Nanostructure of Red Blood Cell Membranes in Premature Neonates with Respiratory Distress Syndrome

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Objective: **to study the nanostructure of red blood cell membranes in premature babies with neonatal respiratory distress syndrome (NRDS), by applying atomic force microscopy. *Subjects and methods*. The investigation included 27 newborn infants, of them 13 premature babies with NRDS formed a study group. The mean gestational age was 33.1±2.3 weeks; their birth weight was 1800±299.3 g. A comparison group consisted of 14 full-term babies with 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 examined using an atomic force microscope. The objects to be examined were residual umbilical cord blood (RUCB) from the premature infants; central venous blood after 7 hours of birth and neonatal venous blood taken on day 7 of life. *Results*. RUCB from full-term babies contained planocytes that were a major morphological type of red blood cells. In physiological pregnancy and acute fetal hypoxia, the morphological composition of red blood cells in premature neonates with NRDS was close to that in full-term babies. The planocytes are also a major morphological type of red blood cells in the premature infants; the frequency of their occurrence varies. Stomatocytes are typical of all the neonates in the NRDS group; their frequency levels vary greatly: from 8 to 65% of the total number of erythrocytes. The examination revealed that the premature infants of 31—36 weeks gestation were characterized by abnormal erythrocyte shapes that showed a high variability. At birth, the premature babies were found to have changes in the nanostructure of red blood cell membranes, which were influenced by intrauterine hypoxia. The first-order value reflecting flickering in the red blood cell membrane varies to the most extent. *Conclusion*. Atomic force microscopy showed that the greatest changes in the structure of red blood cell membranes were found in RUCB. The premature babies with NRDS had intrauterine poikilocytosis caused by unfavorable factors, as confirmed by the presence of multiple correlations. Analysis of the nanostructure of red blood cell membranes revealed that the first-order value reflecting flickering in the red blood cell membrane was most sensitive; this indicator showed a slow normalization.**

Key words: **red blood cell membrane, nanostructure, planocytes, stomatocytes, premature newborn infants, respiratory distress syndrome.**

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The aim of our study was to characterize morphofunctional patterns of erythrocytes performing oxygen — transport function. A variety of actual methods used with the same purpose included light and transmission electron microscopy. However, only atomic force microscopy (AFM) enabled to explore the structure of their membranes in different critical illness [1, 2].

It is known that erythrocytes are disc-shaped, but this shape is common for rheticulocytes [3, 4]. External cell membranes of these cells seem equal, but the protoplasm content of erythrocyte is characterized by higher optical density on electronograms than that of rethiculocytes. Intracellular organelles are often detected in rethiculocytes. In newborn rat erythrocytes because of osmotic loads the prevalence of non-disk erythrocyte are considered as patterns of necrobiosis [5].

Different forms of erythrocytes possess a number of morphological features. In discocytes a thick zone of outer lipid bilayer of cell membrane was observed. In planocytes ring-shaped redistribution of hemoglobin granules associated with the change of cytoskeletal proteins was detected. Spectrophotometric study have demonstrated that autofluorescence of erythrocytes occurs at the wavelength of 480—490 nm; the intensity of the radiation of the toroidal field in the ultraviolet rays can differ by 1,5—3 times. The ultrastructure of discocytes is characterized by regular cell membrane, regular intracellular distribution of hemoglobin and lack of folds.

The ultrastructure of stomatocytes is heterogeneous forming double cell membrane. Chain-shaped cytosceletal proteins with irregular concentration of hemoglobin granules around are detected inside the cells. A large number of local damages of cell membranes is common in stomatocytes [6].

Special attention has been given to studying pre-term neonates. The neonate immaturity is considered to affect blood cells. However, in general the blood system of a preterm infant with an extremely low weight is ready for an outside life. Individual qualitative and quantitative changes of erythrocytes mostly depend on the cause of a preterm delivery [7—9].

Cord blood is a suitable source of information about the processes that occur in a fetus body during the birth [10—13].

The aim of the study: to study the morphological structure of erythrocytes in pre-term infants with respiratory distress syndrome (RDS) by AFM.

Materials and methods

The study included 27 newborns of which 13 pre-term infants were diagnosed with RDS. Anthropometric characteristics of neonates, features of amniotic fluid and duration of mechanical ventilation (MV) are presented in Table 1. The average gestation age was 34,1±1,7 weeks, birth weight 2065,4±304,8 g. At birth the condition of the neonates were severe. All pre-term infants were under the MV because of the severe respiratory insufficiency caused by RDS. Surfactant Curosurf® was applied to all newborns 3—5 minutes after the birth via the endotracheal route. The average dosage of the preparation was 153±24 mg/kg. The average duration of MV was 68±15,6 hours.

In all the cases the course of pregnancy was unfavorable with the threat of interruption. The second half of the pregnancy was complicated by severe preeclampsia resistant to pharmacotherapy. The latter was the reason of a preterm delivery. The majority of the pregnant women were carried on an emergency caesarean cessation by the Gusakov's method.

Erythrocytes of 14 full-term newborns with normal course of pregnancy and urgent delivery were separated from the cord blood. The average gestational age of the newborns was 39,4±0,4, the score of the first minute of life was 8±0,4 points (Apgar), the birth weight was 3131,7±588,8 g. 78.5% of the newborns were delivered by vaginal route, and in 21,4% of cases the emergency caesarean cessation was used. At birth all the infants had no signs of acute hypoxia, the early period of adaptation was normal, they were not carried out MV.

Methods included the following:

(1) Atomic force microscopy (AFM). Cell preparations included erythrocytes of cord blood of pre-term neonates, central venous blood of the newborns 7 hours and 7 days after birth. The blood samples were taken immediately after crossing the umbilical cord. They were taken in special 0,25 ml tubes containing EDTA as a preservative. The blood was mixed with the preservative, then settled for 30 minutes, diluted to 20 ml and a monolayer of erythrocytes on a glass slice was prepared with the aid of a centrifuge StatSpinTM DiffSpinTM 2 Slide Spinner (Fisher Scientific, USA). The picture of erythrocytes was received using the NTEGRA prima Atomic Force Microscope (NT-MDT Development Co., Zelenograd, Russia) in the semi-contact mode. Cantilevers NSG01-A were used as probes. The number of scan dots was 512 and 1024, the area of the scan was 100×100 microns, 10×10 microns. The proportions of various erythrocyte forms were determined with the aid of AFM, and the calculations were performed with 300 cells. The analyses of nanostructures of erythrocyte membranes were performed by using the spatial Fourier transform decomposition of erythrocyte membrane surfaces into three orders according to spectral windows of 1000—600 nm, 80—300 nm 20—60 nm for the first, sec-

Table 1

General characteristics of neonates

Parameters	Values
Neonates with RDS	13
Gestational age, weeks	33,1±2,3
Birth weight, g	1800±299,3
Scores (Apgar scale, first minute of life)	4,9±0,8
Males	7(53,9%)
Females	6 (46,1%)
Clear amniotic fluid	61,5%
Amniotic fluid with blood	30,8%
Amniotic fluid with meconium	7,6%
Duration of MV, hours	68±15,6

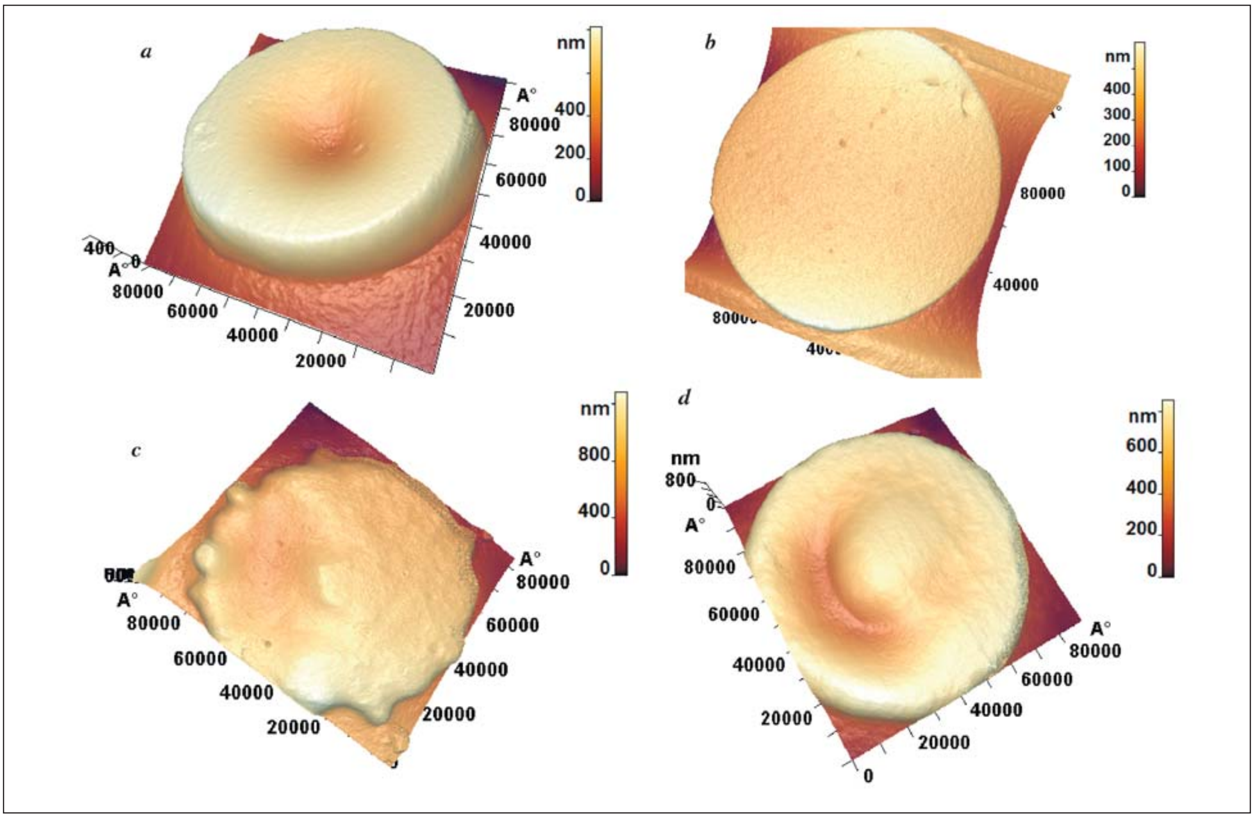

Fig. 1. Shapes of red blood cells.
a — discocyte; *b* — planocyte; *c* — echinocyte; *d* — stomatocyte.

Table 2

Morphological shapes of erythrocytes of pre-term neonates in peripheral blood

Forms of erythrocytes	Values, %
Discocytes	28±16,5
Planocytes	44±16,5
Stomatocytes	26±12,8
Echinocytes	20±8,3
Others	19±8,3

ond and third orders, correspondingly. The first order correlates with the phenomenon of membrane flickering reflecting the macrostructure properties of the membrane. The second and the third orders correlate with the configuration of spectrinal matrix and reflect the conditions of protein connection nodes. The parameters were measured on the plane part of cells of different forms. The method of analysis of nanostructures has been described in details in previously published papers [14,15].

(2) During MV gas composition in capillar blood was routinely performed by Gem Premier 3000 (USA).

(3) Statistical processing of the data was carried out using the standard program Origin 6.1 (Microsoft, USA). Pearson and Spearman correlation coefficients were determined by Statistica 6. The significance of differences were estimated by factor analysis of variance (one-way ANOVA). The differences were considered significant at $p<0.05$.

Results and discussion

The morphology of erythrocytes and clinical conditions of newborns. The shape of erythrocytes is an important informative indicator reflecting the metabolic processes occurring in the body and affecting the conditions of erythrocyte membranes. It is known that mainly discocytes — the double-concave cells with a flat surface circulate in the blood of a healthy person (Fig. 1, *a*).

The study showed that 85% of RB erythrocytes in neonates were presented by flat shaped planocytes (Fig. 1, *b*) and 15% were presented by transformed erythrocytes that include 3% ehinocytres (Fig. 1, *c*), 3% stomatocytes (Fig. 1, *d*) and 9% other abnormal cells.

Erythrocytes whose characteristics are not included in the standard classification were detected. Newborns are characterized by a high number of erythrocytes (intrauterine physiologic erythrocytosis) and a high level of hemoglobin including fetal hemoglobin. Plantocytes might be a transient form in full-term neonates. In this group the correlation between the clinical condition of the neonates and the shape of erythrocytes was not detected.

The morphological structure of erythrocytes of preterm newborns is presented in Table 2. Discocytes and planocytes reached 72% of the total number of erythrocytes in the cord blood of RDS neonates. Discocytes were found in five infants (38,5%), four pregnancies were complicated by acute disturbances of utero-placental blood

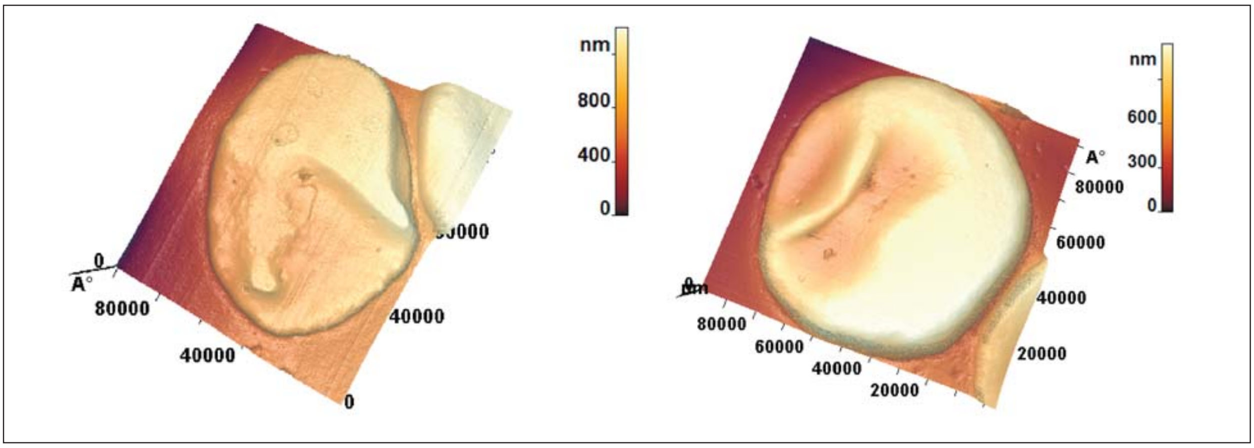

Fig. 2. Abnormal forms of red blood cells

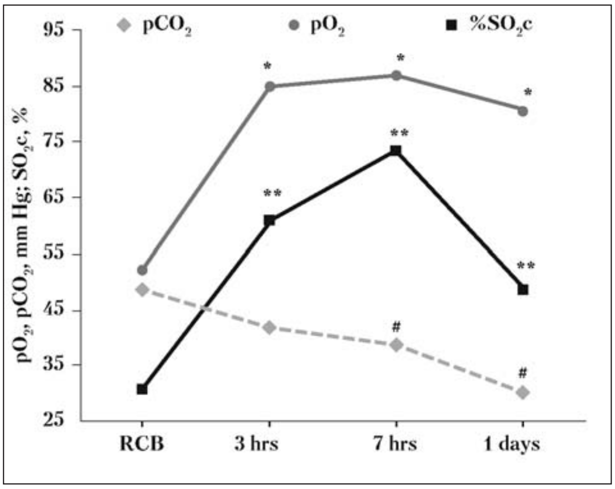

Fig. 3. Dynamics of blood gases in premature infants of RDS group.

*, **, # — reliability of differency in comparison with the residual cord blood.

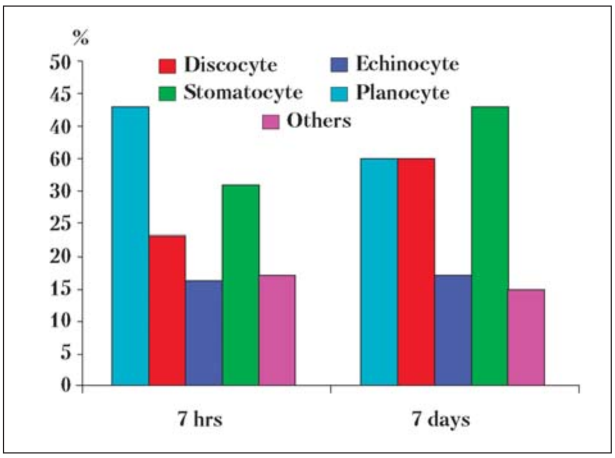

Fig. 4. Morphological shapes of RBC at different study points.

flow (placental abruption), severe preeclampsia, threat of antenatal fetal death in the second half of pregnancy. Discocytes were not found in blood of newborns from multiple pregnancy.

The main morphological shape of erythrocytes in pre-term neonates was presented by planocytes, frequencies of which varied from one per field of view to 90% of the total number of erythrocytes. In normal pregnancies with following acute fetal hypoxia the morphological structures of erythrocytes were similar to ones from full-term infants.

Stomatocytes were common for all RDS newborns, their number varied from 8% to 65% of the total number of erythrocytes. All the pre-term infants with gestational age of 31—36 weeks were characterized by abnormal and variable shapes of erythrocytes. Two of the shapes are presented in figure 2.

The estimation of oxygen status and status of the metabolism of a newborn is important criteria. The dynamics of these indicators is presented in Fig. 3. At birth the median of pH was 7,27 (7,14—7,39) which corresponded to the following parameters of pulmonary gas exchange: the partial pressure of oxygen (PO_2) was 31,3±7.3 mm Hg, the saturation of the hemoglobin by oxygen (SO_2c) was 52,8±22.1%. The data obtained confirms antenatal fetal hypoxia. Three hours after the birth normalization of the oxygen status occurred as a result of MV: PO_2 and SO_2c were increased. Blood pH increased significantly (p<0.05) by the third hour of life and was kept within the physiological norms during the whole study. During the first hours of MV the oxygenation of the blood became normalized.

The following correlation links between morphological forms of CR erythrocytes, indices of metabolism, the nature of amniotic fluid and the scores on Apgar scale in RDS newborns were detected by correlation analysis:

(1) between the number of planocytes and (a) blood pH (r=0,8; p=0,02); (b) nature of amniotic fluid (r=–0,6; p=0,04);(c) the Apgar scale score at the first minute of life (r=–0,6; p=0,02);

(2) between the numbers of stomatocytes and the scores on Apgar scale within the first minute of life (r=0,5; p=0.05);

(3) between the numbers of other abnormal cells and the way of delivery (r=0,7; p=0,03).

Moreover, strong correlations were detected between the duration of delivery, the anhydrous period of delivery and morphological composition of erythrocytes in cord blood.

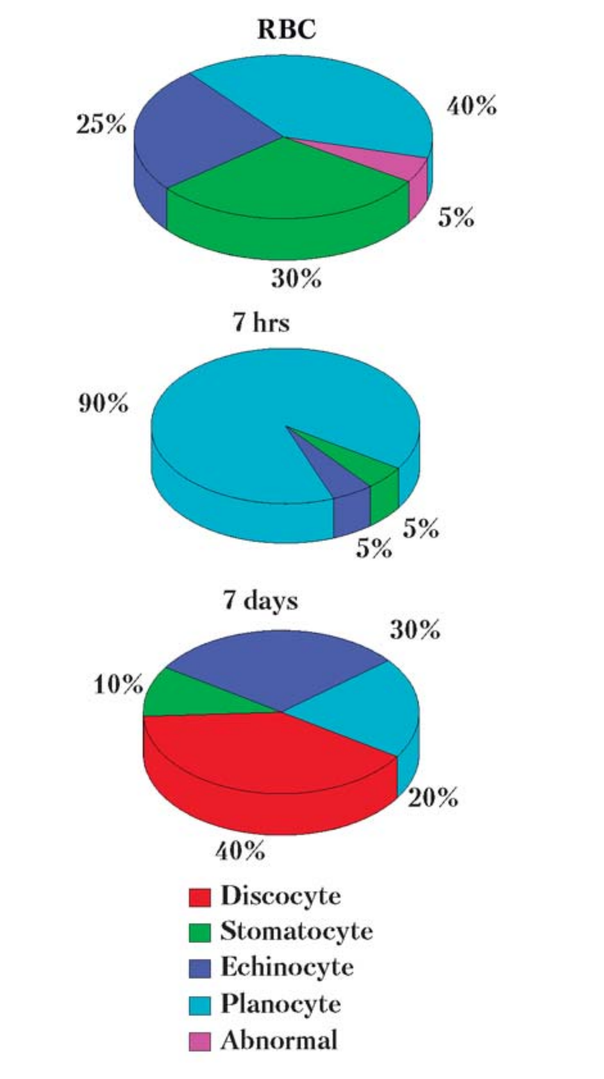

Fig. 5. Morphology of RBC of newborn M. at different study periods.

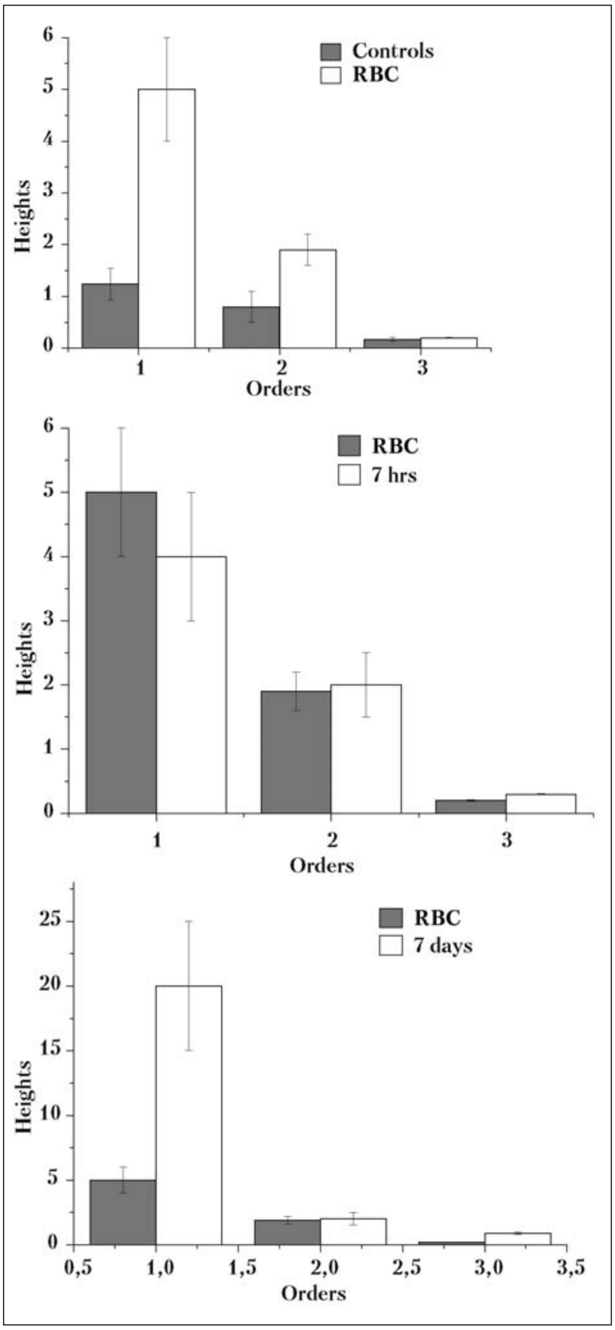

Fig. 6. Histograms of h_1, h_2, h_3 parameters of erythrocyte membranes newborn M. at different study periods.

Seven hours after the birth almost half of erythrocytes of RDS neonates was presented by planocytes, and another half contained stomatocytes (30%) and discocytes (20%). By the seventh day of life the major portion of erythrocytes was presented by planocytes and discocytes while the number of stomatocytes were increased (Fig. 4). The gas exchange was normalized, independent breath recovered and the neonates were extoubated.

Analysis of nanostructures in erythrocyte membrane. The value and period of the order allowed the performing an individual quantitative estimation of nanostructural parameters of erythrocyte membrane in each neonate. Numerous changes in nanostructure of erythrocyte membranes were detected at birth in RDS pre-term infants. The value of the first order (h_1) (reflecting the membrane flickering) was mostly changed and significantly exceeded the corresponding parameter of full-term infants (p<0.05). In six pre-term newborns the value of this order was 3.8—10 times more than in full-term infants. In four cases the value of the first order increased 1.5—3.2-fold. In three infants this indicator was the same as for full-term newborns.

In half of pre-term infants the value of the second order (h_2) exceeded the one in control group by 1.5—2.5 times, spectrin matrix changed in twins born from mothers with severe preeclampsia and placental previa. In five children changes in spectrin matrix were combined with the phenomenon of flickering of erythrocyte membranes. The values of the third order did not differ significantly between the groups (p>0.05).

Seven hours after the birth in three RDS newborns the value of the first order were increased demonstrating

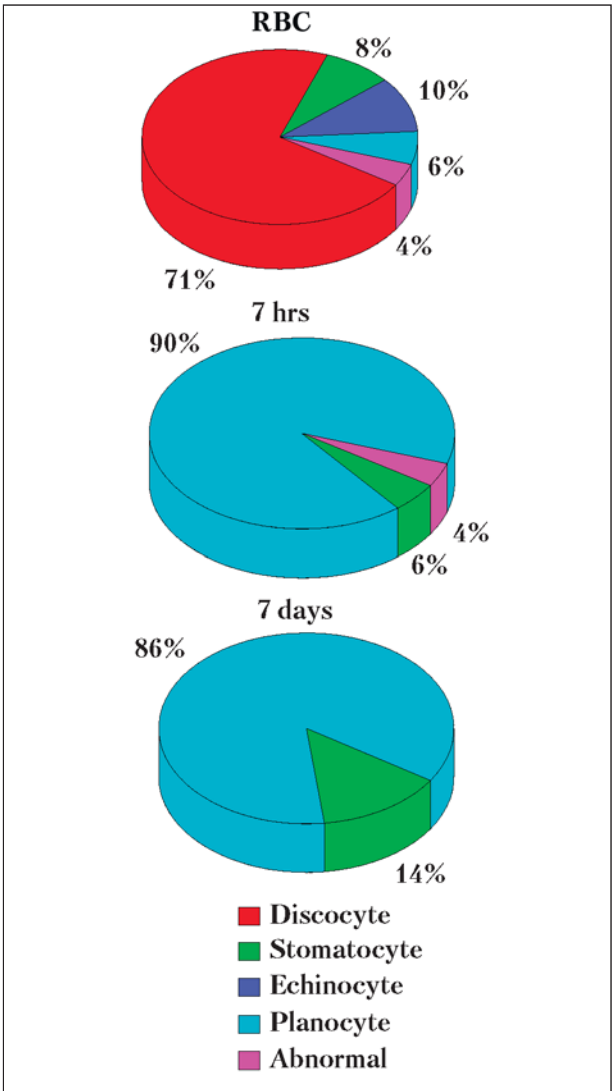

Fig. 7. Morphology of RBC of newborn C. at different study periods.

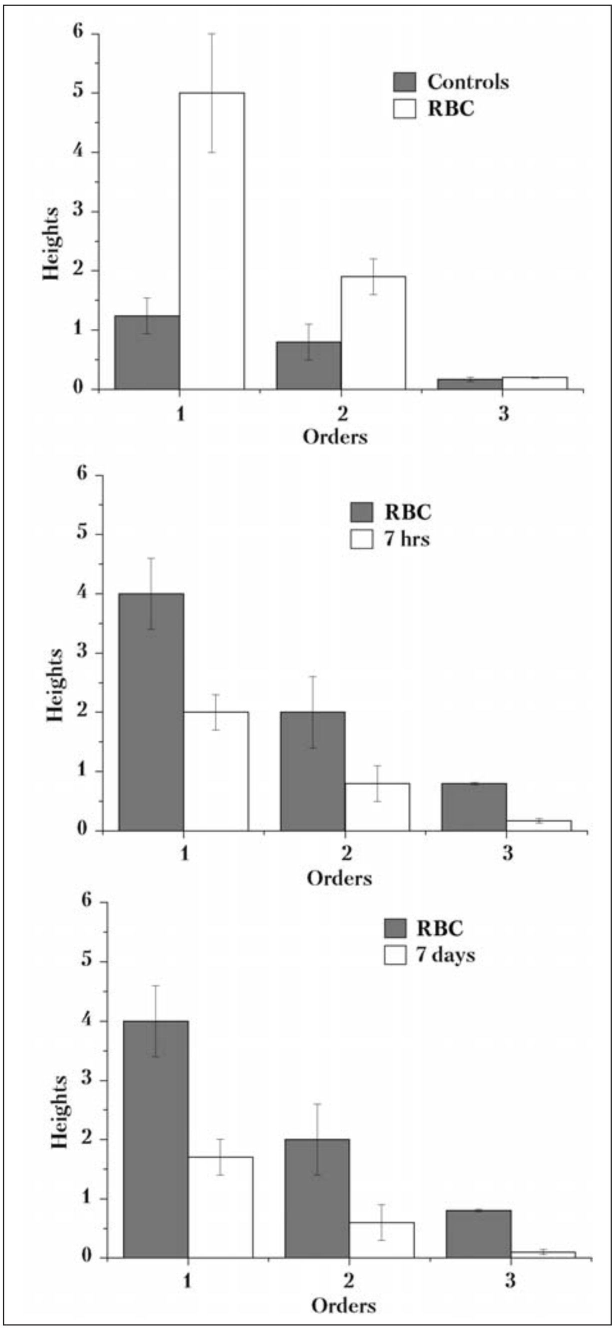

Fig. 8. Histograms of h_1, h_2, h_3 parameters of erythrocyte membranes newborn C. at different study periods.

that the erythrocyte membrane flickering remained whereas the values of the second order changed in one newborn. In other infants the values of the latter parameter were decreased. By the seventh day of life the value of the first order increased in four pre-term neonates.

Seven hours after birth the parameters correlated as follows:

(1) between the number of stomatocytes and (a) pCO_2 of blood (r=0,6; p=0,049);(b) concentration of bicarbonates in blood: (r=0,6; p=0,03); (c) total concentration of carbon dioxide in blood (TCO_2) (r=0,6; p=0,03); (d) saturation of hemoglobin with oxygen (r=–0,6; p=0.02); (e) total concentration of bases in blood (r=0,5; p=0,048);

(2) between blood pH and the number of other abnormal cells (r=0,8; p=0,002); (3) between pO_2 and the height of the second range(r=0,6; p=0,04).

Therefore, correlation links between indicators of metabolism, oxygen condition of blood and the configuration of spectrin matrix were detected.

Conclusions

Using the atomic force microscopy technology the patterns of nanostructural changes in cord blood erythrocyte membranes were detected. Prenatal poikilocytosis due to the influence of multiple adverse factors was common for RDS pre-term infants.

This study established that the nanostructural patterns of the erythrocyte membranes characterized by the values of the first order (reflecting the phenomenon of erythrocyte membrane flickering) were the most sensitive to confirm the prenatal irregularity of membrane nanostructure. Changes in spectrin matrix of erythrocyte membranes were observed at birth, however, stabilization of the matrix was relatively fast. Nanostructural analysis of erythrocyte membranes with the aid of AFM revealed the stability of nanostructural parameters of exposed proteins.

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