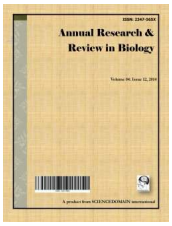

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Physiological Dynamics of Spontaneous Erythrocytes' Aggregation of Rats at Last Ontogenesis

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

In modern biology we still have the actual demand of further investigation of aging aspects of mammals and human beings. Great attention in this investigation is devoted to different aspects of blood rheological peculiarities and its regular elements, i.e. in aging aspect, in normal state and in some pathology conditions and, also, on the background of many variants of correctional impacts on an organism. The purpose is to establish the age dynamics of microrheological particular properties of aging rats' red blood cells. The study involved 95 healthy Wistar male rats that included 32 rats of 18 months of age, 29 rats of 24 months and 34 rats of 30 months of age. We used biochemical, hematological and statistical methods. The control group was represented by 27 healthy Wistar male rats. Examined animals on the background of aging were noted to have activity increase of freely radical lipids' oxidation in the liquid part of blood at the decrease of AOA from 30.70±0.32% at 18 months to 24.40±0.29% at 30 months. Analogical values in the control group were 1.440±0.007 D_{233}/1 ml, 3.460±0.016 umol/l and 34.8±0.010%, correspondingly. Similar picture of POL dynamics

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of examined animals was also found in erythrocytes: Levels of AHP and MDA in them gradually increased – from 18 months of life to 30 months of life on 27.2% and 26.1%, correspondingly. At the same time experimental rats between 18 and 30 months of life were noted to have increase of erythrocytes' aggregation activity with the rise of their summary inclusion into aggregates and quantity of aggregates at the lowering of free erythrocytes' number (228.70±0.31), in comparison with control rats. Found increase of their aggregation is mostly provided by appearing changes in the charge of erythrocyte membranes due to degradation of some negatively charged glycoproteins on them under the influence of increasing with age lipid peroxidation. Aggregation also increases due to inhibition of adenylate cyclase in erythrocytes what leads to the amount decrease of cyclical adenosine phosphate in them and to the stimulation of Ca^{2+} inflow into them. Development of oxidative damage in plasma globular protein "bridges" between erythrocytes, providing their aggregation, increases erythrocytes' disaggregation threshold due to the increase of their connection in aggregates. Age increasing erythrocytes' aggregation in rats negatively influences microcirculation and contributes significantly to marked with aging morbid aggravation due to strengthening a body's sensitivity to negative impacts of environmental factors which promote the realization of hereditary predisposition to various diseases.

Keywords: Aging; rat; erythrocytes; aggregation; microcirculation.

1. INTRODUCTION

In modern biology we still have the actual demand of further investigation of aging aspects of mammals and human beings [1]. The realization of these aging aspects has in its basis genetic [2] and environmental components which allow aging processes touch all the systems of a body [3] progressively worsening their functioning and making its death more and more probable [4]. Great attention in this investigation is devoted to different aspects of blood rheological peculiarities and its regular elements [5,6], i.e. in aging aspect [7], in normal state and in some pathology conditions [8,9] and, also, on the background of many variants of correctional impacts on a body in pathology [10,11].

Being one of the most important microcirculation elements erythrocytes, thanks to their ability to aggregation, mostly define hemodynamic and metabolic tissue homeostasis at the level of capillary channel and influence the realization of many adaptive body's reactions [12]. At the same time their rheological features can change in different physiological, border-line and pathological states [13,14]. Older age is rather vulnerable in this plan as at the appearance of pathology in aging bodies, developing changes of regular blood elements' rheological features negatively influence microcirculation on the whole, deepening the functioning of developing dysfunctions [6,15,16].

At the same time while looking for different therapeutic impacts at many pathological states of a human being [4], it's impossible to do without the application of different experimental models being fulfilled with different kinds of animals including rats [5,7]. Taking into consideration the importance of erythrocytes' aggregation in the development of many disturbances [17] including aging thrombophilia and the necessity of finding approaches to its suppression, its investigation in case of aging rats becomes really urgent. Received data can serve the basis for the further experimental search of approaches for optimization of erythrocytes' microrheological features at the elder age with subsequent cautious shift of received data into gerontological investigations of a human being [3,4]. In this connection the purpose of the present work was the following –to establish the peculiarities of age dynamics of erythrocytes' spontaneous aggregation in case of aging rats.

2. MATERIALS AND METHODS

2.1 Materials

The work was made in strict accordance with ethical principles established by the European Convent on the protection of mammals used for experimental and other scientific purposes (adopted in Strasbourg 18.03.1986 and confirmed in Strasbourg 15.06.2006) and it was also approved by local ethical committee of Kursk Social Education Institute (a branch of Russian State Social University). The experimental group was composed of 95 healthy male-rats of Wistar line that included 32 rats of 18 months of age, 29 animals of 24 months and 34 rats of 30 months of age. The control group was composed of 27 healthy male-rats of Wistar

line at the age of 6 months. The selection of age groups for investigation and control was made with the help of casual getting rats out of cages where animals of the same age were kept. The light was turned off at this moment for the removal of the researcher's subjective factor.

All the animals were healthy before the start of investigation, haven't participated in any experiments before, and haven't suffered any illnesses. All the rats were taken at the age of 2 months from the farm of laboratory animals FIBX RAN (Moscow region, town Puschino). All the animals were kept in vivarium in spacious cages (the area of cage bottom for 1 animal was 200 cm^2). In each cage we kept not more than 8 rats. We changed cages twice a week, so animals were put into clean disinfected cages. On the bottom of cages we put ground litter of 5-10mm width (sawdust, cutting chip or litter turf) which was before application autoclaved in the dry heat unit at the temperature of 150-180℃. The ground litter was changed daily. We used natural light, provided the temperature of 18-22℃ and relative humidity 50-65%. In vivarium the maximum allowed concentration of ammonia in the air was considered to be 0.01 mg/L, carbonic acid in volume - 0.15%, at the rate of air circulation (volumes in an hour) – drawing-out 8, inflow – 10. Rats got combined feeding with full-ration for laboratory animals PK-120 (SLR "Laboratorkorm", Moscow). Water was freely available.

2.2 Methods

We appreciated the common state of animals, weighed them on laboratory scales VM 512 (OKB Vesta, Russia) and carried out common biochemical and hematologic blood analyses. For the fulfillment of biochemical and hematologic investigations animals' blood was taken with the help of a thick needle from caudal vein in the quantity of 1.4 ml in the morning into two 0.7 ml test tubes. In the first test tube as an anticoagulant we used ethylenediaminetetraacetic acid (1 mg/ml) which is also an inhibitor of freely-radical lipids' oxidation. In the second test tube as an anticoagulant we used citrate of natrium in the ratio to blood volume 1:9. Blood in both test tubes was centrifuged for 10 min at 3000 turns/min. Plasma and the layer of leucocytes and thrombocytes were removed separately. Plasma with ethylenediaminetetraacetic acid was used for the investigation of plasma products' POL (peroxidation of lipids) level with the help of thiobarbituric acid concentration (TBA)-active products by the set "Agat-Med" (Russia), acylhydroperoxides (AHP) [18]. For the estimation of plasma AHP level we used as anticoagulant ethylenediaminetetraacetic acid (1 mg/ml). To 0,2 ml of plasma we added 4 ml of heptane-isoproponol mixture (1:1) and shook for 15 minutes with the help of laboratory shaker. Then we added into the tube 1 ml of HCl solution with pH 2.0 and 2 ml of heptane, shook hard and after sedimentation of the mixture for 30 minutes collected heptane level, in which we measured extinction at the wave length 233. The essence of the estimation method of (TBA)-active products level is in the estimation of malonic dialdehyde (MDA) level, which is made by measuring fluorescence on fluorimeter Clinifluor (Hungary). Fluorescence is given by fluorescencing complex formed by MDA with thiobarbituric acid. In erythrocytes received out of it after their washing off and resuspending we defined the quantity of intracellular products POL –MDA and AHP and activity of erythrocyte antioxidant enzymes – catalase and superoxide dismutase (SOD) [19]. The estimation of AHP and MDA levels in erythrocytes was carried out according to the methods mentioned above. Blood plasma taken into citrate was used for the appreciation of its antioxidant activity (AOA) [18], and erythrocytes from this blood after their washing off and resuspending were used for the appreciation of their spontaneous aggregation. For this purpose erythrocytes were resuspended by a standard phosphate buffer in the ratio of 1:4 with the consequent centrifuging for 10 min at 3000 turns/min and removal of supernatant liquid to wash them off all the plasma remnants. After this the existing erythrocytes were resuspended in physiological solution. The principle of estimation method of catalase level is in the fact that it ruins H_2O_2 substrate, and left unbroken part of hydrogen peroxide reacts with natrium molybdat. As the result of this reaction the solution gets yellow color and is analyzed on a spectrophotometer. The principle of estimation method of plasma AOA is based on the ability of analyzed plasma to suppress POL process in homogenate suspension of a rat's brain. Aggregation activity state of washed off and resuspended erythrocytes was found with the help of light microscopy in Goryaev's box by registration of erythrocytes' aggregates' quantity, number of aggregated and disaggregated erythrocytes [20].

For estimation of erythrocytes' aggregation blood from vein was taken into natrium nitrate in ratio 9:1 and centrifuged for 10 minutes at 3,000 turns/min. In the planchette with 96 basins we filled two basins by 0.2 ml of experimental rat's plasma. We removed all the plasma and leucocytes' layer out of the tube. Erythrocytes were re-suspended by standard phosphate buffer in ratio 1:4 with consequent centrifuging for 10 minutes at 3,000 turns/min. It allowed washing them off plasma remains at the removal of supernatant liquid. After that we took 0.02 ml of erythrocytes and re-suspended in the first filled by autologous plasm basin of the planchette with 96 basins. We received 10% hematocrit. Then out of this basin we took with the help of clean dry pipette 0.02 ml of the content and put it into the second filled basin what allowed us to get 1% hematocrit. After that we filled one network in Gorjaev's box by received erythrocytes' suspension, kept for 3 minutes for development of spontaneous aggregation and calculated free erythrocytes (including 2 erythrocytes together) and aggregates (beginning from 3 erythrocytes connected as «coinage columellas») in two large squares of the camera (objective x 40, ocular x 10). We calculated the quantity of «coinage columellas» and quantity of erythrocytes involved into them.

To find out the reliability of differences between experimental groups and control one we used Student's t-test. Statistical significance of differences was proved at $p<0.05$.

3. RESULTS

Experimental rats while aging were found to have the rise of body mass and the increase of characteristic outer aging signs – dimmed and fragile hair, decrease of activity and appetite, absence of interest to the environment, paleness of visible mucous membranes.

Examined animals on the background of aging were noted to have activity increase of freely radical lipids' oxidation in the liquid part of blood (at 18 months AHP 1.600±0.024 D_{233}/1ml, TBA – active products 3.800±0.016 umol/l, at 2.5 years 1.870±0.058 D_{233}/1 ml and 4.280±0.032 umol/l, correspondingly) at the decrease of AOA from 30.70±0.32% at 18 months to 24.40±0.29% at 30 months. Analogical values in the control group were 1.440±0.007 D_{233}/1 ml, 3.460±0.016 umol/l and 34.8±0.010%, correspondingly.

Similar picture of POL dynamics of examined animals was also found in erythrocytes: Levels of AHP and MDA in them gradually increased – from 18 months of life to 30 months of life on 27.2% and 26.1%, correspondingly. At the same time the activity of erythrocyte catalase of experimental rats while aging reached 7450.0±23.4 ME/10^{12}er., SOD of erythrocytes – 1430.0±11.34 ME/10^{12}er., having yielded to the values of the control group on 24.5% and 12.3%, correspondingly (Table 1).

At the same time experimental rats between 18 and 30 months of life were noted to have increase of erythrocytes' aggregation activity with the rise of their summary inclusion into aggregates and quantity of aggregates at the lowering of free erythrocytes' number (228.70±0.31), in comparison with control rats.

4. DISCUSSION

Structure and functions of a body providing its vitality undoubtedly depend on its genetic programme [21] and different external and internal factors [22]. Among them we should especially note hemostatic and rheological blood features [23,24] mostly determining the inflow volume of nutrients and oxygen to tissues. It is inevitably changed at the stage of ontogenesis under the influence of many reasons [3]. An important role in microcirculation dynamics is played by the activity of blood platelets which is under serious impact of a vascular wall and POL processes in their membranes and in blood plasma [25].

It was established that aging rats have got progressively decreasing antioxidant plasma activity causing the rise of AHP and TBA-products concentration in it that negatively influences metabolism in tissues. Active POL in plasma damages vessels' endothelium and receptors on the external membranes of regular blood elements including their most numerous population–erythrocytes, negatively influencing their functions [26]. It causes in erythrocytes of rats between 18 and 30 months of life the decrease of antioxidant protection together with activation of lipids' peroxidation in them.

Found in aging rats increase of erythrocytes' aggregation is mostly provided by developing changes of their membranes' charge. It happens because of degradation of some glycoproteins on them under the influence of active POL. The intensification of active oxygen forms' generation provides aging rats in such conditions with oxidative alteration of membrane's structures at

Table 1. Accountable plasma and erythrocytes indices of aging rats

Registrated parameters	Experimental group, n=95, M±m			Control, 6 months, n=27, M±m
	18 months, n=32	24 months, n=29	30 months, n=34	
weight, g	274.7±0.14*	323.1±0.18**	346.6±0.22**	234.7±0.19
AHP, D_{233}/1 ml	1.600±0.024	1.820±0.033*	1.870±0.058**	1.440±0.007
TBA-compounds, umol/l	3.800±0.016	4.220±0.042*	4.280±0.032**	3.460±0.016
AOA, %	30.70±0.32	26.20±0.27	24.40±0.29*	34.80±0.010
acylhydroperoxides of erythrocytes, $D_{233}/10^{12}$ erythrocytes	2.830±0.019	3.320±0.022*	3.890±0.017**	2.810±0.016
malonic dialdehyde of erythrocytes, nmol/10^{12} erythrocytes	1.160±0.009	1.380±0.010*	1.570±0.008**	1.140±0.005
activity catalase of erythrocytes, ME/10^{12} erythrocytes	8820.0±18.6	8010.0±20.6*	7450.0±23.4**	9870.0±21.0
activity superoxidedismutase of erythrocytes, ME/10^{12} erythrocytes	1600.0±16.28	1530.0±9.25*	1430.0±11.34**	1650.0±12.95
sum of all the erythrocytes in an aggregate	32.90±0.15	37.80±0.13*	43.60±0.18**	30.10±0.09
quantity of aggregates	6.40±0.08	7.10±0.11*	8.00±0.09**	6.10±0.06
quantity of free erythrocytes	285.20±0.28	242.60±0.29*	228.70±0.31**	293.00±0.34

*Notation conventions: reliability of indices' differences between control and aging rats - *<0.05;**- p<0.01*

simultaneous damage of plasma globular proteins which are able to be connected as "bridges" between erythrocytes and fulfil their aggregation [27]. At the same time POL products rise the threshold of erythrocytes' disaggregation because of the stimulation of erythrocytes' adhesion in aggregates, speed rise of the given process because of oxidative damages of their lipids' membranes [6,12].

We should suppose that found in aging rats increase of erythrocytes' aggregation is mostly provided by the impact of catecholamines. If any abnormalities (including aging) happen in a body, their concentration can significantly rise [4]. On the background of α_1-receptors' aggregation the main mediator turns out to be the system of Ca^{2+}-calmodulin and cascade of intracellular phosphatidyl inositolreactions. Activation of α_2-adrenoreceptors leads to adenylatecyclase suppression because of physiological impact from receptors to Gi-proteins causing the decrease of AMP quantity in a cell and stimulating Ca^{2+}inflow into it providing the increase of erythrocytes' aggregation [11].

Quantity increase of aggregates freely circulating in blood of aging rats causes damage of their endothelium lining providing uncovering of subendothelium structures. They stimulate homeostasis processes significantly worsening the processes of blood rheology [9]. Increasing quantity of freely circulating aggregates is able to block vasa vasorum part that plays a great role in vascular haemostatic control decrease [10] and disaggregational impacts on erythrocytes as a result of disaggregants' output decrease in endothelium [22].

5. CONCLUSION

Healthy aging rats are noted to have gradual increase of erythrocytes' aggregation activity that inevitably leads to number growth of their circulating aggregates of different sizes. It makes perfusion of animal's capillaries difficult deepening aging dystrophic tissue changes. The situation contributes to morbid aggravation (marked with aging) rising a body's sensitivity to negative impacts of environmental factors.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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