DOI 10.1007/s10517-021-05241-0
Bulletin of Experimental Biology and Medicine, Vol. 171, No. 4, August, 2021 GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Changes in Hemoglobin Isoforms in the Peripheral Blood of Rats with Experimental Posthemorrhagic Anemia

B. G. Yushkov[1,2,3], S. A. Brilliant[1,3], and A. S. Minin[2]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 171, No. 4, pp. 424-428, April, 2021
Original article submitted February 2, 2021

Six hemoglobin isoforms were identified in the peripheral blood of male Wistar rats by PAAG electrophoresis. Erythrocytes can be separated into 6 fractions by fractional centrifugation; the fractions differ by the content of reticulocytes and cells with fetal hemoglobin. Cells in each fraction contain one light and one heavy hemoglobin isoforms, and their combination is specific to each fraction. Changes in the hemoglobin isoforms in the whole blood in case of blood loss can be associated with changes in physical and chemical properties of circulating erythrocytes, as well as with the formation of various cell populations resulting from activated erythropoiesis.

Key Words: *hemoglobin isoforms; hemoglobin heterogeneity; fetal hemoglobin; hemorrhagic anemia; fractional centrifugation erythrocytes*

Hemoglobin diversity has been actively studied in the last decades. The primary structure has been described for more than 600 hemoglobin variants. Several hemoglobins, with similar or varying structure and functions, can be found in the blood of the same individual [3,6,9,10]. Differences in affinity of different hemoglobin isoforms to O_2 observed in birds and reptiles suggest a potential mechanism of modulation of blood affinity to oxygen via changes in the proportion of hemoglobin isoforms in response to changes in oxygen supply or demand [11,12]. Chronic hypoxia of various origins results in increased production of fetal hemoglobin (HbF) [2,7]. Similar increase in HbF levels in adult body can weaken the pathological effects of sickle cell disease [5,7]. We can conclude that chronic hypoxia can cause shifts in hemoglobin isoforms toward isoforms with higher oxygen affinity [1,5].

However, it is unclear whether similar shifts in the proportion of hemoglobin fractions occurs in response to short-term extreme stimuli (hemorrhage, hypoxia, *etc*.). The profile of hemoglobin fractions in various erythrocyte populations under conditions of activated erythropoiesis remains poorly studied. In this study we chose a rat model, because the highest amount of hemoglobin isoforms (6) has been described in rats.

We studied changes in hemoglobin fractions with various molecular weights and the distribution of hemoglobins and erythrocyte fractions in the whole blood of rats with experimental posthemorrhagic anemia (PHA).

MATERIALS AND METHODS

We used 30 male Wistar rats with a body weight of 250-300 g. The experiments were performed in compliance of Directive 2010/63/EU of the European Parliament and the Council of the European Union (On the Protection of Animals used for Scientific Purposes) and approved by the Ethics Committee of the Institute of Immunology and Physiology (Protocol No. 01/20, April 8, 2020).

Massive blood loss in rats (n=15) was induced by a single phlebotomy of the caudal vein under ether anesthesia (2.0-2.5% body weight, 35-40% of the circulatory volume) [1,4]. Blood samples were collected before and on day 2 after blood loss. Intact rats served

[1]Institute of Immunology and Physiology, Ural Division of the Russian Academy of Sciences, Yekaterinburg, Russia; [2]Insitute of Natural Sciences and Mathematics, B. N. Yeltsin Ural Federal University, Yekaterinburg, Russia; [3]Institute of Cell Technologies in Medicine, Yekaterinburg, Russia. ***Address for correspondence:*** svetlana.brilliant@bk.ru. S. A. Brilliant

0007-4888/21/1714-0421 © 2021 Springer Science+Business Media, LLC

as controls (n=15). Animals of both groups were anesthetized by diethyl ether; blood samples (6 ml) were collected from the caudal vein. Erythrocytes were separated into 6 fractions by fractional centrifugation [8]. In each fraction, qualitatively characteristics of cells were evaluated on a Celly 70 analyzer (Biocode-Hycel). In blood smears, erythrocytes containing fetal hemoglobin (HbF) were detected by the Kleihauer—Betke method [8,13]; reticulocytes were stained with cresyl blue brilliant. Reticulocytes and HbF-containing erythrocytes were counted per 1000 cells. Bone marrow samples were used for counting nuclei-containing cells and myelogram (stained by Romanovsky—Giemsa method). Hemoglobin isoforms were detected in peripheral blood hemolysate prepared by the standard method [8] and separated by electrophoresis in PAAG as described previously [8].

Statistical processing was performed using Statistica 8.0 software (StatSoft, Inc.). The data are presented as $M\pm m$. The groups were compared using nonparametric Mann—Whitney U test; the differences were considered significant at $p<0.05$.

RESULTS

On day 2 after massive blood loss (2% body weight), we observed typical signs of posthemorrhagic anemia (PHA) described in numerous publications: reduced erythrocyte count (6.42±0.36 *vs* 7.51±0.02 g/liter in controls, $p<0.001$) and hemoglobin content (109.5±4.5 *vs* 135.6±1.9 g/liter in controls, $p<0.05$) in the circulating blood and signs of erythropoiesis activation such as increased peripheral reticulocyte count (1.95±0.08% *vs* 1.23±0.06% in controls, $p<0.05$); increased level of HbF-containing erythrocytes (3.55±0.05% *vs* 2.27±0.06% in controls, $p<0.05$); increased content of erythroid cells in the bone marrow (12.15±2.06×10^6/100 g body weight *vs* 7.28±1.03×10^6/100 g of body weight in controls, $p<0.05$) and a shift in the leuko-to-erythroblast ratio toward erythroblasts (1.46:1 *vs* 2.74:1 in controls, $p<0.05$).

The morphological parameters of erythrocytes in the whole blood and fractions were similar in rats with PHA and controls: no significant differences were observed for mean corpuscular volume (MCV; 54.07±0.57 fl *vs* 54.7±0.70 fl in the control) (Fig. 1). Mean corpuscular hemoglobin (MCH) was reduced only in fraction 1 (18.52±0.17 pg *vs* 19.26±0.11 pg in the control, $p<0.05$), which was probably responsible for hypochromia observed in PHA; in the whole blood, this parameter was similar in experimental and control animals (18.39±0.66 pg *vs* 18.57±0.37 pg in the control). At the same time, mean corpuscular hemoglobin concentration (MCHC) was reduced in anemic rats in all fractions except for fraction 3; in the whole blood, this parameter was also similar in both groups (33.1±0.64 g/liter *vs* 35.85±2.13 g/liter in the control) (Fig. 1).

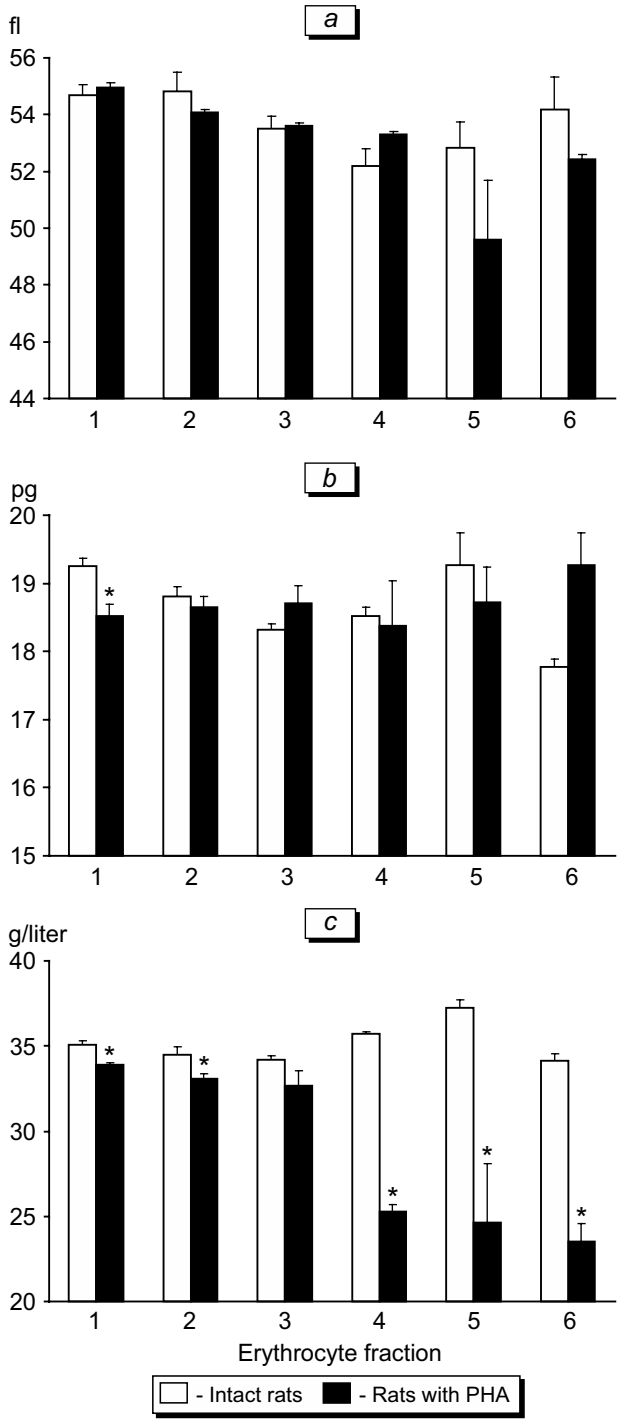

Fig. 1. Morphological parameters of whole blood erythrocyte fractions on day 2 of PHA. *a*) MCV, *b*) MCH, *c*) MCHC. *$p<0.05$ in comparison with the control (intact rats).

Reduced MCHC reflects the impaired functional activity of erythrocytes. This parameter depends on the cell saturation with protein compounds, and its

TABLE 1. Hemoglobin Isoforms in Peripheral Blood Erythrocytes on Day 2 after Blood Loss (n=15; $M\pm m$)

Parameter	F1	F2	F3	F4	F5	F6	Mean for all fractions
Intact rats							
Molecular weight, kDa	52.27±1.33	56.90±1.22	63.00±1.43	70.71±0.32	81.67±0.98	86.86±0.54	68.57±5.60
	(42.1-59.4)	(47.5-63.3)	(52.2-70.6)	(68.6-72.5)	(76.9-89.6)	(83.5-89.8)	(42.1-89.8)
Distribution of isoforms, %	10.93±0.90	13.07±1.10	43.97±0.80	20.52±0.50	8.64±1.30	2.85±0.57	—
Rats with PHA							
Molecular weight, kDa	54.49±0.37	56.53±0.13	69.04±0.14*	71.82±0.14	78.12±0.45	86.04±0.54	69.34±0.30
	(53.0-56.8)	(56.1-57.3)	(68.8-69.8)	(71.2-72.5)	(75.6-79.5)	(82.6-88.5)	(53.0-88.5)
Distribution of isoforms, %	9.11±0.20	10.43±1.00	28.52±1.30*	24.55±0.60*	11.13±0.32*	16.25±1.30*	—

Note. The range of parameter fluctuation is shown in parentheses. *p<0.05 in comparison with intact rats.

TABLE 2. Proportion of Light and Heavy Hemoglobin Isoforms in Erythrocyte Fractions ($M\pm m$)

Erythrocyte fraction		Molecular weight, kDa		Proportion of isoforms in erythrocytes, %	
		heavy isoform	light isoform	heavy isoform	light isoform
F1	intact	74.51±0.84	84.84±1.50	54.00±0.96	46.00±0.96
	PHA	75.80±0.60	88.60±0.26*	43.00±1.08	57.00±1.08
F2	intact	66.68±0.83	83.32±1.05	60.00±0.45	40.00±0.45
	PHA	72.52±0.56*	80.77±0.78	46.25±3.50	53.70±3.51
F3	intact	64.04±1.08	71.89±0.57	33.30±0.76	66.70±0.76
	PHA	67.75±0.88	70.47±0.35	42.00±1.47	58.00±1.47
F4	intact	64.55±0.46	73.46±1.04	43.10±0.88	56.90±0.88
	PHA	56.17±0.71*	67.40±0.52*	36.50±1.50	63.50±1.50
F5	intact	55.30±0.80	61.70±0.52	37.10±1.56	62.90±1.56
	PHA	55.57±0.39	64.20±0.42	26.75±1.70	73.20±1.70
F6	intact	50.76±0.82	59.09±0.75	32.50±1.32	67.10±1.40
	PHA	48.32±2.80	53.80±1.18*	24.25±2.17	75.75±2.10

Note. *p<0.05 in comparison with intact rats.

reduction is associated with decreased hemoglobin synthesis. Therefore, the proportion of hemoglobin isoforms in PHA anemia is of special interest.

Hemoglobin isoforms in the whole blood were separated by PAAG electrophoresis. In the samples of intact controls, 6 isoforms were detected, which agree with published data [5,10]. The most abundant isoform was isoform 3 (F3) with a molecular weight of 63.00±1.43 kDa. In rats with PHA, the molecular weight of F3 was increased, while its percentage was lower; F5 and F6 became more abundant, while the percentage of F1 and F2 remained unchanged (Table 1).

Each fraction of red blood cells contained one light isoform and one heavy isoform. Heavy isoforms were dominant in fractions 1 and 2; light isoforms were dominant in fractions 3-6 (Table 2). In PHA, the proportion of heavy isoforms on day 2 was increased in 5 of 6 fractions, except for fraction 3, where an opposite shift was observed (Table 2).

According to our findings, changes in the proportion of hemoglobin isoforms contribute to the adaptation not only to chronic hypoxia, but also to acute conditions (blood loss). Hemoglobin undergoes polymerization and proteolysis in erythrocytes. Increased weight of hemoglobin isoform 3 and its reduced amount in the blood can be explained by its polymerization. This process occurs in fraction 2 of circulating erythrocytes. At the same time, the level of erythrocyte fraction 5 with high content of this isoform increases in blood. Erythrocyte fractions 4 and 6 have increased amount and reduced weight of heavy hemoglobin isoforms, which can be a result of proteolysis or altered synthesis. Thus, the shifts in the proportion of hemoglobin isoforms in case of blood

loss can be associated with changes in physical and chemical properties of circulating erythrocytes, as well as with the formation of various cell populations of different origins as a result of activated erythropoiesis. However, this area needs further research.

Thus, peripheral blood of rats contains 6 erythrocyte fractions with varying concentrations of hemoglobin isoforms, reticulocyte count, and HbF-containing erythrocyte count. Each fraction contains one light isoform and one heavy isoform of hemoglobin. Activation of erythropoiesis is associated with changes in the proportion of hemoglobin isoforms; the relative content of heavy isoforms increased in all fractions. Changes in the hemoglobin heterogeneity in the whole blood in case of blood loss are associated with changes in physical and chemical properties of circulating erythrocytes, as well as with the formation of various cell populations resulting from activated erythropoiesis.

This study was performed on the equipment of Common Use Centers of the Institute of Immunology and Physiology, Ural Division of the Russian Academy of Sciences, within the framework of the of State Assignment for the Institute of Immunology and Physiology (theme No. AAAA-A18-118020590108-7).

REFERENCES

1. Brilliant SA, Yshkov BG. The study hemoglobine spectrum of rats bone marrow with post-hemorrhagic anemia. Vestn. Ural. Med. Akad. Nauki. 2018;15(4):570-576. doi: 10.22138/2500-0918-2018-15-4-570-576
2. Kriventsev YuA, Kriventseva LA. Hemoglobins of the human as diagnostic markers. Nauch. Obozrenie. Med. Nauki. 2018;(1):16-20. Russian.
3. Manchenko E.A., Kozlova E.K., Sergunova V.A., Chernysh A.M. Homogeneous Deformation of Native Erythrocytes During Long-Term Storage. Obshchyaya Reanimatologiya. 2019;15(5):4-10. doi: 10.15360/1813-9779-2019-5-4-10
4. Ostroushko AA, Danilova IG, Brilliant SA, Gette IF. Application of nanocluster iron–molybdene polyoxometalates for correction of experimental posthemorrhagic anemia. Nanotechnologies in Russia. 2019;14(3-4):159-164.
5. Sumin MN, Rezaikin AV, Yushkov BG. Hemoglobin heterogeneity under conditions of abnormal erythropoiesis. Bull. Exp. Biol. Med. 2003;135(6):563-565. doi: 10.1023/a:1025477202093
6. Topunov AF, Petrova NE. Hemoglobin: evolution, distribution, and heterogeneity. Uspekhi Biol. Khimii. 2001;41:199-228. Russian.
7. Shamratova VG, Usmanova SR, Isaeva EE. Role of fetal hemoglobin in adaptation to hypoxia in case of limited physical activity. Biotechnology-from science to practice. Ibragimov RI, ed. Ufa, 2014. P. 81-85. Russian.
8. Yushkov BG, Brilliant SA. Distribution various isoforms of hemoglobin in rat red blood cells. Ross. Fiziol. Zh. 2020;106(10):1312-1320. doi: 10.31857/S0869813920090095
9. Datta P, Chakrabarty S, Chakrabarty A, Chakrabarti A. Membrane interactions of hemoglobin variants, HbA, HbE, HbF and globin subunits of HbA: Effects of aminophospholipids and cholest erol. Biochim. Biophys. Acta. 2008;1778(1):1-9. doi: 10.1016/j.bbamem.2007.08.019
10. Gell DA. Structure and function of haemoglobins. Blood Cells Mol. Dis. 2018;70:13-42. doi: 10.1016/j.bcmd.2017.10.006
11. Grispo MT, Natarajan C, Projecto-Garcia J, Moriyama H, Weber RE, Storz JF. Gene duplication and the evolution of hemoglobin isoform diff erentiation in birds. J. Biol. Chem. 2012;287(45):37647-37658. doi: 10.1074/jbc.M112.375600
12. Opazo JC, Hoffmann FG, Natarajan C, Witt CC, Berenbrink M, Storz JF. Gene turnover in the avian globin gene families and evolutionary changes in hemoglobin isoform expression. Mol. Biol. Evol. 2015;32(4):871-887. doi: 10.1093/molbev/msu341
13. Smith RD, Li J, Noguchi CT, Schechter AN. Quantitative PCR analysis of HbF inducers in primary human adult erythroid cells. Blood. 2000;95(3):863-869.