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# Early-changes diagnostics of erythrocytes microrheological features in the model of dyslipidemia development in rats at the late stages of ontogenesis


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## ABSTRACT

In our work we traced the dependence of erythrocyte microrheological features dynamics on the age of experimentally developed dislipidemia in rats. The investigation was fulfilled with the help of initially healthy rats of Vistar line. The experimental group was composed of 105 male rats (34 rats, aged 12 months; 32 rats, aged 18 months; 39 rats, aged 24 months) which were exposed to dislipidemia development by alimentary way. The control group was composed of 91 male rats, including 30 rats aged 12 months, 32 rats aged 18 months, and 29 rats aged 24 months. We applied biochemical, hematological, and statistical methods of investigation. Experimental dislipidemia development in rats showed decrease of plasma antioxidant protection and increase of lipids' peroxidation, which deepened with aging of animals taken into the experiment. With dislipidemia development in experimental rats, we noticed that during the aging process there was a rapid rise in the number of erythrocytes that both reversibly and irreversibly lost their biconcave form. In the experimental group of animals, we noticed during the aging process an increase in erythrocytes' aggregation. Development of abnormalities in erythrocytes' microrheological features in experimental rats exceeded the aging-related changes in the control group. Changes thus found contributed significantly to aggravation in morbidity and heightened sensitivity of animals to negative impacts of environmental factors. Therefore, during the aging process, it is necessary to control more and more strictly the level of blood lipids in order to avoid the development of dislipidemia which becomes acutely dangerous especially during aging because of worsening hemocirculation and rise in the risk of thrombophilia development.

**Keywords:** aging, rat, erythrocytes, aggregation, cytoarchitectonics, experimental dislipidemia.
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## INTRODUCTION

Currently, both medicine and biology urgently need to keep pursuing research into the aging aspects of an organism's functional state, both non-human mammals[1,2] and humans.[3,4] It was found out that the basis genetic[5,6] and environmental components allow aging process to adversely affect all the organism's systems progressively, worsening their functioning and increasing the likelihood of death.[8,9] Great attention in this field is devoted to different aspects of blood rheological features and its regular elements, including aging aspect,[10,11] norm and conditions of different pathologies,[12,13] and also on the background of many variants of medicinal impacts on an organism[14,15] taking into consideration the social aspects.[16] Being one of the most important microcirculation elements, erythrocytes, through their cytoarchitectonics and ability to achieve aggregation significantly, define the level of capillary-course hemodynamic and metabolic tissue homeostasis[17] and the level of their influence over organisms' adaptive reactions.[18,19] It was noted that their rheological features can change at physiological, border-line, and pathological states.[20] The second part of ontogenesis is vulnerable enough in this plan, as with pathology it appears that in mature and aging organisms changes that occur in the rheological features of regular blood elements during the developmental process negatively influence microcirculation, on the whole deepening the process of existing pathology.[21,22]

In addition, generating new scientific information about different pathological states of humans[23] would be impossible to do without the application of experimental models that use laboratory animals, more specifically rats.[24] Because of the importance of erythrocytes' microrheological features in the development of abnormalities in an organism,[25] including age-specific thrombophilia, and the necessity of finding approaches to study its suppression there is a great interest in current

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research to investigate the aspects of aggregation and blood platelets cytoarchitectonics in aging rats. The data collected can thus serve as the basis for devising more refined approaches to optimize the development of erythrocytes' microrheological features at later ages, and thus the data transfer will have to be done carefully to extend it to the domain gerontological investigations pertaining to humans.[7]

Modern physiology and medicine pay great attention to the investigations of early stages of different pathology developments and initial mechanisms of its realization.[3,26] Researchers also show interest towards the study of functional and rheological features of different regular blood elements.[27,28] It was established that they play an important role in hemostasis system functioning at different pathologies, including currently the rather widespread cardiovascular abnormalities[29] and other metabolic diseases.[30,31] Atherosclerosis is one of the metabolic diseases that affect a large range of population across the globe, rendering the affected population invalid and contributing significantly to mortality figures among able-bodied population.[32,33,34] A detailed clinical picture of metabolic abnormalities leading to atherosclerosis shows that complications that typically co-occur in this process include the worsening of erythrocytes' microrheological features[35,36] and a weakening of vascular control over them.[37] This situation significantly activates hemostasis, reduces microcirculation efficiency, and metabolic intention in all the tissues.[38,39] In addition, the process of erythrocytes' spontaneous aggregation and cytoarchitectonics at the earliest stages of dislipidemia development that eventually leads to atherosclerosis has not been studied in depth so far.

Taking into consideration that erythrocytes' microrheological features can be easily disturbed and in conditions of weakening with age in many adaptive mechanisms,[38] it can be concluded that such changes lead to the formation of conditions that lead to occlusion of different vessels,[39,40] in which case the fulfillment of experimental work devoted to detailed investigation of dislipidemia's impact on erythrocytes is of great practical interest. The difficulties that clinicians and pathophysiologists encounter are connected with the way of tracing the earliest stages of the appearance of erythrocytes' microrheological abnormalities on a man because people with early stages of dislipidemia seldom come to physicians for examination. It dictates the need for conducting experimental investigations using models that use laboratory animals specifically for dislipidemia at different stages of the second ontogenesis.

## PURPOSE OF INVESTIGATION

Our study aims to ascertain the dynamics dependence of erythrocytes' microrheological features during the aging process of rat models with clinically developed experimental dislipidemia.

## MATERIALS AND METHODS

Research was conducted in strict accordance with ethical principles established by the European Convent on protection of the vertebrata used for experimental and other scientific purposes (adopted in Strasbourg March 18, 1986, and confirmed in Strasbourg June 15, 2006) and approved by the local ethic committee of Kursk Institute of Social Education, branch of Russian State Social University (Record №12 dated December 3, 2015) and the local ethic committee of All-Russian SII of Physiology, Biochemistry and Animals' Feeding (Record №11, dated December 4, 2015).

The investigation used in the experimental animal models rats of Vistar line; the rats were received at the age of 2 months from the farm of laboratory animals FIBX RAS (Moscow, Puschino). Prior to carrying out our experiments, all the rates were checked and verified to be healthy. It was further confirmed they weren't used in any others experiments earlier and haven't suffered any diseases. Experimental group was composed of 105 healthy male rats (34 rats, aged 12 months, 32 rats, aged 18 months, 39 rats, aged 24 months) in which dislipidemia was formed using the alimentary way. The rats were put into small separate cages for 30 days and received a get high-calorie diet consisting of combined feed (47%), sweet condensed milk (44%), vegetable oil (8%), and vegetable starch (1%); the ration thus provided was of the following composition: lipids 29.6%, proteins 14.8%, and carbohydrates 55.6%.[41] Control group was composed of 91 healthy male rats; this group included 30 rats aged 12 months, 32 rats aged 18 months, and 29 rats aged 24 months. They got combined feed PK-120 produced by "laboratorkorm" (Russia) in full volume and experienced no impacts. All the animals were healthy during the whole period of investigation and haven't used in any experiments previously.

Formation of different age groups for experiment and control purposes was made by taking rats of similar ages out of their respective cages after their darkening for the purpose of removing subjective factors.

Concentrations of common cholesterol (CCS) and triglycerides (TG) in animals' blood were found with the help of enzymatic colorimetric methods

**Table 1** **Biochemical and Hematological Parameters in the Second Year of the Life of Rats in the Background of the Experimental Creation of Their Dyslipidemia**

| Registered Parameters | Experimental group, M ± m, n = 105 | | | Control, M ± m, n = 91 | | |
|---|---|---|---|---|---|---|
| | 12 months, n = 34 | 18 months, n = 32 | 24 months, n = 39 | 12 months, n = 30 | 18 months, n = 32 | 24 months, n = 29 |
| Total cholesterol, mmol/l | 260±0.012 | 2.78 ± 0.015 | 3.12 ± 0.018 | 2.18 ± 0.009* | 2.22 ± 0.012** | 2.25 ± 0.010** |
| HDL cholesterol, mmol/l | 1.00 ± 0.010 | 0.95 ± 0.009 | 0.91 ± 0.008 | 1.12 ± 0.008* | 1.11 ± 0.015** | 1.09 ± 0.008** |
| LDL cholesterol, mmol /l | 0.89 ± 0.015 | 1.07 ± 0.016 | 1.41 ± 0.018 | 0.60 ± 0.008* | 0.64 ± 0.007** | 0.68 ± 0.005** |
| VLDL, mmol /l | 0.71 ± 0.014 | 0.76 ± 0.009 | 0.80 ± 0.012 | 0.46 ± 0.007* | 0.47 ± 0.003** | 0.48 ± 0.006** |
| TG, mmol /l | 1.56 ± 0.010 | 1.68 ± 0.014 | 1.76 ± 0.011 | 1.02 ± 0.004* | 1.03 ± 0.006** | 1.05 ± 0.007** |
| AHP, $D_{233}$/1ml | 1.87 ± 0.022 | 1.99 ± 0.016 | 2.46 ± 0.018 | 1.52 ± 0.018* | 1.60 ± 0.024** | 1.95 ± 0.033** |
| TBA-compounds mcmol/l | 3.91 ± 0.019 | 4.78 ± 0.020 | 5.38 ± 0.026 | 3.61 ± 0.022* | 3.80 ± 0.016** | 4.22 ± 0.042** |
| AOA% | 30.3 ± 0.37 | 27.0 ± 0.28 | 20.1 ± 0.32 | 32.6 ± 0.24* | 30.7 ± 0.32** | 26.2 ± 0.27** |
| Alkyl hydroperoxides of erythrocytes, $D_{233}/10^{12}$ erythrocytes | 2.71 ± 0.022 | 3.22 ± 0.024 | 3.67 ± 0.019 | 2.46 ± 0.017* | 2.75 ± 0.028** | 2.99 ± 0.032** |
| Malonic dialdehyde of erythrocytes, nmol/$10^{12}$ erythrocytes | 1.12 ± 0.011 | 1.32 ± 0.012 | 1.49 ± 0.010 | 0.92 ± 0.014* | 1.09 ± 0.010** | 1.19 ± 0.014** |
| Catalase of erythrocytes, ME/$10^{12}$ erythrocytes | 8760.0 ± 17.9 | 7910.0 ± 22.1 | 7200.0 ± 22.0 | 9100.0 ± 19.20* | 8700.0 ± 26.10** | 8330.0 ± 28.39** |
| Superoxide dismutase of erythrocytes, ME/$10^{12}$ erythrocytes | 1700.0 ± 15.19 | 1580.0 ± 9.83 | 1400.0 ± 9.62 | 1890.0 ± 12.93* | 1750.0 ± 14.75** | 1660.0 ± 10.86** |
| Erythrocytes-discocytes,% | 83.2 ± 0.17 | 76.5 ± 0.14 | 69.3 ± 0.15 | 86.6 ± 0.19* | 82.4 ± 0.25** | 80.2 ± 0.29** |
| Reversibly modified erythrocytes,% | 9,6 ± 0.14 | 12.6 ± 0.11 | 14.9 ± 0.16 | 7.4 ± 0.10* | 9.6 ± 0.17** | 10.7 ± 0.23** |
| Irreversibly modified erythrocytes,% | 7.2 ± 0.10 | 10.9 ± 0.13 | 15.8 ± 0.12 | 6.0 ± 0.13* | 8.0 ± 0.16** | 9.1 ± 0.19** |
| Sum of all the erythrocytes in an aggregate | 32.2 ± 0.16 | 37.9 ± 0.18 | 43.9 ± 0.22 | 29.0 ± 0.19* | 32.0 ± 0.27** | 36.1 ± 0.31** |
| Quantity of aggregates | 6.3 ± 0.09 | 7.3 ± 0.12 | 8.2 ± 0.14 | 5.6 ± 0.08* | 5.9 ± 0.11** | 6.4 ± 0.15** |
| Quantity of free erythrocytes | 268.6 ± 0.26 | 240.1 ± 0.31 | 217.3 ± 0.29 | 294.1 ± 0.24* | 286.3 ± 0.34** | 279.3 ± 0.31** |

Note: Reliable differences existed between the experimental and control rats within their age group:
*$p < .05$; **$p < 0.01$.

that used a set produced by "Vital Diagnostikum." The content in plasma CS of high-density lipoproteins (HDLP) was found with the help of a set produced by "Olveks Diagnostikum," again by enzymatic colorimetric methods. The CS content of low-density lipoproteins (LDLP) was determined using the formula reported by W. Friedwald et al. (1972). Concentration of CS in very low-density lipoproteins (VLDLP) was calculated according to the formula: CS VLDLP = concentration TG/2.2.

In plasma we estimated products' quantity of lipids' peroxidation (POL) according to concentrations of thiobarbituric acid–active products with the help of a set named "Agat-Med" (Russia) and alkyl hydroperoxides taking into consideration the level of plasma antioxidant activity (AOA).[42] After washing and re-suspending erythrocytes, we defined the quantity of products POL—malonic dialdehyde and alkyl hydroperoxides—and also the activity of erythrocyte antioxidant enzymes—catalase and superoxide dismutase.[43]

Aggregation activity state of the washed and re-suspended erythrocytes was found with light microscopy in Gorjaev's box by way of registration of erythrocytes' aggregates quantity, that is, number of aggregated and non-aggregated erythrocytes.[44] Cytoarchitectonics of erythrocytes was estimated with the help of phase-contrast microscopy with light microscope "Lumam-R1" (LOMO, Russia) under immersion with typing of red platelets on discocytes in both reversibly changed and irreversibly changed forms.[44]


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Statistical processing of received data was fulfilled with the help of Student's *t* criterion.

## RESULTS

Examined rats while aging were found to have increased characteristic outer signs of aging—dullness of hair, thinning, decrease of activity and appetite, absence of interest to the environment, and paleness of visible mucous membranes.

As for changes with the aging of control-group rats, we didn't find any reliable changes in lipid composition of blood plasma. In the case of experimental rats, we found dislipidemia increased with age and was most evident in animals at 24 months of age (table 1). So, we concluded that these experimental rats had a 1.4 times higher CS content in blood compared with control rats. Atherogenic fractions of cholesterol LDLP and VLDLP in their blood also turned out to be evidently higher with a 1.7 times increase in TG and a 19.7% decrease in CS HDLP.

With the aging of control animals the following changes were noted: increase of free radical lipid oxidation in liquid part of blood (at 12 months alkyl hydroperoxides: 1.52 ± 0.018 $D_{233}$/1ml, thiobarbituric acid–active products: 3.61 ± 0.022 mkmol/l; at 24 months, 1.95 ± 0.033 $D_{233}$/1ml and 4.22 ± 0.042 mkmol/l; correspondingly, a decrease in the antioxidant activity from 32.6 ± 0.24% at 12 months to 26.2 ± 0.27% at 24 months).

The following changes were observed with the aging process of experimental animals: increase of free radical lipid oxidation in liquid part of blood (at 24 months alkyl hydroperoxides 2.46 ± 0.018 $D_{233}$/1ml, thiobarbituric acid–active products 5.38 ± 0.026 mkmol/l and a decrease in the antioxidant activity of up to 20.1 ± 0.32% at 24 months).

Control animals while aging were noted to have in erythrocytes a little increase in alkyl hydroperoxides and malonic dialdehyde levels which then gradually increased from 12 to 24 months of life at 21.5% and 29.3%, respectively. At the same time, activity of erythrocyte catalase of control rats while aging reached 8330.0 ± 28.39 ME/$10^{12}$ er., erythrocyte superoxide dismutase reached 1660.0 ± 10,86 ME/$10^{12}$ er. (table 1).

Experimental animals were noted to have more evident POL dynamics in erythrocytes: levels of alkyl hydroperoxides and malonic dialdehyde in them increased and prevailed at a higher rate in experimental animals aged 24 months at 37.4% and 25.2%, respectively, compared to the control animals. At the same time, activity of erythrocyte catalase of experimental rats at the age of 24 months reached 7250.0 ± 22.5 ME/$10^{12}$ er. and superoxide dismutase of erythrocytes reached 1400.0 ± 9.62 ME/$10^{12}$ er., having given way to the corresponding values of control group at 15.7% and 18.6%, respectively (table 1).

In the blood of control group animals at 24 months of age, we found a decrease in the quantity of discocytes that went down up to 80.2 ± 0.29%, a change accompanied by gradual increase in quantity in relation to both their reversibly and irreversibly changed forms (table 1).

Experimental rats while aging were noticed to have in blood more evident decrease of erythrocytes-discocytes' quantity to 69.3 ± 0.15% in case of animals of 24 months of age (table 1). At the same time, the quantity of reversibly and irreversibly changed erythrocytes increased more evidently in them (in rats of 24 months of age in comparison with the control group of the same age on 39.2% and 73.6%, correspondingly). Rats in the control group aged between 12 and 24 months of life were found to have little increase of erythrocytes' aggregation activity with the increase of their summary inclusion into aggregates and decrease of free erythrocytes' down to 279.3 ± 0.31 (table 1).

Experimental group animals were found to have more erythrocytes' aggregation activity corresponding to age increase along with an increase in their summary inclusion into aggregates and a decrease in the number of free erythrocytes at 24 months down to 217.3 ± 0.29 (table 1).

## DISCUSSION

The aggregate of an organism's signs providing its vitality depends on its genetic program[45,46] and different external and internal factors[47] among which special significance is given to hemostatic and rheological blood features.[48] They mostly define the volume of nutrients' and oxygen inflow to tissues which inevitably changes in ontogenesis under the influence of many causes.[49] An important role in microcirculation dynamics is played by rheological features of the most numerous population of regular blood elements—erythrocytes. Changing of their state while aging and at development of different pathology is of great interest both for fundamental and applied medicine and biology.

During the experimental development of dislipidemia in rats we noticed development of conditions similar to those that occur in humans:[49] hypercholesterolemia and hypertriglyceridemia, weakening of plasma antioxidant potential accompanied by quick rise in it of AHP and TBA-active compounds quantity, and inevitable worsening of metabolism in tissues. In addition, activation of POL processes in plasma caused the

alteration of superficial structures of regular blood elements,[18] including the most numerous of their population—erythrocytes. All this rather negatively influenced their features in the case of the older individuals.

It appeared that during the experiment changes in plasma lipid composition broke the ratio between lipid fractions of erythrocytes' membranes promoting POL activation in them. It quickly changed the activity of receptor and post-receptor mechanisms of erythrocytes' functioning in model rats. It appeared that lipid imbalance in organisms led also to negative dynamics in regulation of ion and antioxidant status in erythrocytes which, no doubt, mostly provided negative changes in their metabolism and structural-functional features of vessels.[30]

In the context of dislipidemia development there happened a rapid increase in the number of erythrocytes as a result of their having lost their biconcave form, which made the process of their circulation in the vessels difficult, especially those of least caliber35]. Coming changes in erythrocytes led to increase in blood of reversibly and irreversibly changed erythrocytes. So, in the blood of experimental rats of 24 months of age there turned out to be the largest quantity of red corpuscles transformed through echinocytosis into spheres with acanthas of different forms in their membranes and into one-sided arched disks through stomacytosis. These erythrocytes were able to fulfil rapid transformation leading to the formation of spheroechinocyte and then spherostomstocyte and finally to spherocyte which was soon lysed.[36] At the same time, level of increase of reversibly and irreversibly transformed erythrocytes in experimental rats' blood led to aggravation along with age increase especially when there were contacts between the animals, which was indicated in blood by an increase in their aggregating ability.

It was found in experimental rats that while aging the increase of erythrocytes' aggregation was more evident than in control group and changes occurred in their membranes' charge because of more active degradation in some glycoproteins under intensive POL impact. Increase of oxygen in active forms in given conditions provided oxidative alteration of membrane's structures in experimental rats while they aged. It was accompanied by a damage to their globular plasma proteins possessing the ability to act as "bridges" between erythrocytes and realize their aggregation as most evident in the oldest animals. At the same time, POL products increased the threshold of erythrocytes' disaggregation as a result of their adhesion stimulation in aggregates and increased speed of the given process due to oxidative damages to their membranes' proteins and lipids.[44]

It becomes clear that during the ageing process dislipidemia conditions increase erythrocytes' aggregation in aging rats, which was mostly a result of the impact of catecholamines' surplus concentration which at different abnormalities in an organism can significantly increase. With $\alpha_1$-receptors' activation, the system $Ca^{2+}$- calmodulin and cascade of phosphatidyl inositol's intracellular reactions occur. Activation of $\alpha_2$-adrenoreceptors leads to adenylate cyclase suppression during physiological impact from receptors to Gi-proteins. It caused in experimental animals a decrease in cyclic adenosine monophosphate quantity in erythrocytes and stimulated input of $Ca^{2+}$ into them providing for the growth of their aggregation.[50]

Evident quantity increase of freely circulating radicals in the blood of experimental rats, that is, erythrocyte aggregates, led to damage of vessels' endothelial lining promoting the exposition of subendothelial structures which stimulated hemostasis processes.[23] As a result, it worsened blood rheology processes more than aging in the control group. Increasing quantity of freely circulating aggregates is able to block a part of vasa vasorum playing a great role in the weakening of vascular hemostatic control[28] and de-aggregating impacts on erythrocytes as a result of decrease in de-aggregates' output in endothelium.[37]

## CONCLUSION

Experimental dislipidemia development in rats of different ages showed weakening of plasma antioxidant protection and increase of POL, which deepened during the aging of experimental animals. Developing abnormalities in experimental rats exceeded aging-related changes of erythrocytes' microrheological features in the control group. Without any doubt ageing can significantly contribute to developing of morbid aggravation and heightened sensitivity of animals' organisms due to negative impacts of environmental factors. It becomes clear that while aging it is necessary to control more strictly the level of blood lipids and avoid dislipidemic situation development in an organism which becomes more dangerous during the aging process because of the worsening of erythrocytes' aggregation and cytoarchitectonics and a higher risk of thrombophilia development.

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