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Comparative studies of changes in hemorheological parameters caused by physical and metabolic effects

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1. INTRODUCTION

Blood plays a special role in the animal world, especially at the top of the evolutionary scale. It enables the delivery of nutrients, hormones, and signal molecules to the target organs, and plays a significant role in the proper functioning of the immune system and the formation of immune responses. For the above-mentioned reasons, it has special physical and chemical properties (i.e., non-Newtonian fluid, pH buffer). Thus, understandably, since the dawn of science, humanity has been interested in the functioning of this strange and special "tissue" and its role in the body.

Among the numerous blood cells (red blood cells, white blood cells, thrombocytes), we must highlight the importance and special properties of erythrocytes. Their main task is to transport oxygen and carbon dioxide between the tissues and the lungs. To ensure adequate oxygen supply to the smallest tissues (ideal tissue perfusion) and to be able to move properly in the body's capillaries, the erythrocytes must be capable of a large degree of shape change and deformability. They owe this property to their cytoskeleton, the ability to control the intracellular ion and water balance, and the surface-to-volume ratio of the cell membrane. These factors can be negatively affected by inherited hemolytic anemias (sickle cell anemia, thalassemia) as well as physical effects, oxidation, and inflammatory processes.

The blood composition (pH, ion concentration, formed elements) of animals belonging to a given taxonomic unit (e.g., class of mammals) is similar, there are sometimes extremely large differences between animal species at different developmental stages within a given species (fetal hemoglobin, adult hemoglobin) or between genders. Because the blood composition, the ratio and number of cells vary in case of different species used in biomedical research (mouse, rat, rabbit, dog, cat, pig) and humans. They all affect the hemorheological properties of the blood, so it is important to examine them and to compare with each other. In addition to the factors mentioned above, many other physiological and pathophysiological processes can also affect the rheology (flow properties) of the blood. For example, tissue damage and hypoxia, which also affect red blood cells due to changed pH and lactate levels, reducing their deformability and increasing blood viscosity. Or the increase in red blood cell aggregation caused by mechanical trauma and reactions involving the release of reactive oxygen species and the processes mentioned above together can reduce microcirculation and thus tissue perfusion.

As well as in all other sciences, in hemorheology it is essential to evaluate the data obtained, compare them, and standardize them. There have been many studies regarding this subject, especially since the 2000s, when the increasingly modern hematological and hemorheological devices provide researchers with more and more detailed data.

2. AIMES OF THE STUDY

We have conducted different experiments to examine and analyze the data obtained from hemorheological and hematological studies, and to study the methodology and data analysis:

1. In our first study, we examined the red blood cell membrane (mechanical) stability result of native and chemically treated red blood cell, obtained from rats. We compared our data analytical methodology to other methods from the literature.
2. The aim of our second experiment was the comparative analysis of the changes in microrheological parameters in human, rat, dog and pig blood samples as a result of in vitro heat treatment and slow heating, as well as the analysis of the effect of the treatments on the rheological parameters of the blood samples, in order to reveal the differences between species.
3. In our third experiment, we studied the effect of the increased cholesterol level caused by the sixteen-week atherogenic diet on the hematological, macrorheological (whole blood and plasma viscosity) and microrheological (red blood cell aggregation, deformability, and membrane stability) parameters in rabbit model.

3. MATERIALS AND METHODS

3.1. Ethical approvals

The experiments on animals were carried out with the approval of the University of Debrecen Committee of Animal Welfare (UDCAW) and the permission of the Hajdú-Bihar County Animal Health and Food Control Station, according to the Hungarian (Act XXVIII of 1998 on the protection and welfare of animals) and European Union (Directive 2010/63/EU) legislation and ethical principles. In the case of human blood samples, we had permission from the Regional Institutional Research Ethics Committee of the University of Debrecen. Registration numbers of individual experiments:

1. Comparative studies of data interpretation methods of red blood cell membrane stability test: 19/2011/UDCAW

2. The effect of increasing temperature on the stability of red blood cells, a comparative analysis: 25/2016/UDCAW, 7/2014/UDCAW, 13/2014/UDCAW

3. Hemorheological effects of a cholesterol-rich diet: 25/2013/UDCAW

For the comparative analysis of the effect of temperature increases on the stability of red blood cells, the authorization number for human blood samples: DE-RKEB 3189-2010.

3.2. Experimental protocols

3.2.1. Comparative studies of data interpretation methods of red blood cell membrane stability test

Five healthy male CD outbred rats (body weight: 478 ± 12.6g) were included in this research. Blood samples (0.5-0.7 mL) were taken from the lateral tail vein, under general anesthesia (60 mg/kg thiopental i.p.) into BD Vacutainer© tubes, containing 1.8 mg/mL K3-EDTA (Becton, Dickinson and Company, USA) with a 26G needle. The laboratory measurements were performed within 2 hours. The animals were only subjected to blood sampling and anesthetization.

In order to compare the membrane stability measurements, three different test samples were prepared from the blood samples (final sample volume of 0.2 mL): native blood, a sample with a reduced hematocrit value (hemodilution) and a sample with reduced deformability (rigid cells):

1. native blood sample that has not received any chemical or physical treatment
2. mixture diluted with 20% (V/V) phosphate-buffered saline (PBS) (200 µL blood sample + 40 µL PBS)
3. red blood cells made rigid with a mixture of paraformaldehyde (PFA) and glutaraldehyde (GA). In this case, we prepared a 2% PFA-GA stock solution. 1 µL of this solution was added to 2 mL of polyvinylpyrrolidone (PVP) solution to obtain a $1x10^{-5}$%(V/V) solution in which red blood cells were suspended, immediately before ectacytometric measurement.

We measured the hematological parameters (red blood cell count (RBC [T/L]), hematocrit value (Hct [%]), hemoglobin concentration (Hgb [g/dL]), mean red blood cell volume (MCV [fL]), mean red blood cell hemoglobin content (MCH [pg]), average red blood cell

hemoglobin concentration (MCHC [g/dL]) and platelet count (Plt [G/L])) as well as red blood cell deformability and membrane stability values.

3.2.2. The effect of increasing temperature on the stability of red blood cells, a comparative analysis

We included 7 healthy men between the ages of 25 and 35 in the experiment. Samples were also taken from 7 male Sprague-Dawley rats (body weight 312.7 ± 70 g), 6 male beagle dogs (body weight: 18.05 ± 2.05 kg) and 6 female Hungahib-39 pigs (body weight: 15.1 ± 1 .2 kg).

In the case of men, samples were taken from the median cubital vein with a 21 G needle into BD Vacutainer© tubes containing 1.8 mg/mL K3-EDTA. From Sprague-Dawley rats from the caudal vein (anesthesia: 60 mg/kg thiopental i.p.), from six male beagle dogs from the cephalic vein (anesthesia: 10 mg/kg ketamine + 1 mg/kg xylazine + 0.25 mg/kg diazepam i.m.) and 6 female Hungahib-39 pigs from the saphenous vein (anesthesia 15 mg/kg ketamine + 1 mg/kg xylazine, i.m. For comparability, the samples were taken in BD Vacutainer© tubes of the same type containing 1.8 mg/mL K3-EDTA down than human samples.

As the first step, we measured the native hematocrit value of the samples. We then separated the cells from the blood plasma by centrifuging at 1000 g for 10 minutes. In the next step, the fraction containing plasma and white blood cells ("buffy coat") was removed, and then the sample was diluted 1:1 with phosphate-buffered saline (PBS). Then, after another 10-minute centrifugation at 1000g, the liquid fraction was removed, and the hematocrit was measured. The obtained hematocrit values were than taken as 100%, we prepared 10% hematocrit suspensions with PBS, and then performed control measurements twice to make sure that the sample was set to 10% hematocrit.

The "stock solutions" were divided into three thirds and immersed in a warm water bath (GFL Water Bath 1031, Scharlab Hungary Ltd., Hungary) for 10 minutes: at 37°C, 40°C and

43°C. The samples were stored in a dry thermostat set at 37°C until the measurements were performed.

Deformability and membrane stability measurements were performed on the LoRRca Maxsis Osmoscan ektacytometer at 37, 38, 39, 40, 41, 42, 43, 44 and 45°C, thus simulating the effect of incremental temperature rise on the blood samples. For all temperature measurements, a new sample (incubated at 37°C) was used.

3.2.3. Hemorheological effects of a cholesterol-rich diet

In collaboration with the Institute of Pharmacology and Pharmacotherapy of the University of Debrecen, twelve 20-week-old male California-New Zealand white hybrid rabbits (CAL/NZW) were included in the experiment (Jurasko Kft. Debrecen, Hungary). The body weight of the animals at the beginning of the experiment in the Control group was 2898 ± 111g (6 rabbits), while in the hypercholesterolemic (HC) group it was 2923 ± 133g (6 rabbits).
The animals were housed in conventional animal facility, with a 12-12-hour light-dark cycle. During the two-week acclimatization period, the animals received normal rabbit chow and water *ad libitum*. After the acclimatization period, we divided the individuals into two groups of 6 each.

This was followed by a sixteen-week period during which the animals in the Control group received normal rabbit chow, while the animals in the HC group received a special chow supplemented with 1% cholesterol and 1% saturated fatty acids. The special, so-called "atherogenic" food was prepared by the Department of Pharmaceutical Technology of the University of Debrecen, Faculty of Pharmacy.

After the sixteen weeks, we took blood samples from the animals and performed the planned hematological and hemorheological measurements.

The blood samples were taken from the rabbit's marginal ear vein (vena marginalis) in 3 mL BD Vacutainer® tubes containing 1.8 mg/mL K3-EDTA anticoagulant and kept at 20°C

until the measurements were performed. All laboratory measurements were carried out within two hours, thus avoiding damage to the samples.

We measured general hematological parameters, whole blood and plasma viscosity, and red blood cell deformability and membrane stability values.

3.3. Laboratory measurements, sample preparation

3.3.1. Hematological parameters

Qualitative and quantitative hematological parameters were determined using a Sysmex K-4500 automatic machine (TOA Medical Electronics Co. Ltd., Kobe, Japan): the red blood cell count (RBC [T/L]), the hematocrit value (Hct [%]), the hemoglobin concentration (Hgb [g/dL]), mean red blood cell volume (MCV [fL]), mean red blood cell hemoglobin content (MCH [pg]), mean red blood cell hemoglobin concentration (MCHC [g/dL]) and platelet count (Plt [G/L]).

3.3.2. Red blood cell deformability

Red blood cell deformability and mechanical stability measurements were performed on a LoRRca MaxSis Osmoscan ektacytometer (Mechatronics BV, The Netherlands). For this, we use 10 μL of blood, which is suspended in 2 mL of polyvinyl-pyrrolidone (PVP)/phosphate-buffered saline (PBS) solution.

The LoRRca ectacytometer uses the laser diffraction principle to measure the shape change of red blood cells as a result of shear force. The red blood cell-PVP suspension is injected into a continuously rotating glass cylinder called a "cup", in which the "bob" containing the laser diode is located. The uniform rotation of the sample placed in the 3-millimeter gap between the "bob" and "cup" creates the liquid mantle that is illuminated by the laser diode (630nm) in the

"bob". The laser reflects off the red blood cells under shearing (diffraction) and creates a diffraction image, which is analyzed by the instrument.

When determining red blood cell deformability, the elongation index (EI) values were analyzed as a function of the applied shear stress (SS [Pa], 0.3-30 Pa interval). In order to compare the elongation index-shear stress curves obtained in this way, the elongation index measured at a shear stress of 3 Pa, the maximum elongation index (EI_{max}), the corresponding shear stress ($SS_{1/2}$) and their ratio ($EI_{max}/SS_{1/2}$) were used. In order to analyze the data, we used the Lineweaver-Burk curve fitting according to the following formula:

$$1/EI = SS_{1/2}/EI_{max} * 1/SS + 1/EI_{max}$$

For the red blood cell membrane stability analyses, the parameters were measured as described above, with the addition of a mechanical stress phase of 100 Pa lasting for 300 seconds, between two deformability measurement.

3.3.3. Whole blood and plasma viscosity

Whole blood and plasma viscosity measurements were performed on a Hevimet-40 capillary viscometer at a shear stress of 90s-1. 0.6-0.7 mL of whole blood or blood plasma is required for the measurements.

The device examines blood samples with two settings. The Cassonian measurement is used for whole blood, and when calculating the results, it takes into account that a non-Newtonian fluid is being measured. The device, which works on the principle of gravity, monitors the flow time of the liquid column in the (temperature-controlled) capillary under constant shear stress, thus calculating the viscosity, which is given in units of mPas (1 mPas = 1 centipoise [cP]).
Since the blood viscosity depends on the hematocrit, we must use the Mátrai-formula for the comparability of the results obtained:

$$VV_1 = PV \left(\frac{VV_0}{PV}\right)^{\frac{Htk_1}{Htk_0}}$$

in which VV_1 is the corrected hematocrit value, PV is the plasma viscosity, VV_0 is the measured whole blood viscosity, Htk_1 is the hematocrit value to which we want to correct, in our case it is 40% and Htk_0 is the native hematocrit value.

3.3.4. Red blood cell aggregation

The Myrenne MA-1 erythrocyte aggregometer (Myrenne Gmbh, Germany) measures aggregation based on the principle of light transmission. The device determines the degree of red blood cell aggregation from the change in the intensity of the infrared light passing through the sample. The 20 µL blood sample is disaggregated by the device (with a shear stress of 600 s-1), and after 5 and 10 seconds we obtain the aggregation index values, with M (stasis or 0 s-1) and M1 (low shear stress 3s-1).

The LoRRca Maxsis Osmoscan ektacytometer (Mechatronics BV, The Netherlands) examines red blood cell aggregation using the syllectomertic principle. The device measures the samples using the Couette system, based on the laser diffraction principle (syllectometric aggregometry). The device creates an intensity-time curve from the intensity-change function of the laser light reflected from the blood sample after disaggregation, so unlike the Myrenne device, the ektacytometer not only presents static aggregation index data, but also provides results on the dynamics of the event.

3.3.5. Statistical analysis

GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses. In general, depending on the distribution of the data, we used one- or two-way ANOVA methods to analyze differences between and within groups, and a t-test or Mann-Whitney test as a simple comparison between groups at a given time. Paired t-test or Wilcoxon test was used to compare EI values before and after mechanical stress, also depending on the distribution of the data.

In each study, values of $p<0.05$ were considered statistically significant.

Regarding the mechanical stability tests, examining which parameter shows the changes most sensitively, the standardized difference values were calculated based on the following formula:

$$\left(average_x - average_y\right) / \sqrt{\sum \left(S.D._x^2 ; S.D._y^2\right)}$$

where: $average_x$ and $average_y$ are the averages of the test groups; and $S.D._x$ and $S.D._y$ are the standard deviations of the groups.

4. RESULTS

4.1. Comparative studies of data interpretation methods of red blood cell membrane stability test

The purpose of the research was to establish what characteristic changes are shown by hemodilution and red blood cells that have become less deformable, and which parameter shows the changes most sensitively. The dilution with PBS solution (0.2 %(V/V)) can be considered successful, since the measured Hct [%] is 19.75 ± 6.58; the red blood cell count [T/L] decreased to 18.45 ± 3.03 and the hemoglobin concentration [g/dL] to 19.2 ± 3.07 compared to the native blood samples.

The deformability measurements before the membrane stability test were considered standard deformability measurements during the tests. The EI values of the constant Hct group were lower compared to the native blood samples. The elongation index ($p = 0.049$ vs. native) and EI_{max} measured at a shear stress of 3 Pa decreased in these samples, $SS_{1/2}$ showed no significant difference. In the case of the PFA-GA treated group, due to the rigidity caused by the agent, reduced deformability was observed: lower EI values (EI at 3Pa: $p = 0.008$ vs. native; $p = 0.004$ vs. diluted), decreased EI_{max} values ($p = 0.017$ vs. native), the $SS_{1/2}$ values increased ($p = 0.016$ vs. native; $p = 0.012$ vs. diluted) and the $EI_{max}/SS_{1/2}$ ratio also decreased ($p = 0.004$ vs. Hct; $p = 0.017$ vs. Constant Hct).

A significant decrease in the EI values after mechanical stress (100Pa 300s) applied in the red blood cell membrane stability measurement was also observed compared to the previous ones, depending on the form of treatment of the samples.

No significant differences were found between the native and constant Hct samples. In the case of the PFA-GA treated samples, the biggest differences were seen when comparing the $SS_{1/2}$ and $EI_{max}/SS_{1/2}$ values after and before mechanical stress. These differences were significant both in absolute ($p < 0.001$) and relative ($p < 0.001$) values compared to the native blood sample. We observed significant differences between the shapes of the curves. Since EI after/before vs.

shear stress curves did not show a linear characteristic in all cases, several curve fitting methods were used.

In the case of the native and PFA-GA treated samples, the majority of the examined parameters showed a standardized difference above 3.0. In addition to the values before the stability test, the largest standardized difference was found in the following cases: EI_{max} after/before ratio (3.09) < $SS_{1/2}$ after/before ratio (3.65) < $EI_{max}/SS_{1/2}$ after/before ratio (3.83) < EI_{max} after stress (4.4) < $SS_{1/2}$ after stress (4.79) < elongation index value at 3 Pa shear stress, after/before ratio (5.27) < $EI_{max}/SS_{1/2}$ after stress (5.38) < elongation index value at 3 Pa shear stress, after stress (6.02).

Both the Constant Hct blood samples and PFA-GA treated blood samples showed lower EI values at constant shear stress during the 300 second mechanical stress period. In all cases, there was a slight increase in EI values during the first 30 seconds of the stress period, after which the values did not increase significantly. The value of the slope (m) of the EI-time curves showed a significant difference ($p = 0.037$ vs. Hct). Furthermore, the standard deviation value of m was 1.58.

4.2. The effect of increasing temperature on the stability of red blood cells, a comparative analysis

4.2.1. Hematological parameters

The rat, dog and pig samples differed significantly compared to the human samples. The white blood cell count was lower in rats ($p < 0.05$), while higher values were found in dogs ($p = 0.0095$) and pigs ($p = 0.001$). The red blood cell count was higher in all species than in humans ($p < 0.001$ vs. rat and dog, $p = 0.0144$ vs. pig). In contrast, higher MCV values were measured in humans ($p < 0.001$ vs. rats, dogs, and pigs). Hct values were lower in rats ($p < 0.001$) and pigs ($p = 0.0371$), but higher in dogs ($p < 0.001$) compared to human samples. The platelet

count showed higher values in all animal species compared to humans (all species: $p < 0.001$ vs. human). The elongation index values measured at a shear stress of 3 Pa showed a significant difference in the case of all examined animal species compared to human samples (all species: $p < 0.001$ vs. human). The EI_{max} values were lower in dogs ($p=0.019$ vs. rats, $p<0.001$ vs. pigs) and pigs ($p < 0.001$ vs. humans and rats). $SS_{1/2}$ values were lower in all animal species compared to human samples (rat: $p < 0.001$; dog $p = 0.007$; pig: $p < 0.001$ vs. human). The $EI_{max}/SS_{1/2}$ ratio was higher for each species (rat: $p<0.0001$; dog; pig: $p = 0.004$) compared to human samples. The red blood cell deformability (EI_{max}, $EI_{max}/SS_{1/2}$) showed a decreasing trend in the whole blood samples, as the tested temperature increased (37-45°C). Between 37-39°C, the decrease was minimal, however, at temperatures higher than 39°C, it became particularly spectacular. The elongation index at a shear stress of 3 Pa and EI_{max} decreased with increasing temperature, while $SS_{1/2}$ increased. The $EI_{max}/SS_{1/2}$ ratio decreased to a different extent with respect to the species under study. As a result of the heat treatment, the human PBS blood suspensions were the most sensitive, while the smallest change occurred in the dog samples.

4.2.2. Red blood cell membrane stability

In the EI values of the PBS-RBC suspensions incubated at 37°C after mechanical stress, the values of the rat, dog and pig samples decreased, on the other hand, an increase was observed in the human samples. After incubation at 40 and 43°C, the values of the rat and dog samples also increased, while the values of the pig samples remained low.

4.3. Hemorheological effects of a cholesterol-rich diet

4.3.1. Weight changes

The body weight data showed significant differences between the experimental groups by the sixteenth week of the experiment. The body weight of the Control group was 3087 ± 56g ($p = 0.004$ vs. base), the HC group was 4121 ± 61g ($p = 0.002$ vs. base, $p < 0.001$ vs. Control).

4.3.2. Hematological parameters

The white blood cell count, the total volume of red blood cells and the platelet count were significantly higher in the HC group by the end of the sixteenth week. There were no significant changes in the general hemoglobin content of the red blood cells. Red blood cell count, hemoglobin, and mean hemoglobin concentration of red blood cells were significantly lower in the HC group compared to Control.

4.3.3. Hemorheological parameters

The whole blood viscosity values were corrected to a haematocrit value of 40%. The values of the HC group were significantly higher compared to the Control group. There was no significant difference in plasma viscosity.

Based on the Myrenne aggregometer, the values of the HC aggregation index increased in all measurement methods compared to the Control group. This increase was significant both at stasis (M 5s: $p < 0.001$, M 10s: $p < 0.001$) and at low shear stress (3 s-1, M1 10s $p = 0.0251$).

Based on the aggregation indices measured by LoRRca, we can say that the aggregation index (AI [%]) in the HC group is significantly higher compared to the Control ($p = 0.0003$),

while the amplitude (Amp [au]) is significantly lower ($p < 0.0001$). The $t_{½}$ [s] value – which describes the kinetics of red blood cell aggregation – did not show a significant difference between the two groups.

Based on the elongation index - shear stress (EI-SS) curves, the HC group showed significantly lower deformability compared to the Control.

The parameters calculated from individual EI-SS curves show similar differences. The EI_{max} values were significantly higher in the Control group compared to the HC. The EI data measured at a shear stress of 3 Pa were lower in the HC group. The $SS_{1/2}$ values were higher, while the $EI_{max}/SS_{1/2}$ ratio was significantly lower in the HC group compared to the Control.

The curves and results of the deformability measurements performed before and after the mechanical stress of 100 Pa lasting for 300 seconds, used in red blood cell membrane stability measurements, show significant differences between the two groups.

Even before the applied mechanical stress, the HC group showed a significantly lower deformability curve compared to the Control group, and this trend remained in the HC group even after the mechanical stress. The differences between the curves before and after were also much larger in the HC group, which indicated a worse stress tolerance of erythrocytes.

The measurement data calculated from the curves showed that the hypercholesterolemic group showed significantly lower EI values, lower EI_{max} values, but higher $SS_{1/2}$ results compared to the Control. And these value differences remained even after the stress, they became even more significant in the $SS_{1/2}$ data of the HC group.

5. DISCUSSION

5.1. Comparative studies of data interpretation methods of red blood cell membrane stability test

The factors affecting red blood cell deformability and aggregation include mechanical trauma to red blood cells, changes in acid-base values, free radical reactions, metabolic changes occurring during pathophysiological processes, as a result of ischemia-reperfusion and sepsis. The degree of mechanical trauma to red blood cells depends to a large extent on the strength and duration of the stress. Measuring the mechanical stability of red blood cells is a useful tool in research and diagnostics, despite this, there are few articles in the literature regarding the analysis and interpretation of the data.

In their research, Szluha et al. investigated the microrheological effects of low-dose single whole-body proton irradiation in a mouse model. In their study, the authors presented the complete EI-SS curves and compared the EI values at a given shear stress as well as the EI_{max} and $SS_{1/2}$ data.

Based on their experiments on an animal model, Tóth and his colleagues pointed out that the insufficiency of smaller grafts was also visible in the changes in red blood cell deformability and membrane stability values. They described that the difference between the EI-SS curves before and after mechanical stress was smaller in the graft group. In addition to changes in the mechanical stability of red blood cells, accompanying hematological and hemostaseological changes were also found. The authors compared the EI-SS curves before and after mechanical stress, using two mechanical stress-shear time content combinations (60 Pa 300s and 100 Pa 300s).

Hemorheological parameters such as whole blood and plasma viscosity, red blood cell aggregation, deformability, and osmotic gradient deformability (osmoscan) also show differences between different species and genders. They also found that red blood cell membrane stability

differs between humans and other species, which is probably shear stress and osmolarity dependent. In these studies, the authors used more in-depth data analysis and comparison methods: the ratios of the EI values before and after mechanical stress were calculated, and the relative changes of the $EI_{max}/SS_{1/2}$ values were also compared in relation to the entire EI-SS curves.

There is little data on the mechanical shear phase in the literature, despite the fact that its analysis can provide valuable data on the mechanical and dynamic changes affecting the cells. Baskurt and Meiselman found in their research that the EI values show an increase at the beginning of the shearing phase. This is probably due to the release of nitric oxide or ATP from the cells as a result of the shear stress.

During the research and laboratory diagnostic measurements carried out at our Department, we experienced several "forms of behavior" during the mechanical stress phase: (1) low, almost linearly decreasing values in the shear phase; (2) initial increase and then decrease in EI values; (3) sudden decline after a stable phase; (4) in some cases, we did not find any tendentious changes in the values. Based on these experiences, we believe that it is advisable not only to compare the curves before and after mechanical stress, but also to monitor and evaluate the behavior of red blood cells under mechanical stress.

5.2. The effect of increasing temperature on the stability of red blood cells, a comparative analysis

A hyperthermic or febrile state can cause a variety of changes in vivo. These physiological or pathophysiological changes depend on the nature of the elevated body temperature, so different differences can occur during, for example, heat stroke, thermoregulatory diseases or fever. In the case of a hyperthermic state, changes in the vascular system can occur relatively quickly. In extreme cases, the heart, lungs, liver, and kidneys can suffer even irreversible morphological changes, which can lead to the shutdown of the given organ. Based on

the data so far, in such cases, it seems that the least affected organ may be the spleen. The heart muscle quickly reacts with vasodilatation, blood stasis develops in the interfascicular vessels, which leads to deterioration of contractility.

The microrheological parameters of red blood cells undergo significant changes with increasing temperature. Under normal conditions, red blood cells are characterized by a high degree of deformability, which shows a high degree of deterioration in case of fever or febrile condition.

The basic premise of this research was that changes in the deformability of red blood cells can occur as a result of heat treatment or measurements at different temperatures. These changes may vary between distinct species. The obtained results and their analysis supported the following: (1) short-term heat treatment (37, 40 and 43°C) in PBS-RBC suspensions (10% Htc) can cause red blood cell deformability that tends to deteriorate; (2) these changes differed in magnitude between species; (3) red blood cells reacted much more sensitively to temperature changes in suspensions compared to whole blood-PVP mixtures; (4) the measurements made at continuously rising temperatures affected the deformability in an inverse proportion and caused different degrees of change between the examined species; (5) the membrane stability test showed a paradoxical result for some measurements related to the increase in temperature.

The difference in red blood cell deformability between species is a complex and not yet fully clarified topic, as cell morphology, membrane composition, viscoelasticity, cell volume and the cell surface-to-volume ratio range between extreme limits in different species.

The temperature of storage and measurements significantly affects red blood cell aggregation and deformability values. Baskurt and Mat, during ektacytometric measurements performed on a rat sepsis model, found that the tests performed at 37°C showed significant differences compared to the control, which differences were not detectable at lower temperatures. Singh and Stoltz showed in human blood samples that the results of red blood cell aggregation and deformability measurements performed at 5°C and 37°C differ significantly. Similar studies have not yet been conducted at temperatures higher than this.

Temperature also affects the proteins of red blood cells and the double membrane layer. Gershfeld et al described that during a 30-hour incubation period at different temperatures, the hemolysis of human red blood cells is temperature-dependent, and this process is a consequence of the so-called "unilamellar-multibilayer" transformation of the cell membrane.

Nitric oxide is known to improve red blood cell deformability. Red blood cells are able to produce nitric oxide, one of the main reasons for which is the shear stress they experience in the blood vessels. In the course of our research, we found that in PBS-RBC suspensions, in measurements after mechanical stress at 37°C, the EI values improved in the case of human samples, while a similar trend was observed only at 40°C and 43°C from rats and dogs in derived samples.

Under physiological conditions, the production of nitric oxide of the red blood cells and the accompanying improvement in deformability could also be detected at lower shear stress values than the one we examined (100 Pa), considering that shear stress values above 10 Pa are rare in the blood vessels. It can be assumed that with the low (10%) hematocrit value in PBS-RBC suspensions, the distribution and transfer of shear forces between erythrocytes differs from the similar properties of normal blood. Furthermore, it is known that the production of nitric oxide increases with increasing temperature in the organs and tissues of the living organism.

The main limiting factor of our research may be that the treatments and measurements were performed in vitro, so we cannot directly apply the results to in vivo research. In the case of the heat-treated PBS-RBC suspensions, the heat stress lasted for a short time, and in the case of the whole blood samples, heating was applied only during the duration of the measurements. In contrast, an increase in core temperature, such as in the case of a fever, can last for days, triggering complex responses in the manner. The warmed whole blood samples were mixed in a PVP-PBS solution so that the measurements could be carried out, which in our case represents a further deviation from the physiological conditions.

5.3. Hemorheological effects of a cholesterol-rich diet

The hypercholesterolemic rabbit model is a widely accepted model for the research of pathological conditions and diseases related to human atherosclerosis and lipid metabolism. Depending on the amount of cholesterol absorbed by the liver, the number of LDL receptors in the liver may decrease in both humans and rabbits. Very low-density lipoprotein (VLDL) receptors are highly expressed on the surface of macrophages in both species.

Even though the rabbit model is excellent for metabolic research, like all experimental animal models, it has its limitations. One such limiting factor is that rabbits kept on a standard diet under laboratory conditions do not develop spontaneous atherosclerosis, due to the low cholesterol content of the diets. More serious pathological changes, such as the deterioration of heart functions, the formation of atherosclerotic plaques, infarction and higher mortality, only occur in rabbits kept on a diet with an increased cholesterol content for a long time. If rabbits are fed food with a high cholesterol content, damage to the aorta may occur, first in the aortic arch and then in the thoracic aorta section; Abdominal aortic involvement is characteristic of humans and occurs only in severe cases. In some cases, atherosclerosis affecting the coronary arteries can also be observed in rabbits.

Another characteristic of the rabbit model is that the damage that occurs in human atherosclerosis, such as plaque rupture, ulceration, or aortic aneurysm, is not or only rarely detected. Plaques in rabbits consist mainly of foam cells with fat deposits and a high macrophage content. Damages in an advanced state can develop after prolonged cholesterol feeding.

The rabbit model used in our research showed significant morphological, functional, and serological changes. The left atrium was thickened, the mass and relative thickness of the left ventricle increased. During the histological analysis, we found the formation of foamy atherosclerotic plaques in the aorta and interstitial fibrosis in the myocardial tissue. The animals also showed signs of diastolic dysfunction. The serum lipid parameters, the atherogenic index and the ApoB/ApoA ratio were significantly higher in the atherogenic group.

Based on our results, we can say that the atherogenic diet influenced many hematological parameters during the 16-week period of our research. Both the red blood cell count and the hemoglobin level decreased in the treated experimental group. Similar changes have been described in rabbits with high cholesterol and LDL levels. According to literature data, a low red blood cell count and significant changes in MCV, MCH and MCHC values can occur even after 6 weeks of atherogenic treatment.

During our experiment, the significantly increased MCV, unchanged MCH and the significantly decreased MCHC values in the HC group indicated the development of hypochromic macrocytic regenerative anemia. The occurrence of anemia can cause a hypoxic state in the body due to the reduced hemoglobin level, which affects several hemodynamic parameters. Changes in clinical and hemodynamic parameters caused by acute anemia are reversible, whereas chronic anemia can lead to progressive heart enlargement and left ventricular hypertrophy. Similar pathophysiological changes were observed in our experiment. An increased MCV may indicate the severity of the atherosclerotic condition and the presence of vitamin deficiency-related atherosclerosis.

The hypercholesterolemic state stimulates an increase in the number of platelets through megakaryopoiesis and myelopoiesis and increases platelet activation. Furthermore, the increased cholesterol level also increases the degree of platelet hyperaggregability. Activated platelets can also form aggregates with neutrophil granulocytes and monocytes. The resulting interaction between platelets and leukocytes plays an important role in the production of inflammatory cytokines, leukotrienes, and reactive oxygen radicals (ROS). Reactive oxygen radicals can promote the production of inflammatory mediators such as C-reactive protein (CRP), which can subsequently activate pro-thrombotic factors and platelets. The increased white blood cell and platelet count, as well as the CRP level we measured, show the inflammatory nature of atherosclerosis.

High cholesterol has a direct effect on blood flow; such as the formation and growth of atherosclerotic plaques in the arteries, the reduction of the lumen of the coronary arteries, the

inflammatory state of the endothelium, and a reduced degree of endothelium-dependent iron relaxation. Together, these worsen myocardial circulation and tissue perfusion. Hypercholesterolemia can also influence rheological factors indirectly: high cholesterol levels increase whole blood viscosity by increasing the number of white blood cells and platelets.

In the case of red blood cell aggregatio, we showed significant differences between the experimental groups, using both the light transmission and syllectometric measurement methods. With the Myrenne aggregometer, we showed that the aggregation indices of the HC group increased significantly compared to the Control in all measurement modes (M, M1). In LoRRca, in addition to the increased aggregation index values of the HC group, we experienced a reduced aggregation amplitude, while there was no significant change in the time dynamics of aggregation. Measurements performed during human clinical studies showed similar aggregation index and amplitude values, using the same measurement methods, in overweight, diabetic and hypercholesterolemic patients. Furthermore, it was established that there is a positive correlation between the total cholesterol level and red blood cell aggregation indices (AI), while the aggregation half-time ($t_{½}$) is negative.

The main cause of increased blood viscosity at low velocity gradients is red blood cell aggregation. A low tissue hematocrit level can cause low local viscosity in the marginal zone of blood vessels, which reduces the frictional resistance with the endothelium. Axial migration promotes the phenomenon of marginalization described for white blood cells and platelets. Marginalization of white blood cells depends on flow properties, axial migration of erythrocytes and their aggregates, local hematocrit value, and red blood cell deformability.

Many physiological and pathophysiological factors affect the deformability of erythrocytes. The membrane stability of red blood cells is also directly affected by the level of LDL-cholesterol. An excessive amount of cholesterol accumulating in the membrane of erythrocytes increases the rigidity of the cells and reduces their deformability. Changes in the cholesterol/phospholipid ratio in vitro can affect the amount of phosphatidylserine on the surface of red blood cells in the blood of patients with hypercholesterolemia and acanthocytosis.

Deterioration of red blood cell deformability was also demonstrated in human clinical studies. Phosphatidylserine is an important trigger for macrophages and thus plays a role in the recognition of aging red blood cells and is also an important factor in the membrane stability of erythrocytes.

Red blood cells with a more rigid membrane are more exposed to the mechanical stress created in the vascular system, so they are more easily damaged (mainly in the capillaries and spleen). Furthermore, more rigid erythrocytes are less deformed by shear forces, so the additional force effect caused by higher viscosity can also contribute to the reduction of red blood cell membrane stability values. Our research results showed that deformability decreased, and membrane stability worsened in the hypercholesterolemic group. The investigated parameters well expressed the differences in deformability (EI, EI_{max}, $SS_{1/2}$ and their ratio) and the decrease in mechanical stability (before and after mechanical stress). The decrease in red blood cell deformability in the hypercholesterolemic group is clearly shown by the decrease in EI_{max}, increase in $SS_{1/2}$ and decrease in the $EI_{max}/SS_{1/2}$ ratio obtained after the parameterization of the EI-SS curves. The deteriorating membrane stability was also reflected in the differences between the EI-SS curves. In the case of the hypercholesterolemic group, lower values were registered even before the mechanical stress, which became even more pronounced as a result of the stress. The $SS_{1/2}/EI_{max}$ ratio calculated here also shows this tendency.

MAIN FINDINGS AND CONCLUSIONS

1. From the point of view of the comparability of studies of changes in red blood cell membrane stability, the following parameters were found to be the most suitable: the slope of the elongation index-time curves during the mechanical stress period, the elongation index before/after ratio in relation to the shear stress (SS), measured at 3 Pa ratio of elongation index after/before, the maximum elongation index (EI_{max}), and the shear stress value ($SS_{1/2}$) corresponding to half of it and their ratio ($EI_{max}/SS_{1/2}$) for both healthy and pathological blood samples.
2. We have shown that even short-term heat treatment significantly reduces the deformability of red blood cells. We described that the red blood cells of different species react differently to varying degrees of heat effects, so human blood samples were the most sensitive, while samples from dogs were the most resistant to the heat effects examined. We have shown that the way the temperature is raised, and the composition of the samples also influence the deterioration of red blood cell deformability.
3. 3. During the examination of the hemorheological effects of the cholesterol-rich diet, we showed that the atherogenic diet causes significant changes: the number of white blood cells, the total volume of red blood cells and the number of platelets increased; the number of red blood cells, hemoglobin and the average hemoglobin concentration of red blood cells decreased. For the first time in the literature, we proved with two types of aggregation measurement methods that hypercholesterolemia significantly worsens the degree of aggregation. We found that the atherogenic diet significantly reduces the deformability of red blood cells. We described that the red blood cell membrane stability showed a significantly lower deformability curve in the HC group compared to the Control group, even before the applied mechanical stress, and this trend remained even after the mechanical stress.

6. PUBLICATIONS

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List of publications related to the dissertation

1. **Tánczos, B.**, Somogyi, V., Bombicz, M., Juhász, B., Németh, N., Deák, Á.: Changes of Hematological and Hemorheological Parameters in Rabbits with Hypercholesterolemia. *Metabolites. 11* (4), 1-12, 2021.
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3. Baráth, B., Somogyi, V., **Tánczos, B.**, Varga, Á., Bereczky, Z., Németh, N., Deák, Á.: Examination of the relation between red blood cell aggregation and hematocrit in human and various experimental animals.
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