ORIGINAL ARTICLE

ERYTHROCYTE REOLOGICAL PROPERTIES IN CHIDREN WITH PERTHES` DISEASE AND TRANSIENT SYNOVITIS OF THE HIP JOINT

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ABSTRACT

Objective: To study parameters of aggregation and cytoarchitecture of erythrocytes in children with Perthes disease (PD) and Transient synovitis of the hip joint(TS).

Material and Methods: This study was conducted at Children Clinical Hospital No 1, Ivanovo, Russia from 01.11.2001 to 10.02.2006. A total of one hundred and thirty six children (sixteen children from control group), and one hundred and twenty one children diagnosed as PD and TS were examined. Determination of aggregation parameters of erythrocytes was carried out by optical method (Int. committee for standartization in haematology, 1988). The following parameters were estimated: average size of aggregate (ASA), parameter of aggregation (PA) and percent of non aggregated erythrocytes (PNE). For an estimation of structurally functional properties of membrane of erythrocytes were investigated their cytoarchitecture. Patients data was processed statistically using SPSS version 14.

Results: Increase in ASA and PA ($p > 0.05$) is revealed in TS in comparison with the CG. In PD there was reliable increase in ($p < 0.05$) contents of ASA and PA in I and II stages (6.2 ± 0.17; 6.1 ± 0.32), (1.7 ± 0.11; 1.6 ± 0.13) accordingly and decrease in percent of PNE (58.6 ± 0.11) ($p < 0.05$) in stage I. Analysis of cytoarchitecture of erythrocytes revealed that the main population of erythrocytes were cells of biconcave form (discocytes) and constituted 71.8 ± 2.56 % in CG. Reversible forms of erythrocytes (RE) composed 18.2 ± 2.07 %, while IE: 9.69 ± 1.76 %. In children with TS discocytes were 72.58 ± 1.66 %, RE – 13.84 ± 1.2 %, IE – 12.81 ± 1.28 % ($p > 0.05$). In patients with of PD, contents of discocytes constituted 73.25 ± 3.01 % and 68.68 ± 4.14 % in stages I and II respectively, while IE were 15.5 ± 3.57 % in comparison with the CG ($p > 0.05$). Contents of RE constituted – 11.25 ± 1.6 %; and 11.37 ± 0.9 % in I and II stages, $p < 0.05$ in comparison with the CG.

Conclusion: Rheological characteristics of erythrocytes in I - II stages of PD are indicated by authentic escalation in aggregation of erythrocytes, reduction in reversible forms and increase in irreversible forms of erythrocytes at II stage of disease. Changes in aggregation characteristics of erythrocytes in children with PD are reliably more expressive, than in patients with TS. .

Key Words: Perthe's` disease, rheological characteristics of erythrocytes, cytiarchitecture of erythrocytes, transient synovitis of the hip joint.

This article may be cited as: Tauseef Raza, Nazarov SB, Pahrova OA, Philosophov AV. Erythrocyte reological properties in chidren with perthes` disease and transient synovitis of the hip joint. J Med Sci 2017; 25: (3) 302-307.

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Date Received: August 11, 2016
Date Revised: September 25, 2017
Date Accepted: October 20, 2017

INTRODUCTION

The diagnosis of PD is carried out basically in late stages of disease when irreversible changes in the head of femur have arised and then the most careful and thorough treatment does not give full recovery. As a result the amount of failures reaches 40 - 80 %, early development of coxarthritis and patients become disabled at early age[1-4].

Though a great number of works are published on the diagnosis of disease, the diagnosis of PD is still established on the basis of clinical and radiological examination of the patient. New opportunities for early diagnostics of PD become accessible with the use of biochemical and rheological methods of investigations[5,6].

Microvasculature depends not only upon the vascular status but also upon the rheological characteristics of blood[7,8]. The shape and elasticity of erythrocytes have a significant value, as the majority of blood forming cells. Deformability of erythrocytes is the key factor maintaining adequate microcirculation, between erythrocytes and capillaries[9-11].

The blood flow in microvasculature significantly depends upon the rheological characteristics of blood[8]. A significant role in the disturbance of microcirculation is mainly due to aggregation of erythrocytes. Acute inflammatory process is accompanied by fibrinogenemia due to release of cytokines from erythrocytes. Fibrinogen and its derivatives have a significant aggregative effect upon erythrocytes, causing increase in blood viscosity, leading to difficulty in perfusion through capillaries. Progression of inflammation is directly related to disturbance in microcirculation and capillary blood flow[12,13].

Rheological characteristics and cytoskeleton of erythrocytes have been studied in different disease processes[14-19]. Contradictions concerning the treatment of PD dictate necessity of studying rheological characteristics and cytoskeleton of erythrocytes during the course of the disease[20].

MATERIAL AND METHODS

This study was conducted in the Department of Traumatology and Orthopedics, Children Clinical Hospital No 1, Ivanovo, Russia from November 2005 to February 2006. Total of 136 children of both genders including 71 children with Perthes disease, 49 children with Transient Synovitis, and 16 healthy children of the control group were included in the study. Age of patients with PD ranged from 5 to 14 years (mean age: 6.5 years), 3 to 10 years in TS (mean age 5.8 years), and 5 to 12 years in the control group (mean age: 6.2 years). Children suffering from any other acute or chronic diseases were excluded from the study. Blood sampling was done from the ulnar vein in strict aseptic conditions. Blood was taken early in the morning before breakfast. Convenient sampling methods were used to collect the data. Laboratory tests were done in the scientific research center of Ivanovo State Medical Academy, Russia. Informed consent was taken and the study was duly approved by the ethical committee of hospital. All the findings were documented on proforma and were subjected to statistical analysis by using software SPSS version 14 and p value of < 0.05 was considered significant. Aggregation parameters of erythrocytes was determined by optical method (Int. committee for standartization in haematology[21]).

Assessment of erythrocyte aggregation: Venous blood is to be taken in the morning after a 14-hour starvation through a thick needle by gravity into a test tube with 3.8% of sodium citrate in 9:1 ratio and be centrifuged for 10 min at 3 000 rpm. Two wells 96-well reaction plate are to be filled with 0,2ml of the patient's plasma in a 96 lunula reaction plate. All plasma and a leucocyte layer are to be removed from the tube. Erythrocytes are resuspended in standard phosphate buffer in 1:4 ratio, followed by 10-min centrifugation at 3000 rpm, allowing to wash them from plasma residues at the supernatant removal. Then 0,02ml of erythrocytes are taken and resuspended in the first well filled with autologous plasma in the 96-well reaction plate to provide 10% hematocrit. Then 0,02ml content is to be taken out of this well with a clean dry pipette and placed into the second filled well to provide 1% hematocrit. Thereafter, one grid in a hemocytometer is to be filled with obtained erythrocyte suspension, kept for 3 minutes till the spontaneous aggregation, and then free erythrocytes (including two erythrocytes together) and aggregates are counted, starting from 3 erythrocytes, combined as "rouleaux") in 2 large squares cameras (lens x 40, ocular x 10). Aggregation activity of erythrocytes can be recorded with a light microscope by counting in a hemocytometer of

Table 1: Parameters of aggregation of erythrocytes in children with TS, PD and CG

Parameter	ASA	PA	PNE
CG n = 16	5,30 ± 0,17	1,2 ± 0,03	77,0 ± 3,12
TS n = 49	5,83 ± 0,24	1,4 ± 0,02	77,2 ± 2,29
PD I n = 8	6,2 ± 0,17*	1,7 ± 0,11*(**)	58,6 ± 5,27* (**)
PD II n = 22	6,1 ± 0,32*	1,6 ± 0,13	72,0 ± 5,32
PD III n = 21	5,6 ± 0,27	1,3 ± 0,06	76,6 ± 3,82
PD IV n = 8	4,6 ± 0,5	1,4 ± 0,17	74,3 ± 8,12

Note: * – reliable difference in comparison with CG, ** - in comparison with TS ($p < 0,05$).

Table 2: Parameters of cytoskeleton of erythrocytes in TS, PD and CG

Parameter	Disco cyte	Acantho cyte	Dacro cyte	Echino cyte	Stomato cyte	Sphero cyte	Schisto cyte
CG	71,8 ± 2,56	7,3 ± 0,62	3,4 ± 0,6	8,9 ± 2,22	8,1 ± 1,68	2,0 ± 0,08	2,1 ± 0,26
TS	72,6 ± 1,66	6,0 ± 0,64	3,8 ± 0,44	5,3 ± 0,77	10,7 ± 1,2	1,9 ± 0,24	2,0 ± 0,20
PD I	73,2 ± 3,01	4,6 ± 0,39*	3,2 ± 0,48	4,7 ± 0,39	11,3 ± 0,95	1,2 ± 0,16	1,2 ± 0,15*(**)
PD II	68,7 ± 4,14	4,7 ± 0,43*	3,1 ± 0,61	4,7 ± 0,58	16,9 ± 0,84*	1,3 ± 0,16*	1,6 ± 0,16
PD III	75,4 ± 2,26	4,6 ± 0,32*	3,4 ± 0,59	5,25 ± 0,76	9,6 ± 1,34	1,8 ± 0,75	1,9 ± 0,26
PD IV	71,6 ± 4,38	6,20 ± 0,80	4,3 ± 0,92*	2,20 ± 0,48	17,8 ± 1,34*	1,7 ± 0,67	1,6± 0,30

Note: * – reliable difference in comparison with CG, ** - in comparison with TS ($p < 0,05$).

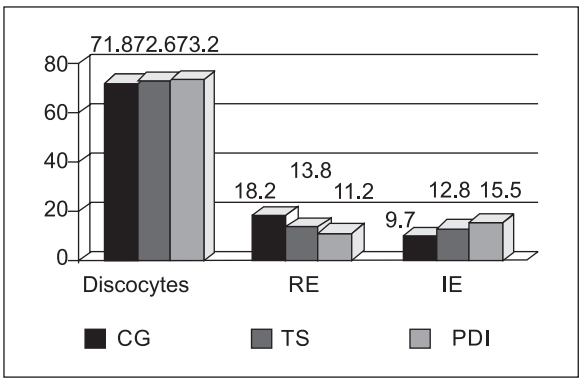

Fig. 1: Parameters of cytoskeleton in children with TS, PD and CG.

erythrocyte aggregates, aggregate and non-aggregate erythrocytes in the washed red blood cells suspension in plasma followed by the calculation of an average aggregate size.

Average size of aggregate (ASA):

ASA=EAA/AQ, where

EAA= amount of all erythrocytes in the aggregate,

AQ= amount of aggregates

Parameter of aggregation (PA): is calculated as follows:

PA= (ASA×AQ+FEA) / (AQ+FEA), where

FEA= amount of free erythrocytes

Percentage of non aggregated erythrocytes (PNE) is determined as follows:

PNE= (FEA × 100) / ASA × AQ + FEA)

Assessment of erythrocyte cytoarchitectonics: To test the surface geometry of erythrocytes, blood is fixed by 1% glutaraldehyde in 199 medium (pH: 7.4) at 4°C for one day, and then a "crushed drop" is prepared and a light phase contrast microscope is applied. Cells are counted by percent per 200 erythrocytes. Recorded erythrocytes are typed by seven groups: discocytes, erythrocytes with reversible changes (ER): acanthocyte, dacrocytes, echinocyte. The first four categories of red blood cells (discocytes, including those with echinocyte transformation signs) are considered to be reversibly deformed due to their ability to spontaneous recovery. The other red blood cells categories (stomatocytes, spherocytes, schistocytes) are related to the group of irreversibly deformed or pre-hemolytic forms.

RESULTS

Data of 136 children (71 patients with PD, 49 with TS, and 16 in the CG) is presented in Table 1. Analysis of parameters of aggregation of erythrocytes in children with TS has not revealed a reliable difference, though increase of the average size of aggregate (ASA) and parameter of aggregation (PA) ($p > 0.05$) is revealed in comparison with the control group.

In children with PD there was reliable ($p < 0.05$) increase in contents of ASA (6.2 ± 0.17; 6.1 ± 0.32), and PA (1.7 ± 0.11; 1.6 ± 0.13) during I and II stages accordingly, and decrease in percent of non aggregated erythrocytes (PNE) (58.6 ± 0.11) ($p < 0.05$) in stage I of PD. Reliable increase in PA and decrease of PNE ($p < 0.05$) is established in comparison with TS.

By determining cytoskeleton of erythrocytes (Table 2) it has been established, that the main form of erythrocyte population in CG were cells of biconcave form (discocytes) and constituted 71.8 ± 2.56 %. Total amount of reversible forms of erythrocytes (RE) in control group constituted 18.2 ± 2.07%, and irreversible forms (IE) – 9.69 ± 1.76%. In TS discocytes were 72.58 ± 1.66 %, RE – 13.84 ± 1.2 %, IE – 12.81 ± 1.28%, ($p > 0.05$) in comparison with the control group.

Parameters of reversible forms of erythrocytes in children with TS do not differ from parameters of children of CG and patients with I stage of PD. Reduction in number of reversible forms of erythrocytes in children with stage I of PD in comparison with control group has been caused by decrease in acanthocytes (4.6 ± 0.39) ($p < 0.05$) and echinocytes (4.7 ± 2.18%). In TS the amount of reversible forms of erythrocytes were

reduced mainly due to echinocytes, while the amount of irreversible forms of erythrocytes were increased due to stomatocytes.

In stage I of PD, contents of discocytes did not differ from parameters of control group 73.25 ± 3.01% ($p > 0.05$). Contents of RE constituted – 11.25 ± 1.6%, that were authentically lower ($p < 0.02$) than similar parameter of the CG (Fig 1). Irreversible forms (IE) of erythrocytes were 15.5 ± 3.57 % ($p < 0.05$) in comparison with CG.

In II stage of PD the amount of discocytes constituted 68.68 ± 4.14%, and did not differ reliably from the parameter of control group ($p > 0.05$). However amount of RE was authentically reduced up to 11.37 ± 0.9% ($p < 0.05$), while the IE contents is increased up to 17.05 ± 1.8% ($p < 0.01$). As in TS, during stage II of PD, the amount of reversible forms of erythrocytes were reduced mainly due to echinocytes, while the amount of irreversible forms of erythrocytes were increased due to stomatocytes.

In stage II of PD was observed authentic decrease in acanthocytes 4.0 ± 0.34% ($p < 0.001$) and increase in stomatocytes – 16.91 ± 0.84 in cosmparison with parameters of CG ($p < 0.05$). During the late stages of PD, no reliable difference in the parameters of cytoskeleton between PD and TS was documented ($p > 0.05$).

DISCUSSION

The analysis of results shows that changes in cytoarchitecture of erythrocytes in patients with I and II stages of Perthes` disease are characterized by authentic decrease in reversible forms and increase in irreversible forms of erythrocytes not only in comparison with the control group but also with TS.

These results confirms the findings of Butnier et al that microcirculation depends not only upon the vascular status but also upon the rheological characteristics of blood, especially erythrocytes[21]. As erythrocytes form majority of blood cells, hence very important will be the shape, elasticity and interaction of these cells. Deformability of erythrocytes is the key factor maintaining adequate microcirculation, between erythrocytes and capillaries[22]. As previous studies revealed, these data are very important in the assessment of the functional state of hip joint in early stages of individual development. It is clear that the state dynamics in human depend on dynamics of microrheological properties of red blood cells, including at the stage of development of a disease process, as well as the duration of recovery[22].

Both in PD and TS, there are different variants of changes in cytoskeleton and rheological characteristics of erythrocytes. Disturbance of rheological charactreristics contributes to slowing down of capillary blood flow, worsens the tissues perfusion – especially of the vessel wall which leads to progressive disturbance of microcirculation[23]. This indicates the great practical importance of the above methods, able to provide accurate information on pathological conditions, when microcirculation can significantly deteriorate because of significant amount of blood of erythrocytes with modified shapes and a large number of their aggregate. Provided that adequate perfusion of the hip joint in many respects defines their overall functional state, rapid assessment of aggregation activity and of the surface geometry of the red blood cells is practically needed to be easy and affordable.

Increase in different types of erythrocytes may play an important role in the pathogenesis of development of ischemic changes in the head of femur and reflect the characteristic disturbances in the organ cells. Increase in number of irreversible deformable erythrocytes leads to difficulty in passage through capillaries, contributes to slowing down of blood flow and cell aggregation. Increased aggregation of erythrocytes is accompanied by increase in average size of aggregate, increase in parameter of aggregation and decrease in percentage of non aggregated erythrocytes. Erthrocytes aggregate are capable of occlusion of microvasculature, formation of cell wall thrombus, leading significant worsening of tissue perfusion and development of perfusion deficiency[24].

Irreversible deformable erythrocytes are pre hemolytic forms, which are prone to preterm hemolysis, not only in the spleen, but also in the circulation, leading to activation of different mediators in the blood flow and contribute to aggregation of formed elements. In comparison with discocytes, the electrostatic force of attraction between the pre hemolytic erythrocytes is greater than the force of repulsion, due to change in their membrane potential[25].

Irreversible deformable erythrocytes don't have the plasticity - enabling them to pass through microvasculature, leading to disturbance of blood flow and transmembrane metabolism. Hence, certain contribution to disturbance in microcirculation can bring changes in rheological charactersistics of erythrocytes and in particular increase in irreversible deformable erythrocytes (pre hemolytic forms)[25].

Thus, the analysis of data confirms presence of close pathogenic connection between increased parameters of aggregation, and changes in cytoskeleton of erythrocytes, as well as clinical-laboratory manifestation of TS and PD, both on systemic and local levels.

Significant role of parameters of aggregation in pathological and physiological processes, in combination with changes in cytoskeleton of erythrocytes provide the unique diagnostic opportunities for timely diagnosis and treatment of Perthes disease.

These methods of assessment of the main microrheological properties of red blood cells do not require expensive equipment and are able to provide sufficient information. Long-term practical application of these methods allows to record changed shapes of insignificant part of red blood cells in healthy people and under pathological conditions.

Thus, the rheological properties of erythrocytes and platelets largely determine the rheological properties of whole blood. In this regard, the role of aggregation and surface geometry is very high; their screening remains very popular in practical biology. Assessment of these microrheological properties of red blood cells can provide accurate information in pathological conditions, when microcirculation can significantly deteriorate. By applying these methods, it is confirmed that only a small portion of red blood cells is characterized by changed shapes in normal people. In pathological conditions, changes in these indicators can be much more pronounced, significantly impairing the microcirculation.

There are few limitations to our study. We are sharing the experience of one centre, so we must have a multicenter data with proper robust study design in order to confirm the findings presented in this study, and to formulate more practical preventive measures in our setup. Well designed, large scale comparative studies are required in our setup to establish these results, and use these markers in the diagnosis and differential diagnosis of other diseases as well.

CONCLUSIONS

1. Rheologic characteristics of erythrocytes in stage and II of Perthes disease are indicated by authentic enhancement in aggregation of erythrocytes.
2. Changes in cytoskeleton of erythrocytes are manifested by reduction in reversible forms and increase in irreversible forms of erythrocytes during II stage of PD.
3. Changes in aggregation characteristics of erythrocytes were reliably more expressive in children with Perthes disease, than in TS.
4. Increase in parameter of aggregation higher than 1,65 international unit and decrease in percent of non aggregated erythrocytes lower than 71 % is necessary to take into account for differential diagnosis between PD and TS.

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CONFLICT OF INTEREST: Authors declare no conflict of interest

GRANT SUPPORT AND FINANCIAL DISCLOSURE NIL

AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

Raza T: Concept and design, acquisition of data.

Nazarov SB: Drafting of manuscript.

Pahrova OA: Data collection and analysis.

Philosophov AV: Overall supervision and bibliography.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.