

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Comparative micro-rheological studies with special emphasis on red blood cell membrane stability

by Viktória Somogyi

Supervisor: Norbert Németh, MD, PhD, DSc



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By Viktória Somogyi, Biotechnology MSc

Supervisor: Norbert Németh, MD, PhD, DSc

Doctoral School of Clinical Medicine, University of Debrecen

Head of the **Examination Committee**: Prof. András Berta, MD, PhD, DSc

Members of the Examination Committee: Béla Juhász, PharmD, PhD

György Nádasy, MD, PhD

The Examination takes place at the library of Department of Ophthalmology, Faculty of Medicine, University of Debrecen; 2018, 11.00 AM

Head of the **Defense Committee**: Prof. András Berta, MD, PhD, DSc

Reviewers: Prof. Lajos Bogár, MD, PhD, DSc

Gabriella Varga, MD, PhD

Members of the **Defense Committee**: Béla Juhász, PharmD, PhD

György Nádasy, MD, PhD

The PhD Defense takes place in the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; 9 July, 2018, 14 PM

1. INTRODUCTION

Rheology is a science that focuses on the flow properties of materials. The pioneer of haemorheology was Alfred L. Copley, who introduced the concept of biorheology (1948) and haemorheology (1951). Copley defined haemorheology as the following: "Macro- and microscopic hydrodynamics of cellular and plasmatic components of blood and the rheology of vessels in contact with blood combined." The goal of rheology is to understand the hydrodynamics of blood, more specifically to understand the microcircular processes and to investigate the pathological changes.

In these days hamorhaeological examinations and researches are widespread thanks to up-to-date measurement methods. In this field of science numerous methods are available based on different principles (ektacytometrial and filtrating methods, techniques for measuring aggregation). Complex development of processes is allowed by the understanding of the circulatory system. In the laboratories of the University of Debrecen, Faculty of Medicine, Department of Operative Techniques and Surgical Research hemorheological and micro-rheological parameters can be measured by numerous measuring equipment. In the last few years our Department prepared multiple measurement standardization studies, and investigated gender differences of hemorheological parameters in laboratory animals.

To standardize experimental animal data to human models, it is imperative to chose an adequate experimental method, to understand interracial differences, and to chose appropriate methods. It is also important to plan and implement the measurements with high accuracy, to be performed by standardized conditions, and to have acceaptable lab animal principles.

Different measuring methods require different sample preparation, sample volume, and the parameters in question can be affected by many factors. Because of the aforementioned agents, great caution is needed to choose the adequate lab animal, and in order to reduce the number of animals used in the study it has to be defined what is the needed versus available sample volume that can be taken in a terminal and in a non-terminal way. If no follow up is needed, and single blood taking is in place, terminal blood taking can be applied. For follow up investigation only non-terminal blood sampling is allowed, where only limited volume of blood can be taken.

To perform comparative measurement standardization study newer methods are indispensable to understand and extrapolate research data. Our comparative studies aim to enhance the following: examination of gender- and age-related differences, optimization of the measuring methods, evaluation of the results and improvement of the safety of laboratory measurements.

2. AIMS AND OBJECTIVES

1. We aimed to conduct a comparative, descriptive study using rat, canine, porcine and human blood samples to investigate the possible diversity of erythrocyte mechanical stability at various combinations in magnitude and duration of shear stress.
2. Optimization of the measurement techniques of the red blood cell osmotic gradient ektacitometry (detailed analysis of elongation index - osmolality curves). To analyze the samples before and after the mechanical stability test at 100 Pa for 300 sec, using 200, 250, 300, and 500 mOsmol/kg PVP solutions. To investigate how and what extent the osmolality may affect the results of mechanical stability measurements.
3. Comparative hemorheological analysis of the gender and age differences with regards of the red blood cell deformability, red blood cell aggregation, membrane stability and osmotic gradient ektacitometry. To examine this phenomenon we created a follow-up study in Crl:WI male and female rats.

3. MATERIALS AND METHODS

3.1. Membrane stability and osmoscan tests at various combinations of shear stress magnitude and duration

3.1.1. Experimental animal and human blood samples

The animal experiment parts were approved and registered by the University of Debrecen Committee of Animal Research (permission Nr.: 19/2011/UD CAR), in accordance with national and EU regulations (the Hungarian Animal Protection Act (Law XVIII/1998) and the Edict 63/2010). Human blood samples were obtained from volunteers under Clinical Ethical Committee approval (permission Nr.: DE OEC RKEB/IKEB 3625-2012).

In the morning hours blood samples were taken from 6 healthy male Sprague-Dawley outbred rats (age: 4 months, bodyweight: 522 ± 42.9 g) via lateral tail vein puncture (anesthesia: 60 mg/kg, i.p. thiopental); 8 healthy male inbred beagle dogs (age: 9-11 months, bodyweight: 13.75 ± 0.78 kg) via cephalic vein puncture; 11 healthy female Hungahib pigs (age: 10-12 weeks, bodyweight: 19.05 ± 2.89 kg) via medial saphenous vein puncture (anesthesia: 15 mg/kg, i.m. ketamine, 1 mg/kg, i.m. xylazine); and 7 female volunteers via median cubital vein puncture (age: 31-48 years). Blood samplings were carried out using 21 G BD Eclipse™ blood collection needle into 3 ml BD Vacutainer® tube containing 1.8 mg/ml K₃-EDTA as anticoagulant (Becton, Dickinson and Company, USA). Laboratory measurements were completed within 2 hours.

Each blood sample was subjected to mechanical stability test (see below) using nine combinations of shear stress magnitude and durations as the followings: 30, 60, or 100 Pa for 100 s, 200 s or 300 s.

Five aliquots of blood samples per each abovementioned species were investigated further. On those samples the mechanical stability test at 100 Pa for 300 sec were carried out using 200, 250, 300, and 500 mOsmol/kg PVP solutions.

3.1.2. Determination of hematological parameters

Hematological parameters were tested by a Sysmex F-800 semi-automated microcell counter (TOA Medical Electronics Co., Ltd., Japan). The device uses aperture-impedance principle to calculate the number of red blood cells (RBC), white blood cells (WBC) and platelets (Plt). The concentration of hemoglobin was measured by photometry. The other parameters are calculated from the above data. A test requires approximately 70 μ l of blood. From the results, red blood cell count (RBC [T/l]), hemoglobin concentration (Hgb [g/dl]), hematocrit (Hct [%]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin (MCH [pg]) and mean corpuscular hemoglobin concentration (MCHC [g/dl]) were presented.

3.1.3. Determination of hemorheological parameters

3.1.3.1. Determination of red blood cell deformability and membrane

Red blood cell deformability and the cell membrane stability were determined by LoRRca MaxSis Omoscan rotational ektacytometer (Mechatronics BV, The Netherlands).

For both deformability and membrane stability, 10 μ l of blood sample was suspended in 2 ml of PVP solution (polyvinyl-pyrrolidon: 360 kDa, Sigma-Aldrich Co. USA; PVP-PBS solution viscosity = 30.83 mPas, osmolality = 298 mOsmol/kg, pH = 7.2).

The LoRRca device consist of two different part, a statical glass cylinder, and a rotating cup part. This is called Cuette-system based ektacytometer. A sample can be insterted via Pasteur pipette into the gap

(~0.3 mm) between the two parts of the machine. The cup rotates around the cup with a predefined angular velocity, while the sample is illuminated by laser. Changes of the diffraction image depends on the elongation of the red blood cells. The diffraction pattern is recorded by a CCD camera, which is analyzed with a computer software.

The EI is equal to $(L-W) / (L+W)$, where L is the length and W is the width of the diffractogramm. For the comparison of individual EI-SS curves Linewaver-Burke analysis ($1/EI = SS_{1/2} / EI_{max} \times 1/SS + 1/ EI_{max}$) were performed, calculating the EI_{max} (the maximal elongation index) and $SS_{1/2}$ [Pa] (the shear stress values at half EI_{max}). In addition, $EI_{max} / SS_{1/2}$ ratio was also calculated. EI_{max} and he increase of $SS_{1/2}$ values suggest degrading deformability.

After regular ektacytometry measurements, the cell membrane stability tests were carried out. The method consists of two regular deformability tests, before and after a shearing period with controlled magnitude and exposure time of the shearing force. Every sample was tested with nine combinations of shearing force and duration: 30, 60 or 100 Pa shear stress for 100, 200 or 300 seconds. Measurements were carried out under the same conditions described for the regular deformability test. For evaluating the effect of the various mechanical stress combinations, the EI-SS curves obtained before and after the shearing were compared with the parameters described above, together with their ratio (after versus before values).

3.1.3.2.Determination of the osmotic gradient ektacytometry

During the osmoscan measurement he elongation index values were determined at a constant shear stress value (30 Pa) while, the osmolality is continously and gradually rising from 0 to 500 mOsmol/kg. The measurements were carried out in PVP-PBS solutions at various

osmolality: 200, 250, 300, and 500 mOsmol/kg (pH ~7.2, viscosity = 29-31 mPas).

For the test 250 μ l blood was gently mixed in 5 mL PVP solution. The parameters given by the device were: minimal elongation index values measured at low-osmotic environment (EI min), maximal elongation index values (maximal EI max at the constant shear stress (not equal to EI_{max}), the osmolality values at which these minimum and maximal values were obtained (O min and O max), the half-maximal elongation index in the hypertonic arm (EI hyper) and the corresponding osmolality at which it occurs (O hyper), and area under the individual elongation index-osmolality curves (AUC). The following were calculated based on those parameters: difference between maximal and minimal EI values (Δ EI), the difference between osmolality values at maximal and minimal EI (Δ O), and their ratio.

3.1.4. Statistical analysis

Data are presented as means $\pm$ standard deviation (S.D.). For comparing EI values before versus after the mechanical stress paired t-test or Wilcoxon signed-rank test was used, depending on data distribution and equality of variances. For comparing the effect of various mechanical stress combinations on deformability impairment one way ANOVA with Bonferroni's post hoc test or one way ANOVA on ranks with Dunn's test were used. For inter-species comparison two sample t-test/Mann-Whitney rank sum test was applied depending on the normality of data distribution. A $p < 0.05$ value was considered statistically significant.

3.2. Gender and age-related studies of micro-rheological parameters

3.2.1. *Experimental animals and blood sampling protocol*

The experiments were approved and registered by the University of Debrecen Committee of Animal Welfare (permission Nr.: 19/2011. UDCAW), in accordance with national (Hungarian Animal Protection Act, Law XVIII/1998) and EU regulations (Directive 2010/63/EU).

Coeval male (n=10) and female (n=10) Wistar (CrI:WI) rats (Toxi-Coop Ltd., Hungary) were followed-up for 15 months. The animals were kept in standard cages in groups of two, with natural light-cycles, and were fed with commercial rodent chow (Bábolna rodent-specific CRLT/N). The temperature during the follow-up period was maintained at about 20-22 °C. Blood samples were obtained by puncturing the lateral tail vein (each time ~0.5 ml, anticoagulant: K3-EDTA) at the age of 3 months (base value, tested in March), and later when the animals were 4, 5, 9, 12, 15 and 18 months old.

The phase of the estrus cycle was determined by vaginal swab smear technique. The samples were taken during the morning hours between 7-9 am. After that, we let the smear samples to air-dry for overnight on the slides. The staining was a slightly modified Giemsa-staining protocol. The samples were fixed with absolute methanol for 30 seconds. The effective staining commenced after the fixation of the samples. We used stock Giemsa-stain solution (J.T Bakers' histology/cytology Giemsa 3856.1000) on the samples for 1 minute, then rinsed the slides in distilled water until all the residual stain was washed off. The stained samples were air-dried for a night in a clean, dustless container and then observed under light microscope. The following vaginal cytology classification was used: proestrus- nucleated epithelial cells, estrus- cornified squamous epithelial cells, metestrus- clustered cornified squamous epithelial cells and polynucleated leukocytes and diestrus-circular leukocytes.

3.2.2. Hematological parameters

Hematological parameters was investigated by Sysmex F-800 (TOA Medical Electronics Co., Ltd., Japan) according to chapter 3.1.2.. Red blood cell count (RBC [$10^6/\mu\text{l}$]), white blood cell count (WBC [$\times 10^3/\mu\text{l}$]), monocyte+granulocyte % (Mo+Gr% [%]), hemoglobin concentration (Hgb [g/dl]), hematocrit (Hct [%]), platelet count (Plt [$\times 10^3/\mu\text{l}$]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin (MCH [pg]) and mean corpuscular hemoglobin concentration (MCHC [g/dl]) are presented.

The measurement of red blood cell deformability, membrane stability and osmoscan by LoRRca MaxSis Osmoscan rotational ektacytometry were according to chapter 3.1.3.1. and 3.1.3.2. Polyvinylpyrrolidone (PVP) – phosphate buffered saline (PBS) solution was used as high-viscosity suspending media (PVP: 360 kDa, Sigma-Aldrich Co. USA; PVP-PBS solution viscosity = 23.7-35.9 mPas, osmolality = 296-319 mOsmol/kg, pH = 7.0-7.2).

During the osmotic gradient ektacytometry (osmoscan) test, the elongation index values were determined at a constant shear stress value (30 Pa) (3.1.3.2.).

3.2.3. Hemorheological parameters

3.2.3.1. Determination of red blood cell aggregation by light transmission principle

A light-transmittance method was used for determining red blood cell aggregation with the Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany).

The 20 μl anticoagulated blood samples required by the measurements are applied in a bubble-free from on a motor rotated, 2° angle cut glass lens. Underneath the lens an infrared detector can be found. By closing the lid, the blood sample is spread by a slide. On top of it an

infrared diode is placed. The determined aggregation index parameters are the followings: M5 s, M1 5 s, M10 s, M1 10s. The principle of the device: first the red blood cells in the sample are disaggregated (600 s^{-1}). Then suddenly the shear rate drops to zero (stasis, M mode), or to low value, to 3 s^{-1} shear rate(M1 mode). Then the device measure the light transmission changes in the 5th and 10 th of the aggregation process. The M and M1 increase with enhanced aggregation. The equipment is not tempered, the measurements are carried out without heat control.

3.2.4. Statistical analysis

Data are presented as mean values $\pm$ standard deviations (S.D.). Differences within the groups (age-related changes) were analysed by one-way ANOVA followed by post-hoc Bonferroni or Dunn where appropriate. For inter-group comparison (males versus females) t-test or Mann-Whitney rank sum test were used, depending on the normality of data distribution. A p value of <0.05 was considered as statistically significant. Statistical analysis was made using SigmaStat (Systat Software Inc., San Jose, California, USA) software.

4. RESULTS

4.1. Osmoscan and red blood cell membrane stability at various combinations of shear stress magnitude and duration

4.1.1. Hematological and red blood cell deformability parameters

Red blood cell count was the highest in the rat, then in an order of dog, pig and human. Mean corpuscular volume was the highest in the human, then in an order of dog, pig and rat. Besides dog mean corpuscular hemoglobin concentration, all parameters were significantly different from the human values (for rat hemoglobin concentration: $p=0.032$, for the other parameters: $p<0.001$).

Red blood cell deformability describing elongation index (EI) – shear stress (SS) curves showed inter-species differences both in the shape of the curves and in the EI values. Generally, the highest EI values were measured in rat blood and the lowest in the human. The shape of the canine EI – SS curves was the most similar to the human ones but with higher values. Rat and porcine EI values ran parallel to each other at lower shear stress levels, and then above 3 Pa the slope of the porcine curves became flatter. The calculated parameters (Table 1) reflected well the inter-species differences of the EI – SS curves. All values, except for canine $SS_{1/2}$ and $EI_{max} / SS_{1/2}$ values, were differed highly significantly from the human values ($p<0.001$).

4.1.2. Red blood cell membrane stability

Using smaller shear stress and by shorter duration, the differences were diminished, but not in the same manner in the investigated species.

Due to the 30 Pa mechanical stress rat erythrocytes did not show significant EI impairment, as the ratio of EI values measured after and before the mechanical test – as a representative parameter for the magnitude of EI impairment – was close to 1 and its value did not change as exposure time increased.

Canine erythrocytes showed improved deformability –higher EI values– after the mechanical stress of 30 Pa. At 100-second duration significantly higher EI values were measured generally at the 10-30 Pa shear stress zone, while in case of 200 and 300-second duration at all measured shear stress levels. The magnitude of the improvement significantly depended on the exposure time (under 10 Pa).

In case of the pig the lowest combination of the mechanical stress (30 Pa for 100 seconds) already caused significant lowering in the EI values measured generally at the 10-30 Pa shear stress zone, but its magnitude –EI after/before– was independent on the exposure time.

Human erythrocytes expressed slight, but not obvious deformability improvement that could also be seen at 100 and 200-second duration time.

The application of the mechanical stress at 60 Pa caused significant impairment in the EI values measured at all shear stress values, except for dog, in which blood samples this shear stress did cause important changes in EI values. The magnitude of the EI impairment was basically independent from the length of the exposure time. However, slight difference was seen in the human, and also in the rat at lower shear stress levels.

The highest mechanical stress (100 Pa for 300 seconds). The shape of the EI – SS curves were highly irregular under 0.95 Pa, mostly in the human and the least in the rat. Due to the 100 Pa shear stress applied for 300 seconds human erythrocytes showed the largest deformability impairment, the elongation index dropped by 17.6-42.4% between the 0.95-10 Pa range and by 5.5-12.6% between 10-30 Pa (except at 0.95 Pa shear stress, $p<0.001$ at all tested shear stress values points: 1.69, 3, 5.33, 9.49, 16.87 and 30 Pa). The same values in rat were 8.6-21.5% and 2-5.2% ($p<0.001$ at all tested shear stress values), in dog 14.5-20.8% and 9.2-12.3% ($p<0.001$ at all shear stresses, except for 0.95 Pa: $p=0.007$), and in

pig 10.6-26.8% and 3.3-7% ($p < 0.001$ at most of the shear stress levels, except for 16.87 and 30 Pa where $p = 0.002$ and $p = 0.024$ values existed).

The erythrocytes' capacity of resistance against increased in the four investigated species by the ratio of the $EI_{\max} / SS_{1/2}$ values determined from EI-SS curves after and before the mechanical stress. The values did not change obviously with the exposure time when we used the shearing protocol at 30 Pa. In canine blood even a slight improvement was seen. Using 60 Pa shearing, the values moderately decreased in rat, increased in dog and decreased in porcine and human blood. In all the four species $EI_{\max} / SS_{1/2}$ values decreased with the increase of exposure time when tested at 100 Pa stress level.

4.1.3. Osmolality-dependent alterations of red blood cell membrane stability data

Osmolality changes both in hypo- or hyperosmolar direction strongly influenced the mechanical stability test results (100 Pa for 300 s). Interestingly, the tests carried out on using hypoosmolar PVP-PBS solution (200 mOsmol/kg) showed improvement of the EI values after the mechanical shearing in all the four investigated species, most expressedly in rat and porcine blood. Tests at 250 mOsmol/kg reflected the deterioration described above in the main results, except for porcine blood, in which still an improvement was seen. At 500 mOsmol/kg no obvious changes were detected, because of the irregular curves caused by the presence of shrunken cells.

Comparing the ratio of $EI_{\max} / SS_{1/2}$ values of EI-SS curves determined after and before the mechanical stress, inter-species differences could be observed. The highest ratio values were observed at low osmolality, while 250 and 300 mOsmol/kg data showed similar results, the hyperosmolar condition triggered a decrease of the values, except for pig, where it rather increased. In rat blood the difference between the values

tested at 250 and 500 mOsmol/kg was the smallest, and it hardly changed. While in canine blood the 200-300 mOsmol/kg range was relatively stable, and at higher osmolality the values dropped. Human data changed in the smallest range, but with obvious direction: decreasing values as osmolality increased.

4.2. Gender and age-related studies of hematological and hemorheological parameters

4.2.1. Bodyweight changes

During the 15 months of the study there was a significant increase in the body weight of the animal. There was also a significant difference between the weights of male and female rats from the second month of the observation. The female's average weight increased by 89,9 %, the male's by 133,4 % by the end of the follow-up study, when the animals reached the age of 18 months.

4.2.2 Hematological parameters

The white blood cell count declined over the first 9 months in females and was constant thereafter. In males it declined between 5 to 9 months and slightly increased afterwards. Consistently higher white blood cell counts were measured in the male rats. Monocyte+Granulocyte percentage gradually increased till the age of about 12-months in female and male rats.

Hemoglobin was significantly higher in male than in female rats at 3 ($p=0.047$), 4 ($p=0.002$), 5 ($p=0.016$), 9 ($p<0.001$) és 15 ($p<0.001$) months of age, and Hct higher at 5, 9 and 15 months. By 15-18 months, Hgb and Hct tended to be higher than at 3 months.

Mean corpuscular volume (MCV [fl]) did not show consistent gender differences but was significantly higher in females compared to the males at ages of 4, 9 and 18 months ($p<0.001$; $p=0.035$; $p=0.002$).

In the 5, 12 and 18th months, the mean corpuscular hemoglobin content (MCH) values were higher in the female group.

The mean corpuscular hemoglobin concentration (MCHC) values decreased in both genders at 18 months compared to 3 months.

4.2.3. Hemorheological parameters

4.2.3.1. Red blood cell aggregation

Red blood cell aggregation index values were initially lower in male than in female animals. At start higher M1 5s and M1 10s aggregation indices were found in females, and higher M 5s and M 10s values in males. By the age of 4 and 5 month old these values changed significantly (in april male vs female M 5s $p=0,011$; M1 5s: $p=0,001$; M 10s: $p<0,001$; M1 10s: $p<0,001$; may M 5s: $p<0,001$; M1 5s: $p<0,001$; M 10s: $p<0,001$; M1 10s: $p<0,001$). During winter we observed a similar pattern, in females the aggregation indices increased, while decreased in males. At the end of the observing period M10s and M1 10 s values were higher than that of the young animals. Altogether in the case of there 4 parameters no constant behaviour was observed, increases and decreases were both seen.

4.2.3.2. Red blood cell deformability, membrane stability (normal and osmotic gradient ektacytometry)

The elongation at 3Pa shear stress was significantly higher for females than males. From the age of 9 months, this index was lower than at 3 months. The EI_{max} also tended to be higher for the females and was significantly different compared to male values at the ages of 5,9 and 12 months ($p<0,001$; $p<0,001$; $p=0,029$). EI_{max} also tended to decrease with age. The $SS_{1/2}$ values increased from 9 months until the end of the follow up period, and tended to be higher in males. The $EI_{max}/SS_{1/2}$ was significantly higher in females from age 3 month to 9 month (3rd month: $p=0,004$; 4th month: $p=0,001$; 5th month: $p<0,001$; 9th month: $p<0,001$).

Between the 12th and 18th month $EI_{\max}/SS_{1/2}$ values were decreased compared to 3 months both in male and female groups.

Maximal elongation (EI max, at osmolality close to the isotonic level) or minimal elongation (EI min, at low osmolality) did not vary consistently with age or between genders. EI max showed decreased values between ages of 9-12 months, while EI min values increased significantly by the end of the observation period in both genders. The O min and O EI max values in males were lower than in females in every month, with the differences at 4, 5, 9 and 15 months statistically significant ($p=0.009$; $p=0.002$; $p=0.027$; $p=0.024$). EI hyper values reflected the changes in EI max, as they calculated from the latter parameter. O hyper values did not show consistent variation with age or between genders over the first 9 months, but then decreased so that at the end of the observation period O hyper was significantly decreased compared to the base (3-month) data. In males, AUC was lowered at 9 and 12 months, but otherwise, no clear trends were evident. The ΔEI and ΔO and their ratio may provide additional information about the hypo-osmolar part of the osmoscan curve (between EI min and EI max, O min and O EI max). Cells are swelling in this region until their rupture. We found significant decrease in ΔEI in the blood samples of 9- and 12-month males and 12-month females. ΔOI values were higher in females than males at most ages. After increasing between ages of 5-12, values slightly decreased in samples of 15- or 18-month male animals. The ratio of $\Delta EI/\Delta OI$ values was almost unchanged throughout.

5. DISCUSSION

5.1. Red blood cell membrane stability and osmoscan at various combinations of shear stress magnitude and duration

Mechanical stability of red blood cells is essential for their survivor in the circulation. Under physiological circumstances the shear stress on erythrocytes are generally under 5 Pa and usually not exceeding 10 Pa. However, pathophysiological processes or non-physiological circulatory conditions can cause the increase of shear stress, which can lead to membrane injury of erythrocytes. Extent of the mechanical injury –that causes sub-lethal and later hemolytic trauma to the red blood cells– depends on the magnitude and exposure time of the shear stress, as well as on the mechanical stability of the cells. Hereditary membrane disorders and enzymopathies of the erythrocytes or any pathophysiological processes that causes injury to the red blood cells result impaired membrane stability and lower capacity of resistance against increased shear stress. The end point of the mechanical injury is the lysis of the cells due to membrane rupture. The mechanical trauma that does not cause hemolysis yet but results in deterioration of cells' micro-rheological parameters, such as deformability and aggregation, is called sub-lethal trauma. It was firstly mentioned by Brinsfield et al in 1962, and they experienced a decrease in red blood cell count after extracorporeal circulation lasting 10-48 hours in experimental animals. It causes impaired red blood cell deformability and increased aggregation, which have a negative effect on microcirculation and tissue perfusion. Through several cascade-like mechanisms and by effect on leukocyte- and platelet functions supra-physiological shear stress causes alteration in the hemodynamic parameters that will lead to further increase in the shear stress, and the process turns into a vicious circle. For investigating various pathophysiological processes that may alter shear forces in the circulations, and for developing-testing intravascular devices

(e.g., stents, grafts, vascular prostheses, artificial valves and hearts, devices for extracorporeal circulation, special intravascular circulation-supporting devices, etc) *in vivo* studies are necessary.

It is known that and already widely investigated that like other physiological parameters, hemorheological ones also show interspecies differences. Red blood cell deformability is determined by several cellular factors, and one of the most important in the maintenance of mechanical stability is the integrity of spectrin-based membrane skeleton. Interspecies differences in the mechanical stability of red blood cells can be partly explained by the quantitative and qualitative difference in the spectrin-network and the levels of protein phosphorylation, especially for the protein 4.1R that modulates spectrin and actin affinity and membrane stability of erythrocytes. However, it should be taken under consideration that this mechanical stability measurement cannot be performed *in vivo*, and although all measurements were completed within 2 hours under the same protocol, different species red blood cells are sensitive to *in vitro* conditions at a different manner. Changes in the erythrocytes' metabolic state may also cause membrane stiffening due to reduced skeletal junction phosphorylation.

Similarly to our findings on dog erythrocyte deformability improvement, recently it was reported by Meram et al. that a very brief (few-second) duration of 5-20 Pa shear stress may even improve deformability of human red blood cells up to by 8% . Simmonds et al. also observed improved deformability on human red blood cells under physiological shear stress and they found that the sub-hemolytic threshold for human erythrocytes was 30-40 Pa with 300 s exposure time. However, Arwatz and Smits, investigating two whole blood samples using a custom-made Taylor-Couette apparatus, found only 1-2% hemolysis when 50 Pa shear stress for 50 seconds was applied, 5% at 50 Pa for 300 seconds, and

10-12% at 200 Pa for 300 seconds. In our experiment elongation index – shear stress curves became more and more prominently irregular under 0.95 Pa shear stress as the magnitude and exposure time of the applied mechanical stress increased. It was probably due to increasing amount of erythrocyte fragmentation and hemolysis.

We have not found explanation in the literature for the strange observation on osmolality-dependency of the mechanical stability results together with their inters-species differences of this study. In hypotonic environment the cells are swelling, their shape become more spherical and their surface-to-volume ratios are changing accordingly resulting in decreased deformability and increased stretching-straining of the membrane. If a shear stress is applied on this condition it might cause altered shear stress distribution on the cells compared to a discocyte formation. It is hypothesized that due to the elastic characteristics of the cells (membrane), the stretching effect of mechanical shearing might be more expressed under this condition (mechanical shearing at low osmolality).

Rat and pig erythrocytes, having smaller MCV, showed more expressed ‘improvement’ during mechanical stability test at 200 mOsmol/kg compared to dog or human. Previously we also observed significant difference in of the elongation index – osmolality (osmoscan) curves were shifted to right compared to rat, dog or human. It seems that the interspecies diversity of hemorheological factors become much more complicated as we investigate with further and further techniques.

5.2. Gender and age-related hemorheological parameters

According to the literature the red blood cell deformability and aggregation values vary between different species. However, little is known about the gender and age-related differences. Earlier we studied red

blood cell deformability and aggregation in rats and dogs, and it has been showed that female animals have higher red blood cell deformability index values in rats, while in dogs the male animals possess higher elongation index. In the case of red blood cell aggregation we can see a different trend in humans. According to numerous articles human males have increased red blood cell aggregation and lower deformability scores.

Red blood cell deformability is determined by intracellular viscosity, membrane viscosity and elastic properties, surface area to cell volume ratio, and cell morphology. Red blood cell aggregation depends on cellular (cell morphology, deformability, properties of the cell surface glycocalyx) and plasmatic factors (fibrinogen and other protein levels), besides the shear forces. In addition to all these factors, hormonal and metabolic aspects may also affect these parameters.

Several studies have shown that hemorheological factors are significantly affected by aging. Majority of the studies have reported increased plasma and whole blood viscosity, enhanced red blood cell aggregation and impaired deformability in older age. However, the age-related hemorheological results are controversial. Some authors found either no correlation or concluded that not age itself, but certain risk factors are responsible for the alterations, involving obesity, hypertension, smoking or certain medication. The discrepancy may be not only due to the health status, but also to the different methods used for the measurements, sex and the use of different age categories. Kameneva et al. demonstrated a statistically significant difference in male versus female hemorheological parameters. Men had higher hematocrit, increased blood viscosity, red blood cell aggregability and red blood cell rigidity, as well as lower deformability. Based on experimental and clinical data Simmonds et al. in their review discussed the altered blood rheology in aging and the related mechanisms, involving the enhanced oxidative stress and the pro-

inflammatory condition of aged individuals. Hemorheological aspects of aging and gender in rats have not been completely revealed yet.

When evaluating these results, several influencing factors and limitation have to be taken into consideration, such as possible strain-specific properties, estrus cycle, and seasonal effects, among others.

The estral cycle of the rats was observed by simple, old method (since 1922), investigating the vaginal smears under a light microscope. The animals were kept in a conventional animal house, so the Lee-Boot effect (suppression or prolongation of the estrus cycle when the females are held together without males in the room) was excluded, but not the Whitten effect (pheromones of male animals induce the synchronization of the estrus cycle in adult females). The menstrual cycle can modify the hematological parameters as red blood cell and total leukocyte count in primates. Also the different phases of estrus cycle (diestrus versus all another phase) can change the hematological values like hemoglobin, red blood cells and eosinophils in beagle dogs. Cetin et al. have investigated the effect of sex, pregnancy and season on blood parameters in Angora rabbits, finding significant differences between gender, physiological status and periods of year. Female rats' estrus cycle is about 3-5 day-long (polyestrus animals). Aging may have effects on rats' cycle by either prolonging or shortening it. Therefore we cannot directly link these hemorheological results to the estrus cycle. We tested the cycle with vaginal smear technique, showing just a "cross-section" of the estrus cycle stat in the group. It is one of the limitations of our study.

The effect of season cannot be excluded; however, we could not provide evidence on this issue. At the beginning of the follow-up period, in March, all the rats were 3-month old. The highest increase in aggregation index values of males was observed around the 4th and 5th months (still in spring), that was followed by a decline. During the winter, this tendency

reversed itself, again the male values were higher, and the female values were lower in comparison. In parallel, leukocyte count also decreased.

Other limitation is the relatively rare sampling. We did not plan to perform blood sampling more frequently, due to the effects of blood-loss and the necessary recovery time. Our main concern was the animals' well-being during the study period. Furthermore, as majority of the studies are conducted on 3-4 rarely up to 6 month old rats, over this period we did not wanted to affect the animals more. Blood samplings were performed under short general anesthesia. We experienced that with aging the effect of anesthesia can be also altered, so we also wished to reduce the risk to lose the animals. Also due to the limited blood sampling volume, we could not investigate further parameters, such as fibrinogen concentration, routine blood chemistry, enzymes and hemostaseological parameters.

„Most of the researchers used to relate human and rat age by simply correlating their life span, which is not acceptable, because, for a specific research work, one uses a particular developmental phase of rat-life. Thus one should consider different phases of their life to have an accurate correlation”. Laboratory rats live about 2-3.5 years. Considering the whole life-span, about 26.7 human days is equal to 1 rat day, and about 13.8 rat days is correlated to 1 human year. However in „puberty”, adult age, reproductive senescence and post senescence periods, the correlations can be different. We followed-up the animals up to their age of 18 months, only. It can be correlated to about 45 years in human. It means that we have just still investigated the hemorheological alterations till a middle-aged human as it is correlated. We plan further studies to extend the follow-up period, altogether with regular blood pressure monitoring as well.

6. SUMMARY OF MAJOR RESULTS AND CONCLUSIONS

1. Red blood cells' membrane stability data showed well-identifiable changes in relation to the examined species (rat, pig, dog and humans). We have concluded that using the applied combinations of mechanical stress pig erythrocytes proved to be the most sensitive. As the magnitude and the duration of the mechanical stress increased, the shape of the elongation index – shear stress curves became more and more prominently irregular under 0.95 Pa, and its magnitude was different among the species.
2. Osmolality changes both in hypo- or hyperosmolar direction strongly influenced the mechanical stability test results in all species. We were the first to describe that following mechanical stress, in hypoosmolar range the EI values improved in all investigated species, mostly in rat and porcine blood. This phenomenon was not observable at 250, 300 or 500 mOsmol/kg.
3. For the first time in the literature in a follow-up study we analyzed the age-related changes of the micro-rheological parameters (red blood cell aggregation, deformability) in CrI: WI rats concerning the gender differences. The red blood cell aggregation index values showed irregular changes during the follow-up period, while red blood cell deformability and membrane stability values worsened by aging, mostly in male animals.



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List of publications related to the dissertation

1. **Somogyi, V.**, Pető, K., Deák, Á., Tanczos, B., Németh, N.: Effects of aging and gender on micro-rheology of blood in 3 to 18 months old male and female Wistar (CrI:WI) rats.
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IF: 1.679





List of other publications

3. Mester, A., Magyar, Z., Molnár, Á., **Somogyi, V.**, Tanczos, B., Pető, K., Németh, N.: Age- and gender-related hemorheological alterations in intestinal ischemia-reperfusion in the rat.
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