of fewer primitive erythroid cells in the pig fetuses' blood (on the 45-55th days of embryonal development) is explained by that.

Conclusions. In the given research the methods of cytomorphology, cytomorphometry and cytospectrophotometry the cellular content of swine embryo's erythroid population was studied, started with the yolk hemopoiesis till the postnatal period, including the one-day, one-month and three-month aged piglets. The observations of the present study clarified periodization of the erythropoiesis in pigs and described erythroid cells and their development in ontogenesis of the swine.

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