ти плаценты, второго и третьего ребенка из тройни, причем величина показателя увеличивается от первого к третьему.

4. Коллизия близнецов сопровождается мембранным flickering высокой интенсивности у обоих детей и высоким стоматоцитозом.

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Поступила 24.08.2013

The Specific Features of Red Blood Cell Membranes in Premature Neonates Due to Multiple Pregnancy

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Objective: **to reveal the specific features of red blood cell membranes and their nanostructures in premature babies born from multiple pregnancy.** ***Subjects and methods.*** **The investigation enrolled 28 newborn infants, including 14 premature babies born from multiple pregnancy. The mean gestational age was 34.1±1.7 weeks; their birth weight was 2065±304.8 g. A comparison group consisted of 14 term neonates born from favorable pregnancy and term labor. The mean gestational age of the babies was 39.4±0.5 weeks; their birth weight was 3131.7±588.8 g; the infants had a one minute Apgar score of 8±0.4. Their red blood cells were exam-**

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ined using an atomic force microscope (AFM). The objects to be examined were residual umbilical cord blood (RUCB), central venous blood 7 hours after birth and venous blood had been taken from the premature neonates on day 7 of life. The placentas were also examined. The data were compared with gestational age, birth health status, and clinical symptoms. *Results*. In babies at birth, planocytes are a major morphological type of red blood cells; at the same time, there are also stomatocytes and other abnormal cells that are intermediate reversible forms. The first-order surface is the least stable and prone to pathological changes on the red blood cell membrane and the spectrin matrix and protein clusters are unchanged. The partial pressure of carbon dioxide and hemoglobin oxygen saturation affected the heights h_1 and h_2 of the red blood cell membrane in residual UCB. The internal, cytoplasmic surface is resistant to long-term hypoxia exposure, as the heights h_1 and h_2 of the red blood cell membrane at birth corresponded to the similar heights of healthy neonates and remained unchanged throughout the study. The first postnatal life hours are characterized by the active transformation of red blood cells from one form to another. The fact that diskocytes are lacking in premature neonates during the early neonatal period suggests that the resistance of red blood cell membranes to unfavorable intrauterine factors is diminished. The consequences of prenatal exposure of red blood cell membranes are retained for a certain time and are beyond the early neonatal period. A baby's birth stops a cascade of pathological reactions, but there is no normalization in the indicators in question by postnatal day 7. The high membrane flickering is conserved; the morphological composition of red blood cells does not persist. *Key words:* multiple pregnancy, membrane nanostructure, red blood cells, premature neonates, placenta.

Alterations of erythrocytes may contribute to mechanisms of critical illness development [1—7]. A great attention has currently been given to the issues of perinatology because the rate of premature and sick children birth is still high. Special attention has been given to multiple pregnancies in which there is a high risk of antenatal and intranatal deaths of fetuses and birth of pre-term infants including the ones with low and extremely low birth weight [8, 9].

Along with the complex examination of the newborns the study of red blood cells (RBCs) and other cells in residual umbilical cord blood (RUCB) remains an important source of information, especially in multiple pregnancy and preterm birth, because the detection of abnormal cells and cell elements could be due to the process of adaptation to the labor or a certain disease.

At the same time, hypoxia is an important factor affecting the RBCs condition. Acute hypoxia was found to increase the number of RDCs and hemoglobin concentration. Intrauterine hypoxia increases the number of normoblasts in cord blood, which indicates the stimulation of erythropoiesis [10].

Active hemopoiesis in extremely pre-mature infants with extremely low body is a necessary condition for the adaptation in the early neonatal period. The lack of bone marrow hemopoiesis, low hemoglobin content, low number of RBCs, low hematocrit, change of RBCs morphometric characteristics — poykilocytosis — are negative prognostic symptoms. The normalization of erythropoiesis is observed only at the end of the 1—2nd month of life in pre-mature infants with favorable process of adaptation [11]. The morphological structure of RBCs, the structure of their membranes, the detection of reversible and irreversible changes have diagnostic significance along with their quantitative characteristics. The oxygen transport function of RBC depends on the functional state of its membrane [12]. Studing oRBCs morphology using light immersion microscopy showed that echinocytes, ovalocytes, spherocytes, shistocytes were the most often common forms of RBCs in newborns with multiple organ failure by the 7th day of life, which may be caused by the influence of various factors, including effects of oxidative stress on RBCs membrane [13, 14].

Studying the RBC ultrastructural characteristics using light and electron microscopy shows that the individual ultrastructure of diskocytes and planocytes is not always homogeneous. The thickening of the outer zone of cell membrane lipid bilayer is typical for diskocytes. The ultrastructure of stomatocytes is more heterogeneous if compared with diskocytes and planocytes.

When forming, less optical density is typical for stomatocytes and increases during further cell transformations. Double cell membranes are formed on the surface of stomatocytes. In some cases the transformation of diskocytes into stomatocytes causes additional exit of hemoglobin and parts of the inner content from the cell through small areas of cell membrane lipid bilayer. The ultrastructure of stomatocytes is characterized by less homogeneous membrane the larger number of its local damages and less even intracellular distribution of hemoglobin if compared with diskocytes;. The results obtained show that the reversible transformation «diskocyte → stomatocyte» and back cannot be exactly the same because the transformation of stomatocyte into diskocyte will require so called «healing» of local destructive changes in cells [15]. Atomic force microscopy allows to study the microstructure of RBC membranes in various diseases and critical conditions [16, 17].

Objective: to study the morphology of RBCs and the nanostructures of their membranes in premature children born from a multiple pregnancy.

Materials and methods

A study of RBCs membrane nanostructure of 28 newborns including 14 premature newborns born from a multiple pregnancy, which formed the study group, was performed. The basic indicators of pre-mature infants are presented in table 1. The mean gestational age was 34.1±1,7 week, birth weight 2065,4±304,8. 4 spontaneous twins (3 bichorionic biamniotic and 1 monochorionic biamniotic) and 2 trichorionic triamniotic triplets, received by the method of in vitro fertilization (IVF) were included in the study.

4 (28,6%) neonates with respiratory distress syndrome (RDS) needed artificial lung ventilation (ALV). The duration of artificial lung ventilation was 52±23 hours.

In most cases the amniotic fluid was transparent, in two cases there was blood due to the placental abruption. The male gender was prevaliant.

Exclusion criteria: newborn isoimmunisation by the ABO and rhesus factor.

The data on the newborn mother's health condition, the course of pregnancy, the way of delivery are very important. The medical anamnesis of the mothers is presented in table 2.

Table 1

Basic parameters of the newborns

Parameter	Values ($M\pm\sigma$), % n=14
Gestational age, weeks	34,1±1,7
Body weight, g	2065,4±304,8
Twins	4
Bihorionic biamniotic	3
Monochorionic iamniotic	1
Triplets	2
Score (Apgar's scale, the 1st minute of life)	6,2±1,0
Score (Apgar's scale on the 5th minute of life)	7,2±0,7
Males	11 (78,6)
Female	3 (21,4)
The number of the newborns on ALV	4 (28,6)
Transparent amniotic fluid	12 (85,7)
Amniotic fluid with blood	2 (14,3)

In 83.3% of cases the obstetric history was complicated: the pregnancies followed previous medical abortions, spontaneous abortions, which gave the background for the threat of interruption and pre-term delivery from the earliest days. The physiologic cause of pregnancy was observed in one case.

The primary and secondary infertility, multiple pregnancy, obtained by the method of IVF are the risk factors for pathological cause of pregnancy. In our study 2 pregnancies, obtained with the help of high reproductive technologies, were under the threat of interruption from the earliest days. One of them was complicated by isthmic-cervical insufficiency, the other one was complicated by gestational diabetes, preeclampsia of the second half of a pregnancy, which was the cause of pre-term delivery. In one case the third child was born without any signs of live birth, the intranatal fetal death was confirmed.

The causes of preterm birth were premature pouring out of amniotic fluid, the detachment of low lying placenta, rapid delivery, preeclampsia of the second half of pregnancy, isthmic-cervical insufficiency. The majority (5—83,3%) of pregnant women held an emergency caesarean cessation by Gusakov's method.

All the pregnant women included in the study received treatment aimed at the prolongation of pregnancy and improvement of maternal-placental blood flow.

The RUCB of 14 full-term newborns with favorable course of pregnancy and urgent delivery was used to obtain normal RBCs in the field of AMS.

The mean gestational age of the infants was 39,4±0.5 weeks, the birth weight — 3131,7±588,8 grams, one minute Apgar score of 8 ±of 0.4 points. 78.5% of the newborns were born by vaginal delivery, 21,4% — by emergency caesarean cessation. At birth all the infants had no signs of acute hypoxia. Pathological changes of the placenta were not detected during the morphological examination.

The following methods were used:

1. The study of RBCs of the newborns in the field of the atomic force microscope (AFM).

The objects to examine were: RUCB of premature neonates, central venous blood after 7 hours of birth and venous blood of premature infants taken on day 7 of life. The samples of RUCB were taken immediately after crossing the umbilical cord. They were taken in special 0,25 ml tubes containing EDTA as preservative, the blood was mixed with the preservative, settled within 30 minutes, then a monolayer of erythrocytes for further processing in the field of the atomic force microscope was prepared: 20 μl of blood were put on a slide glass and a monolayer of erythrocytes with the aid of centrifuges Diff Spin 2 (USA) was prepared. The picture of erythrocytes was received using the atomic force microscope «NTEGRA prima» (Russia) in semi-contact mode. Cantilevers NSG01-A were used as probes. The Number of scan dots was 512 and 1024, the area of scan was 100×100 microns, 10×10 microns. The analysis of RBC membrane nanostructures was performed using a spatial transformation with the use of the Fourier decomposition of RBC membrane surfaces into three orders according to spectral windows of 1000—600 nm., 80—300 nm., 20—60 nm. for the first, second and third orders, correspondingly.

The first order correlates with the phenomenon of flickering surface «waves» on the erythrocyte membrane and reflecting macro-structural properties of the membrane; the second and the third orders correlate with the configuration of spectrin matrix and reflect the condition of protein connection nodes. The method of analysis of nanostructure has been described in details in previously published papers [18, 19].

2. Hystological examination of the placenta. The morphological data was compared with gestational age, the condition of the children at birth, clinical symptoms and the morphology of RBCs.

3. The statistical processing of data was carried out using the standard program of Origin 6.1.,which is included in Microsoft Office (USA) set. The significance of the differences was estimated by the factor analysis of variance (One-way ANOVA). The difference was considered to be significant at (p<0.05).

Table 2

The obstetrical anamnesis of the mothers

Parameter	Values ($M\pm\sigma$), % n=6
Women's age	31,2±5,4
Primary and secondary infertility	2 (33,3)
The first pregmancy	2 (33,3)
Normal course of pregnancy	1 (16,7)
Medical abortions, spontaneous abortions, previous pre-term deliveries	4 (66,7)
IVF pregnancy	2 (33,3)
The threat of termination	5 (83,3)
Severe hystosis of the second half of pregnancy	1 (16,7)
Istvico-cervical insufficiency	1 (16,7)
Vaginal delivery	1 (16,7)
Delivery via surgery	5 (83,3)

Results and discussion

RUCB is the most important object to be examined in newborns. The results indicate the antenatal and intranatal condition of the fetus. The study showed that in case of normal pregnancy and urgent delivery in RUCB of newborns there were 85% of flat shape RBCs — planocytes and 15% of transformed RBCs, 3% of which were presented by ekchinocytes and stomatocytes, 9% — by other abnormal cells representing intermediate forms of erythrocytes associated with the severity of intrauterine poykilocytosis (Fig. 1).

Different forms of RBCs were detected studying the morphology of RBCs of premature infants born from multiple pregnancies. Diskocytes as the basic physiological form of RBCs were not typical for the infants of this group. They were present in the blood 3 newborns, only in one of which (the second child from bichorionic biamniotic twins) the content of diskocytes reached 43% of the total cells number; in other infants only isolated cells were visualized. Planocytes were visualized in 12 (85,7%) infants, but the frequency of their occurrence was different: in 9 children the content of planocytes was from 41 to 89.4%, so they were the prevailing form of RBCs; in 3 infants the frequency of their occurrence was 5—29% and in 2 infants — planocytes were not detected.

Stomatocytes were found in 12 (85,7%) infants, but their number varied from 3 to 95%. Significant stomacytosis was diagnosed in the first child of bichorionic bianmiotic twins with the collision of twins in childbirth.

Echinocytes were almost never detected in the cord blood. 35% of echynicytes were found in one child from bichorionic biamniotic twins; in four infants isolated echynocytes were visualized. They were not found in newborns of monochorionic biamniotic twins and triplets.

In twins the morphological features of RBCs didn't not depend on the placenta chorionality. There were three types of cells in the RUCD: planocytes, stomatocytes and other abnormal cells associated with the severity of fetal poykilocytosis. This phenomenon was typical for all the

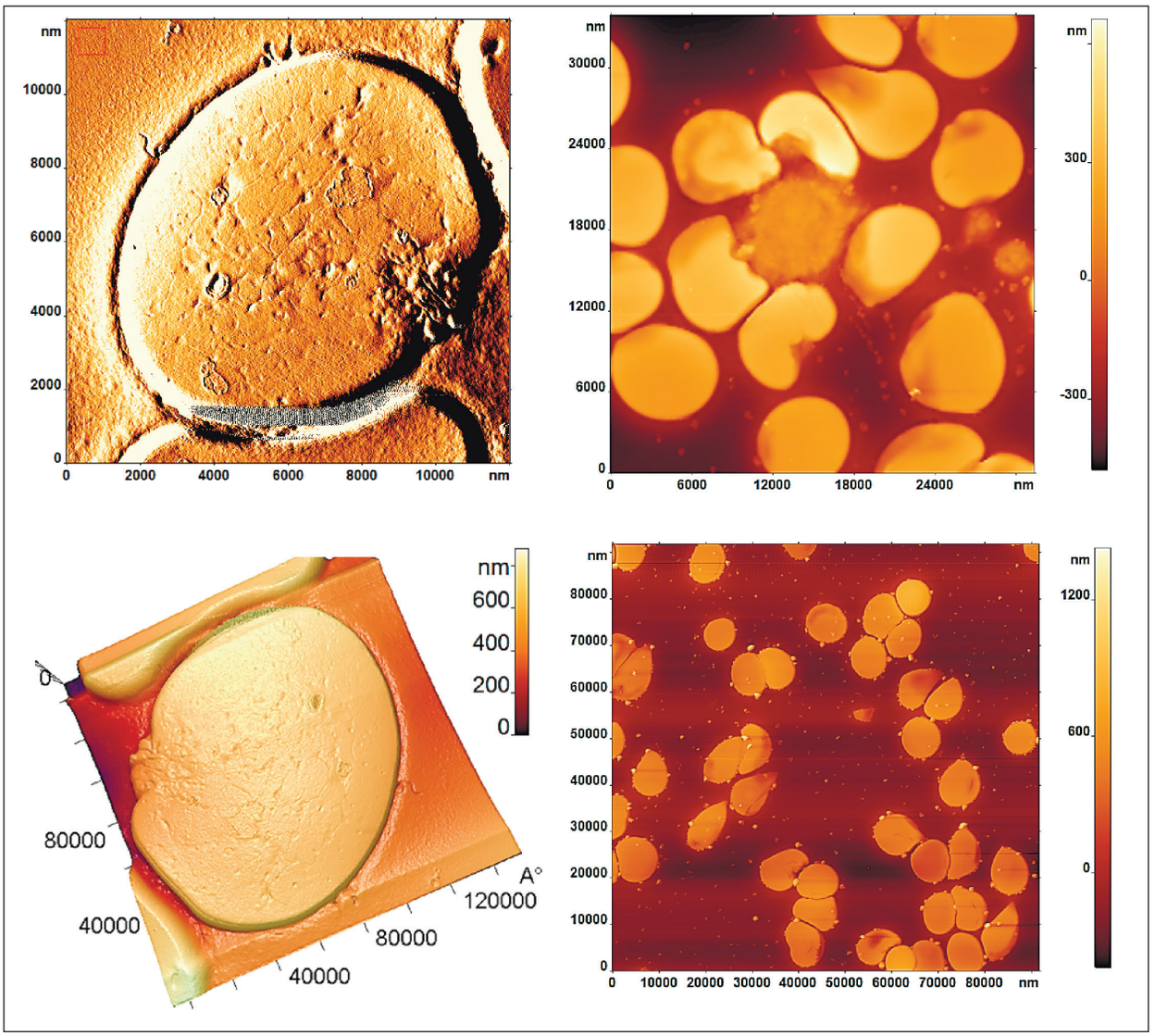

Fig. 1. Abnormal forms of red blood cells due to intrauterine poykilocytosis (the image was received by the method of AFM)

infants included in the study. The frequency of their occurrence was 30,4±22,3% of the total number of RBCs.

In triplets one half of RBCs was presented by planocytes, the second half — by stomatocytes and other abnormal cells. When intrauterine death of the third fetus of the triplet occurred, 55% planocytes and 45% of other abnormal cells were detected in his RUCB, which indicated his recent death.

4 newborns needed mechanical lung ventilation. 2 of them were newborns from bichorionic biamniotic twins and the third and the fourth ones were the second and the third infants of trichorionic triamniotic triplet, respectively. The twins were born with rapid delivery at 32 weeks of gestation, which resulted in acute hypoxia and intrauterine respiratory distress syndrome. The morphological structure of RBCs in RUCB of the first infant was as follows: 89,4% of planocytes and 10,6% of stomatocytes; of the second newborn — 41,3% of planocytes, 22,6% of stomatocytes and 35,5% other abnormal cells. The same morphological structure of RBCs was observed in two newborns of a triplet, who needed artificial lung ventilation.

Thus, planocytes were found to be the main morphological form of RBCs, typical for preterm newborns.

The presence of stomatocytes and other abnormal cells indicated prenatal poykilocytosis. Planocytes are transient physiological form of erythrocytes of newborn regardless of gestational age and the number of fetuses.

Analyzing the nanostructure of RBCs membranes, the height of the first order (h_1) was found to exceed the indicator of the infants in the group of comparison ($p<0.05$) in 8 (57.1%) newborns, that indicated in utero violation if RBCs membrane (membrane «flickering»).

It was tyipcal for the second child of the twins and for the second and third child of a triplet regardless the placenta chorionality; the value of this indicator significantly ($p<0.05$) increased from the first newborn to the third one. For example, h1 for the first child was 3 nm,for the second one — 5 nm, for the third one — 7.5 nm; that might be due to hypoxia, which is often experienced by infants in multiple births. The collision of twins was associated with membrane flickering in both infants, but the height of nanostructure h_1 was 12 times greater in the first newborn and it was associated with almost absolute stomacytosis (Fig. 2).

The heights h_2 and h_3 did not differ significantly from the corresponding indicators of children in the group of comparison, i. e. the spectrin matrix and the structural state of RBCs proteins were stable.

The equation of regression was received the following correlation links were detected by statistical correlation analysis in RUCB:

- a feedback link of medium strength between hemoglobin saturation with oxygen ($\%SO_2c$) and the height h_1 (r=a-0.7; p=0.03); y=7,18181672 — 0,0527131533*x;
- a feedback link of medium strength between the partial pressure of carbon dioxide (pCO_2) and the height h_2 (r=-0,6; p=0.04); y=3,31985946 — 0,0370085327*x;
- a direct link of medium strength between the fifth minute Apgar score and the height h_1 7 hours after the birth (r=0,6, p=0.02); y = -15,0731707 + 2,97926829*x.

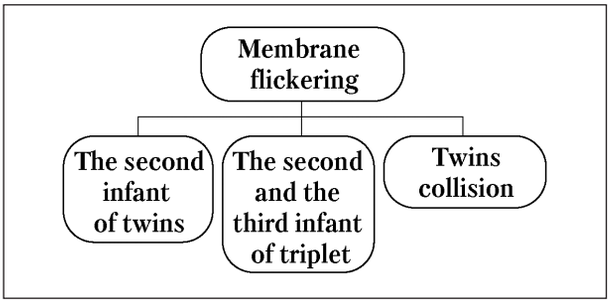

Fig. 2. Groups of newborns for membrane flickering studies.

Other indicators of RUCB affected RBCs membranes, for example pCO_2, the content of which was physiological and reached 47,5±10,4 mm hg., that favorably affected the spectrin matrix of RBCs.

Low intranatal hemoglobin saturation with oxygen affected the value of membrane flickering. Low Apgar score indicated acute intranatal hypoxia, which was not observed in the majority of the newborns in our study.

The placenta it was found to match the term of gestation; the combination of chronic subcompensated and acute placental insufficiency, moderate disturbances of water-salt metabolism (swelling of the decidual tissue, fiber's stroma Vartanov's jelly) were registered. Other dystrophic changes were also registered: fibrin, fibrinoid, calcifications; some fibers were immured in fibrinoid. The vascularization was poor.

Insufficient vascularization of the fibers and the obliteration of vessels, moderate dystrophic changes of the vessels walls were also were observed. The following signs of acute placental insufficiency were seen: vascular paresis, hemorrhages. In case of low placentation and premature detachment of the low lying placenta the vascularization of fibers was absent.

Individual morphological changes of placenta and abnormality of umbilical cord was typical for triplets. In one case the placenta corresponded to the age of gestation of the first and the second fetuses, there were not violations of the fiber maturation. Moderate dystrophic changes were identified: fibrin, calcifications, pseudoinfarctions, cell dystrophy. There were small hemorrhages in intrafiber space and blood circulation disorders. Diffusive leukocyte infiltration was registered in decidual layer. Paracentral attaching of the umbilical cord of first fetus and regional attaching of umbilical cord of the second fetus were also found. The umbilical cord of both fetuses was short.

The third fetus (intranatal death) revealed decompensated placental insufficiency: significant dystrophic changes, calcified sinticial nodes, violation of maturation and branching of fibers, sclerosis of fiber stroma, violation of vascularisation, acute placental insufficiency (vascular paresis, focal hemorrhage, regional attaching and short umbilical cord) were registered.

In other case there was violation of maturation and branching of fibers and their chaotic arrangement.

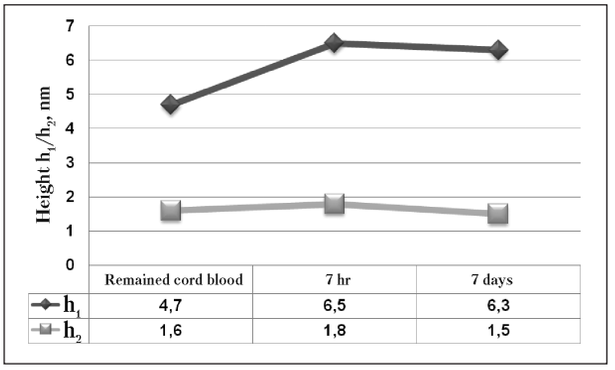

Fig. 3. The dynamics of the heights h_1 and h_2 of RBCs membranes in different periods of the study.

In other case there was prevalence of small fibers and dystrophic changes (fibrin, calcifications, pseudoinfarctions; cell degeneration), focal hemorrhage in intrafiber space, blood circulation disorders, partial obliteration of fiber supporting vessels.

The changes in placenta were accompanied by disturbances of blood circulation, and promote the development of hypoxia, adversely affected the prenatal state of RBCs, causing the damage of membrane nanostructure.

At birth the cord blood parameters of pulmonary gas exchange reflected the intranatal state of the neonates: the partial pressure of oxygen (pO_2) — 39,3±16 mm Hg, the saturation of hemoglobin by oxygen (SO_2c) — 58,6±23.7%. The clinical condition of 10 (71,4%) neonates was stable, there was not respiratory failure, oxygen dependence was moderate, infusion therapy, oxygen therapy through the front mask with oxygen fraction = 30—40% were carried out. The following early period of adaptation was uneventful.

7 hours after the birth morphological structure of RBCs was almost unchanged. Three types of RBCs were determined in newborns: stomatocytes, planocytes and other abnormal cells. A significant change in the number of these cells, in comparison to RUBC, was not revealed (p>0.05).

Single diskocytes were visualized in three children regardless the way of delivery, placenta chorionality and other factors. The same was for ekchynocytes. Thus, in the early hours of postnatal life the morphological composition of erythrocytes was stable.

The dynamics of h_1 и h_2 of RBCs membranes in the following stages of the study is presented on the graph (figure 3). 7 hours after the birth there was an increase in h_1, but the changes were not statistically significant (p> 0,01).

The following changes of the research indicators were observed: in 8 (57.1%) infants with low h_1 in RUBC this indicator increased up to 4,5—12 7 days after the birth, that meant that the violations or RBCs membrane nanostructure remained.. In other cases h1 decreased to 1.5—3 nm.

The state of spectrin matrix and protein clusters was stable. Significant changes were not observed (p<0,01).

The early neonatal period was characterized by an increase of the stomatocyte number and the reduction of planocyte number (p<0.05), the lack of discocytes and ekhinocytes.

Stomatocytes were found in 13 (92,9%) newborns, their quantity varied from 20 to 100%, a high number of stomatocytes was identified in 8 (57.1%) infants.

By the 7th of life the indicators h_1 and h_2 of RBCs membranes didn't change (p>0.01).

The changes of the membrane flickering were different: in 5 newborns (35,7%) the value of h_1 decreased. In 2 newborns with high h1 there was a wave-like change of h_1 in RUBC: it was decreasing by the 7th hour after the birth and then the indicator increased dramatically; in other cases, the height of h1 was practically the same.

By the 7th day of life the condition of the newborns was stable, there were not signs of respiratory failure and oxygen dependence in the infants, the infusion therapy in the majority of the infants were cancelled on the 4—5 days after the birth.

In all the 4 newborns the course of the RDS was favorable, by the 5th day of postnatal life the respiratory insufficiency was resolved, however all the RBCs of the second newborn of the bichorionic biamniotic twins were transformed into stomatocytes by the 7th day of life; the stomacytosis was 74,6% in the 1st newborn; in the 3rd newborn of triplet the stomacytosis was 51%.

It was not possible to identify the relationship between stomacytosis and the duration of mechanical ventilation due to small size of sample.

In case of multiple pregnancy the onset of preterm birth was the result of chronic and acute placental insufficiency, the abnormality of the umbilical cord, which was confirmed by morphological study and indicators of gas exchange at birth. That resulted in perinatal change of RDCs memranes of different severity. The main RBCs form at birth were planocytes, stomaticytes and other abnormal cells, which represented intermediary, revercible forms were also identified. Changing of RBCs shape may be due to changing the ratio of external and internal area of monolayers. It is a reversible phenomenon.

Prenatal injuries of RBCs outer membrane — flickering- remained, spectrinal matrix and protein clusters were intact, the surface of the first order, reflecting the damage to the membrane at this level was the least stable indicator.

Other indicators (pCO_2, % SO_2c) of RUCB affected the height h_1 and h_2 in RBCs membrane.

A correlation between the indicators and the research heights of RBCs membranes was also found.

The inner cytoplasmic surface was resistant to prolonged hypoxia, because the height h_2 and h_3 of RBCs membranes were similar to the corresponding indicators of healthy newborns at birth and were not changed later.

The first hours of postnatal life were characterized by active transformation of RBCs from one form to another. The lack of diskocytes in premature infants in the early neonatal period indicateed a reduced sustainable stability of RBCs membranes to the influence of unfavorable intrauterine factors.

The effects of prenatal exposure to the RBCs membrane remained for a certain period of time, and lasted longer than early neonatal period.

Baby's birth of interrupted the cascade of pathological reactions, but the normalizing of research indicators didn't occur by the seventh day of life. High membrane flickering remained, no stabilization of RBCs morphological composition occured.

Conclusion

1. Using atomic force microscopy we confirmed that the damage of RBCs nanostructure occurs in pre-term newborns during complicated multiple pregnancy.

2. The early period of adaptation was found to be characterized by the change of morphological forms erythrocytes: planocytosis were replaced by stomacytosis, other abnormal cells were also detected. They appeared due to the active process on the RBCs membranes.

3. Membrane flickering was mostly diagnosed in the second newborn of twins, the second and the third child of triplets; it did not depend on the chorionality of placenta, its value increased from the first infant to third one.

4. The collision between the twins was associated with membrane flickering and significant stomacytosis in both children.

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Submitted 24.08.2013