THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

COMPARATIVE STANDARDIZING INVESTIGATION OF LABORATORY ANIMALS' MICRO-RHEOLOGICAL PROPERTIES FOR EXPERIMENTAL SURGICAL RESEARCH STUDIES

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

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The Examination takes place at the Library of the Department of Ophthalmology, Medical and Health Science Center, University of Debrecen
at 11 a.m. 13 January 2012.

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen at 2 p.m. 13 January 2012.

1. INTRODUCTION

In 1951 Alfred L. Copley defined hemorheology as the science dealing with the macro- and microscopic flow properties of the corpuscular and plasmatic components of blood as well as the vessel wall being in contact with the blood.

Besides hematocrit, plasma viscosity and fibrinogen concentration the micro-rheological parameters such as red blood cell deformability and red blood cell aggregation have an important role determining whole blood viscosity.

The deformability of red blood cells can be described by the passive changes in the shape of the cells due to shear stress.

The importance of proper deformability is the most in the zone of individual flow (vessel diameter < 8 μm), since in the human body there are several regions (heart muscle, brain) where through the 3-5 μm or even less diameter micro-capillaries only the properly deformable red blood cells are able to pass. The importance of red blood cell deformability measurements is in that, since the injured, becoming rigid red blood cells cause further damage and failure in the tissue microcirculation.

The deformability of red blood cells is determined by several cellular factors which are highly sensitive for the changes of cell homeostasis. The major determinants of deformability are the cytoskeletal structure, the cell morphology, the surface-volume ratio, the inner viscosity (hemoglobin-content) as well as the own viscosity of the cell membrane.

The other important micro-rheological property of the biconcave erythrocytes is the aggregation which is a reversible connection under stasis or low shear rate. The aggregation of red blood cells is determined by both plasmatic and cellular factors. Among the plasmatic factors the fibrinogen concentration, as well under in vitro circumstances other branching structured macromolecules, has an important role. The cellular factors are the cell shape, the deformability and the hardly determinable structure of the glycocalyx.

Due to enhanced red blood cell aggregation in the zone of mass flow the particulum size is growing so the viscosity is elevated in the lower shear rate zones. In the microcirculation higher disaggregating energy is needed to break the aggregates therefore the tissue hematocrit can be impaired.

By now with the spreading of modern rheological measuring instruments it hemorheology became a real multidisciplinary field of science from base and applied research to clinical tests. The investigated problems naturally originated from clinical questions, to which the answer is often can be given by animal models used in experimental medicine. Whole blood and plasma viscosity, hematocrit, red blood cell deformability, red blood cell aggregation, fibrinogen show pathological, often signaling changes in cardiovascular and cerebrovascular clinical pictures, peripheral vascular diseases, gerontological aspects, metabolic diseases, inflammatory-, ischemia-reperfusion and septic processes, in addition in case of the depletion or loss of splenic function.

In research field the validity, appreciability and extrapolability of results are as keystones that the planning and execution of the established research model should be carried out regarding to up-to-date laboratory animal science principles and standardized conditions, including the choice of proper laboratory/experimental animal race, the determination of case numbers, gender ratio, blood sampling site, blood sample volume as well as sample handling and sample preparation conditions. Without these principles and standards research results of various laboratory animal races, different research teams cannot be securely compared and cannot be properly extrapolate back to the uprising problems of the human clinical practice.

There are growing numbers of data available about the racial hemorheological differences, although the gender differences of laboratory animals are poorly known. In human aspect the gender hemorheological differences are well traceable, which can be significant. The investigation of this

issue can be important in experimental models in the way of gender distribution in the formed experimental groups.

The different measuring-devices can determine the changes in laboratory animal blood samples by altering sensitivity. Supposedly, the race and gender differences of red blood cell deformability are also more sensitively detectable by increasing the viscosity of the applied solutions used for ektacytometric red blood cell deformability measurements in case of both normal and heat treated, rigid red blood cells which are used for comparative investigations.

We assumed that the differences caused by altered sample handling and measurement-technical conditions can reach or even mask in the pathophysiological processes detectable, notable, often significant hemorheological changes that was induced between experimental groups.

Parts of our research aimed to analyze important issues in the establishment of experimental animal hemorheological measurement-technical standards by comparative investigations of sample handling and instrument-sensitivity in order to serve data for the planning of animal models used in experimental surgical research and for the execution of well-comparative-results-providing hemorheological measurements.

2. AIMS AND OBJECTIVES

1. Among laboratory animal races we aimed to analyze the possible gender differences of the main hemorheological parameters (whole blood viscosity, plasma viscosity, red blood cell deformability and red blood cell aggregation) of the CD outbred rat and the inbred beagle dog, with the control, base data of healthy animals tested at the hemorheological research-laboratory of the Department of Operative Techniques and Surgical Research.
2. Regarding to experimental animal research there is only a few data available about blood sample storage in hemorheological aspects, therefore we aimed to investigate the possible changes of rheological parameters at different storage temperature along storage time in CD rat and beagle dog blood samples.
3. By using slit-flow ektacytometry, able to determine red blood cell deformability in modern way, we aimed to analyze comparatively the data gained from the usage of various viscosity red blood cell suspending media in order to show more sensitive detection of the hemorheological race and gender differences of Sprague-Dawley rats and beagle dogs.
4. We aimed to perform the comparative investigation of deformability parameters of intact and heat treated red blood cells, by determining the sensitivity of slit-flow ektacytometry using different viscosity suspending media on Sprague-Dawley rat and beagle dog blood samples.

3. MATERIALS AND METHODS

3.1. Hematological and hemorheological analysis of gender differences in laboratory animals

3.1.1. Experimental animals and blood sampling protocol

This comparative study focused on adult, middle-weight outbred CD rats and inbred beagle dogs. Our data-pool of healthy control values of hemorheological variables in various experiments and regular control tests measured at our hemorheological research laboratory was particularly used for analyzing the gender differences. All the experiments were approved by the Committee of Animal Research at University of Debrecen (permission Nr.: 6/2000, 7/2006, 34/2007 and 37/2007).

Blood was always collected in a closed system: on dogs using Vacutainer® tubes and puncture of the cephalic vein (needle size: 22 G); on rats using syringes and puncture of the lateral tail vein (needle size: 24–26 G), or under general anesthesia via cardiac puncture (needle size: 22 G).

For hematological, red blood cell aggregation and ekytacytometry tests K3-EDTA was used as anticoagulant (on dogs: 7.5%, 0.04 ml, BD Vacutainer® tubes; on rats: syringes with 1.5 mg/ml). For testing blood viscosity and for filtrometry tests sodium-heparin was used (on dogs: 143 IU, BD Vacutainer®; on rats: syringes with 10–15 U/ml), while fibrinogen concentration was determined in plasma from blood anticoagulated with sodium-citrate (on dogs and rats: 0.129 M, BD Vacutainer® tubes).

3.1.2. Determination of hematological parameters

Hematological parameters were determined using a Sysmex F-800 microcell counter (TOA Medical Electronics Co., Ltd, Japan). The tests require approximately 70 µl of blood. For the comparative analysis data of 106 male and 43 female CD rats, and 82 male and 86 female beagle dogs were used.

3.1.3. Investigation of hemorheological parameters

3.1.3.1. Determination of whole blood and plasma viscosity

Blood and plasma viscosity [mPa.s] were measured in a Hevimet-40 capillary viscometer (Hemorex Ltd, Budapest, Hungary).

The Hungarian-developed instrument contains two pieces of a 500 mm long and 0.6 mm caliber vertical capillary measuring chamber, which bedded in a tempered oil tub to secure the constant 37 °C during measurements. The measurements require 1ml of blood or plasma sample. During measurements the pressure-gradient is supplied by the hydrostatic pressure of the fluid.

For the comparative analysis plasma viscosity data and whole blood viscosity values at 90 s^{-1} shear rate of 20 male and 12 female CD rats, and 32 male and 27 female beagle dogs were used.

3.1.3.2. Measurement of fibrinogen concentration

Fibrinogen concentration (Fbg [g/l]) in plasma was determined using a Sysmex CA-500 coagulometer (TOA Medical Electronics Co., Ltd, Japan) based on Clauss's method. For the comparative analysis data of 20 male and 12 female CD rats, and 32 male and 27 female beagle dogs were used.

3.1.3.3. Determination of red blood cell deformability

3.1.3.3.1. Filtrometry

As a bulk filtration method a Carat FT-1 filtrometer (Carat Ltd, Hungary) based on the St. George's filtration technique was used.

For the measurements the red blood cells are required to be prepared in normal phosphate-buffer solution (PBS; osmolarity: 295 ± 5 mOsm/kg; pH: 7,4) to 5% hematocrit level. The RBC–PBS suspension flows through a 5 μm average pore-sized polycarbonate Nucleopore® filter at constant filtration pressure (4 cmH_2O). By the position-time detection with photodetectors the filtration velocity can be determined, from which the applied software can calculate the

initial relative filtration rate (IRFR), and with knowing the hematocrit of the suspension the relative cell transit time (RCTT) can be calculated, too:

$RCTT = [(IRFR^{-1} – 1)/Hct] + 1$

For the comparative analysis data of 20 male and 12 female CD rats, and 32 male and 27 female beagle dogs were used.

3.1.3.3.2. Ektacytometry

For the ektacytometric measurement of red blood cell deformability a Rheoscan-D200 slit-flow ektacytometer (Sewon Meditech Inc., Korea) was used. By the instrument vacuum-generated shear stress (SS; 0,5-20 Pa) the blood sample flows through a 200-220 µm high, 40 mm long slit. The measurement is based on the analysis of the diffracted laser images pattern from the elongated red blood cells by shear stress.

During sample preparation 6 µl of blood sample was placed into 600 µl high-viscosity -generally above 20 mPa.s- isotonic polyvinyl-pyrrolidone (PVP, 360 kDa) solution. The high viscosity solution is needed to supply properly the transfer of the applied shear stress to the red blood cells making their elongation possible. The instrument quantifies the red blood cell elongation index (EI) values at known shear stress (SS) levels by the analysis of the laser-diffractogram. EI increases parallelly with the deformability of the cell.

For the comparison of EI–SS curves EI values at 3 Pa, as well as, from the Lineweaver-Burk analysis calculated maximal elongation index (EI_{max}) and the shear stress at half maximal elongation index ($SS_{½}$ [Pa]) were used.

$$1/EI = SS_{½} / EI_{max} \text{ x } 1/SS + 1/EI_{max}$$

The $SS_{½}$ value increases with the decrease of cell deformability.

For the analysis data of 15 male and 18 female CD rats, and 24 male and 22 female beagle dogs were used.

3.1.3.4. Determination of red blood cell aggregation

For testing red blood cell aggregation a Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) was used, based on light transmission method. The measurements require approximately 20 µl of blood.

The instrument contains a transparent glass plate and a 2º glass lens spun by the shear flow producing rotor. Above the glass plate there is a infrared diode and under the lens there is a infrared detector.

During measurements the sample is disaggregated by 600 s^{-1} for a few seconds then suddenly the shear rate drops to zero (M mode) or to low value, to 3 s^{-1} shear rate (M1 mode). Then the instruments calculates the aggregation index value by the light transmission changes of the sample at the 5^{th} or 10^{th} second of the aggregation process. The indices increase with enhanced red blood cell aggregation.

For the analysis data of 38 male and 18 female CD rats, and 18 male and 42 female beagle dogs were used.

3.1.4. Statistical analysis

Data are presented as mean values and standard deviations (means±S.D.), as well as median values and 25^{th}/75^{th} percentiles (25%/75%). For comparison Student's t-test or Mann-Whitney rank sum test were used, according to the normality of data. A p value of <0.05 was considered to be significant.

3.2. The effect of storage time and temperature on the hematological and hemorheological parameters of laboratory animal blood samples

3.2.1. Experimental animals and blood sampling

The experiments were approved by the Committee on Animal Research, University of Debrecen (Permission Nr.: 37/2007. UDCAR).

Healthy female CD outbred rats (n=5, bodyweight: 329±83 g) and beagle dogs (n=5, bodyweight: 12.1±2.13 kg) were involved into the investigation.

6-8 ml of blood was obtained with closed system into vacuum tubes containing K_3-EDTA (7.5% 0.04 ml, BD Vacutainer®): from rats via cardiac puncture under general anesthesia (sodium-pentobarbital, 35 mg/kg, i.p.) and from beagle dogs via cephalic vein puncture. From beagle dogs further 6 ml of blood was withdrawn into sodium-heparin containing vacuum tubes (143 IU, BD Vacutainer®) for viscosity tests.

3.2.2. Storage and sample preparation protocol

Blood samples from each animal were divided into 10 aliquots. Seven were kept at room temperature (22-23 °C) in air-conditioned laboratory and tested at 0 hours (i.e., 10-15 minutes after sampling) and at 2, 4, 6, 24, 48 and 72 hours.

The rest three aliquots were stored on “wet ice” in a small insulated container. The ice was in plastic bags and the sample tubes were in a tray on the ice bags, and thus the tubes did not directly contact the ice. The temperature inside the container was continuously monitored: 4.4 °C at the start, 6.1 °C at 24 hours, 6.8 °C at 48 hours and 9.8 °C at 72 hours.

Samples were tested at 24, 48, and 72 hours after sampling. Prior to testing, the cooled samples were at room temperature for 20 minutes, then each blood sample was gently mixed and measured.

3.2.3. Laboratory tests

The hematological parameters were determined from every aliquots of all samples by Sysmex F-800 microcell counter according to chapter 3.1.2. The changes of mean cell volume (MCV [fl]) values are presented.

The determination of whole blood and plasma viscosity was according to chapter 3.1.3.1. and the measurement of red blood cell deformability by bulk filtration was according to chapter 3.1.3.3.1. These tests were only carried out on the cool stored beagle blood samples due to the required sample volume.

By ektacytometric way (Rheoscan-D200 slit-flow ektacytometer) the room temperature and cool stored K_3-EDTA anticoagulated blood samples were tested according to chapter 3.1.3.3.2.

Red blood cell aggregation was investigated by Myrenne MA-1 erythrocyte aggregometer according to chapter 3.1.3.4.

3.2.4. Statistical analysis

Data are presented as mean and standard deviation (SD). Differences from fresh samples at 0 hour were evaluated by one way ANOVA tests (Dunn's and Bonferroni's methods). Differences between room temperature and cooling were tested by Student's t-test or Manny-Whitney rank sum test according to the data distribution. A p value <0.05 was considered as statistically significant.

3.3. The effect of different viscosity suspending solutions on the sensitivity of laboratory animal red blood cell deformability measurement

3.3.1. Further ektacytometric investigations of gender differences

3.3.1.1. Experimental animals and blood sampling

The experiments were executed with the University of Debrecen Committee of Animal Research's approvement (Permission Nr.: 37/2007. UDCAR) on 10 male (455.6 ± 38.1 g) and 10 female (284.8 ± 14.3 g) 8 months old healthy Sprague-Dawley (SD) rats and on 10 male (12.9 ± 1.6 kg) and 10 female (12.3 ± 2.3 kg) healthy 3 to 4 years old beagle dogs.

Blood sampling occurred in the morning hours (8 a.m.-10 a.m.). From rats 0.5 ml of blood was drawn via puncturing the lateral tail vein, using 26 G needle and a connecting syringe that contained sodium-EDTA (1.5 mg/ml). In case of dogs blood was drawn via cephalic vein puncture, using vacuum tubes containing K_3-EDTA (7,5% 0,04 ml; BD Vacutainer®; 2-ml tube).

Each blood sample was tested by ektacytometry using all three different viscosity polyvinyl-pyrrolidone (PVP) solutions as described below

3.3.1.2. Sample preparation and red blood cell deformability measurement

In normal phosphate buffered saline (PBS) polyvinyl-pyrrolidone (PVP, 360 kDa, Sigma Aldrich) solutions were made at three different viscosities: 14.57, 20.11 and 30.51 mPa.s. For simplifying the comparison in the followings we write ‘15’, ‘20’ and ‘30’ mPa.s. The osmolarity of the PVP-PBS solutions was 295-327 mOsmol/kg, pH was 7.34-7.37.

Further sample preparation and the determination of red blood cell deformability occurred according to chapter 3.1.3.3.2.

3.3.2. The sensitivity investigation of ektacytometric measurements with laboratory animal heat treated red blood cells

3.3.2.1. Experimental animals and blood sampling

8 female, healthy Sprague-Dawley rats (334 ± 55.8 g) and 8 female, healthy beagle dogs (11.05 ± 1.41 kg) were subjected into the investigations.

In case of rats blood sampling was carried out under general anesthesia (sodium-thiopental 60 mg/kg, i.p., Thiopenthal®, Biocheme GmbH, Austria): after performing midline laparotomy the caudal caval vein was gently explored, and punctured using a 26 G needle and a connecting syringe that contained sodium-EDTA as anticoagulant (1.5 mg/ml). Approximately 1.5 ml blood was withdrawn. At the end of blood sampling, the animals were euthanized by exsanguination.

From beagle dogs blood sampling occurred via cephalic vein puncture, using vacuum tubes containing K_3-EDTA (7.5% 0.04 ml; BD Vacutainer®, 2-ml tube)

3.3.2.2. Sample preparation and heat treatment

After testing native blood samples, samples were centrifuged at 1000 g for 10 mins at 15 °C, then the red blood cells (RBC) were washed twice in normal PBS (700 g, 10 mins, 15 °C).

From the washed RBCs 10% Hct RBC-PBS suspensions were prepared. Each suspension was tested in the ektacytometer, then for heat treatment the suspensions were immersed in a temperature controlled water bath at 48 °C for 9 mins while the samples were gently mixed.

After heat treatment RBC-PBS suspensions were immediately cooled to room temperature (22 °C) then the samples were tested for red blood cell deformability.

3.3.2.3. Hematological and morphological investigations

Among the hematological parameters of native samples and RBC-PBS suspensions hematocrit (Hct [%]) and mean corpuscular volume (MCV [fl]) values were analyzed. For checking the cell morphology changes in RBC-PBS due to heat treatment samples were dripped on a degreased slide then covered with an escutcheon. A Nikon Eclipse E200 microscope was used for the investigation.

3.3.2.4. The determination of red blood cell deformability

The native samples and the normal and heat treated RBC-PBS suspensions were tested by ektacytometry according to chapter 3.1.3.3.2., parallelly in 15, 20 and 30 mPa.s viscosity PVP-PBS suspending solutions.

3.3.2.5. Statistical analysis

Data are presented as mean ± standard deviation (S.D.).

Differences between the usage of 15, 20 and 30 mPa.s media (like intra-group-like comparison) were evaluated by one-way ANOVA tests using Dunn's or Bonferroni's method.

The comparative male and female data were evaluated by Student's t-test or Mann-Whitney rank sum test, according to the normality of data distribution.

Biological variation was expressed as the coefficient of variation (CV%) of the data in male and female animals in both species.

Comparison of native samples versus RBC-PBS suspensions were tested by Student's t-test or Mann-Whitney rank sum test. Differences before versus after heat treatment were evaluated by paired t-test or Wilcoxon signed rank test, according to the normality of data distribution. A p value <0.05 was considered as statistically significant.

In case of the before and after heat treatment results the standardized difference was also calculated. The difference in red blood cell deformability between normal and heat treated, more rigid cells can be well detected.

The bigger this difference what the instrument measures the more sensitive is the instrument itself. The comparative parameter for this purpose is the *standardized difference*, calculated according to Cohen's method:

$$\text{standardized difference} = \text{average}_{\text{control}} - \text{average}_{\text{treated}} / \text{pooled S.D.}$$

The pooled standard deviation is calculated as the square root of the mean of squared standard deviations of the two groups being compared.

4. RESULTS AND DISCUSSION

4.1. Hematological and hemorheological analysis of gender differences in laboratory animals

4.1.1. Hematological parameters

White blood cell count (WBC [x10^3/μl]) showed significant gender difference in case of rats, being higher in males. In beagle dogs there was no obvious gender difference.

Platelet count (Plt [x10^3/μl]) was higher in females, which was significant in rats.

Red blood cell count (RBC [x10^6/μl]) showed significantly higher values in male animals of both races that resulted in higher hematocrit (Hct [%]) values, accordingly.

Hemoglobin concentration (Hgb [g/dl]) and mean corpuscular volume (MCV [fl]) did not differ significantly.

The mean corpuscular hemoglobin content (MCH [pg]) was significantly higher in both races female animals. Thus the mean corpuscular hemoglobin concentration (MCHC [g/dl]) values were only higher in female beagle dogs, showing significant gender differences.

4.1.2. Hemorheological parameters

4.1.2.1. Whole blood and plasma viscosity

In female rats whole blood viscosity (WBV [mPa.s]) values at shear rate of 90 s^{-1} were higher than in males but without significant difference. In beagle dogs, males showed slightly higher blood viscosity values.

Plasma viscosity (PV [mPa.s]) was significantly higher in female rats compared to males, which was accompanied by a significantly larger fibrinogen concentration (Fbg [g/dl]). In beagle dogs both plasma viscosity and fibrinogen concentration were similar, without any gender difference

4.1.2.2. Red blood cell deformability

Filtration parameters of red blood cell – PBS suspensions did not show significant differences between genders, however, in female animals of both races the relative cell transit time (RCTT) values were slightly lower, referring to a slightly better red blood cell deformability The difference of gender averages was 2.2% in CD rats and 8.7% in beagle dogs.

Using ektacytometer, the gender differences were more expressed. In CD rats the females showed better, i.e. higher elongation index (EI) values compared to males, while in beagle dogs the males had higher EI values.

By the parameters, which are appropriate for the comparison of EI-SS curves, in rats the EI values at 3 Pa were significantly higher in females, while EI_{max} did not differ. $SS_{1/2}$ [Pa] values were lower in females, however did not reach a statistically significant level.

In beagle dogs the situation was inverse: the male animals had better EI values, although being without statistically evidenced significant difference.

4.1.2.3. Red blood cell aggregation

In CD rats the females showed higher aggregation index values, giving pronounced gender differences (M 5 sec: 77.8%; M1 5 sec: 25%; M 10 sec: 87%; M1 10 sec: 36.3%) In case of beagle dogs higher aggregation index values were experienced in males. Although the gender differences were remarkable but it did not reach the rates which were measured in rats (M 5 sec: 39.5%; M1 5 sec: 41.7%; M 10 sec: 4%; M1 10 sec: 16.5%).

Concerning hemorheological gender differences in human several data can be found in the literature. The male blood has higher viscosity, hematocrit and fibrinogen concentration, the red blood cell aggregation indices are higher, the deformability is lower compared to same aged female blood.

Although growing number of data reveals hemorheological inter-species differences in experimental laboratory animals, the gender differences are poorly known and no organized comparative study can be found.

The magnitude of the alterations in the hemorheological gender differences did not correlate with each other in the comparison of the two races. Oppositely to human data the enhanced red blood cell aggregation was not associated with lower red blood cell deformability in males. A solid explanation for the alterations was not found, probably the complex differences of these two micro-rheological parameters determining plasmatic and cellular factors and their interactions can be in the background.

4.2. The effect of storage time and temperature on the hematological and hemorheological parameters of laboratory animal blood samples

4.2.1. Hematological parameters

Among the hemorheological parameters the mean cell volume (MCV [fl]) values of both races did not show significant changes during the first 6 hours at room temperature. After 24 hours the MCV increased in case of both races, by 25% in rats. The MCV values of beagle dogs were continuously increasing under the whole investigation time (24 hours: 9%, 48 hours: 19%, 72 hours: 28%).

Cooling prevented these increases in both races, although in dogs MCV showed a 5% increase by 72 hours.

4.2.2. Hemorheological parameters

4.2.2.1. Whole blood and plasma viscosity

An increase in the whole blood viscosity was experienced already at the 24^{th} hour of cooled storage. An opposite tendency change was noted in the plasma viscosity, which showed significant decrease at the 48^{th} hour of storage.

4.2.2.2. Red blood cell deformability

A continuous decrease was detected in the initial relative filtration rate (IRFR) values during storage, which was leading to significantly lower values at the 72th hour of storage compared to the base values.

The relative cell transit time (RCTT) values showed increase during storage, which was already significant at the 24th hour of storage.

Although the cooled storage the changes of these two parameters referred to impairing red blood cell deformability as time passed by.

The ektacytometricly determined red blood cell deformability in rat blood stored at room temperature was unaltered during the first 6 hours, then markedly decreased during storage. Changes at 24 hours were seen only at the highest investigated shear stress values (10, 20 Pa), while at 48 and 72 hours significant deformability impairment was experienced at all stress levels. Cooled storage could prevent any decrease of EI over the entire 72 hour period.

EI values of beagle dogs were also stable for 6 hours at room temperature. But unlike rat cells, dog RBC showed a slight increase of deformability at lower shear stress levels at 24 and 48 hours, while at the 72th hour of storage significant EI values were noticed at all investigated shear stress levels. Cooled storage could prevent the changes of EI values.

4.2.2.3. Red blood cell aggregation

Rat blood stored at room temperature exhibited a bi-phasic change of M and M1 measured in 10-sec mode with storage. Already after 2 hours a significant decrease, a 40-60% decrease in aggregation indices was experienced, which continued at 4 and at 6 hours. But after 24 hour the aggregation index values started to increase, approaching then reaching and in case of M1 parameter at 72 hours passing their base values. Interestingly, by storage at

4-8 °C the M and M1 values remained at a significantly lower level compared to the base values.

In beagle dog blood samples a gradual decrease in the aggregation indexes was noticed by storage time. Up to 6 hours no significant change was noticed neither in M nor in M1 parameter but at the 24th hour of storage marked aggregation index decrease was experienced. The cooling after 24 hours could slow down but was not able to prevent the aggregation index decrease.

According to our results the time and temperature of sample storage affect remarkably the micro-rheological parameters, in rat blood samples within a shorter period of time and in a larger way than in beagle dog blood samples.

For parts of the experienced micro-rheological changes can serve explanation the changes occurring in the red blood cell deformability determining factors, such as in the cell surface - cell volume ratio, the cell morphology, cell size. The MCV value increase in the room temperature stored samples could play a role also in the red blood cell deformability decrease.

Supposedly, in the background of red blood cell aggregation changes can be partly the plasma protein degradation, altered surface charge and glycocalyx changes, although the morphological changes can have a key role. The rat red blood cells are very sensitive to environmental changes. The presence of echinocyte forms results deformability decrease and furthermore these cell shapes are not or just hardly capable for aggregation. The sensitivity of rat blood cells against environmental changes leads to notable echinocyte-formation, therefore the aggregation index decrease is understandable. As storage time passing, when the cells are not able to maintain their volume due to hypoxia and energy depletion they start to swell and therefore the number of echinocytes decreases.

4.3. The effect of different viscosity suspending solutions on the sensitivity of laboratory animal red blood cell deformability measurement

4.3.1. Further ektacytometric investigations of gender differences

Both in Sprague-Dawley (SD) rats and dogs the elongation index values were the lowest in PVP solution at 15 mPa.s viscosity, and the highest in 30 mPa.s viscosity media. The difference between 15 and 20 mPa.s viscosity medias was near the same in magnitude as between 20 and 30 mPa.s viscosity solutions.

The widest variability was experienced in both races when measurements were completed in the 30 mPa.s viscosity solution.

In both species and both genders when using 15 and 20 mPa.s viscosity medias the EI values at shear stress of 3 Pa were significantly lower ($p<0.001$) compared to the usage of 30 mPa.s viscosity solution. Significant gender difference was found in rats using 20 and 30 mPa.s solutions ($p=0.027$ and $p=0.01$, respectively).

In case of 15 and 20 mPa.s viscosity medias significantly lower EI_{max} values were measured compared to the results gained with the 30 mPa.s viscosity solution. In dog blood samples the highest EI_{max} values were observable in 20 mPa.s viscosity media, which was significant in male animals compared to the 30 mPa.s viscosity solution ($p=0.007$). The $SS_{1/2}$ [Pa] values in 15 and 20 mPa.s viscosity solutions were significantly higher versus 30 mPa.s viscosity solution (SD rats: $p=0.001$ and $p=0.04$ in males, $p<0.001$ and $p=0.037$ in females; beagle dogs: $p<0.001$ and $p<0.001$ in males, $p<0.001$ and $p=0.004$ in females, respectively).

Significant gender difference was found only in rats using 20 and 30 mPa.s viscosity solutions ($p=0.017$ and $p=0.005$, respectively).

4.3.2. The sensitivity investigation of ektacytometric measurements with laboratory animal heat treated red blood cells

4.3.2.1. Hematological and morphological changes

In case of both animal races the red blood cells in the suspensions were mostly of intact morphology, only a few echinocytes were noted in the microscope. After the heat treatment the number of distorted shape cells and echinocytes was increased, parallelly with the decrease of normal discocytic morphology cell number.

4.3.2.2. Red blood cell deformability changes

In case of both races elongation index values were significantly lower in RBC-PBS suspensions compared to native, normal blood samples.

After heat treatment the EI values were significantly lower in every PVP solution. Also in this investigation process the lowest values were observed in the 15 mPa.s and the highest values in the 30 mPa.s viscosity solution.

In rats after heat treatment of samples the EI-SS curves were significantly distorted at shear stress of 1 and 2 Pa when 15 and 20 mPa.s viscosity solutions were used, while in the 30 mPa.s viscosity media the EI-SS curves were of regular shape at the lower shear stress levels, too.

The differences of the results before and after heat treatment were the largest when 30 mPa.s viscosity solution was used.

Although the lowest EI values were observed in 15 mPa.s viscosity solution, the standardized differences showed the highest variability in this one. Below shear stress of 1 Pa, the lowest values were seen in 15 and 20 mPa.s viscosity medias. The standardized difference increased till 3 Pa then tended to decrease. The values of 30 mPa.s viscosity suspension were relatively stable, and not moved in wide range as the other suspensions' values did. Over 5 Pa the values in 20 and 30 mPa.s viscosity suspensions were closely similar to each other.

In beagle dogs it was also observable that EI values in red blood cell-PBS suspensions were significantly lower versus the native blood samples ($p<0.001$).

The heat treatment caused significant decrease in all parameters, however, the magnitude of the changes was smaller than it was in rats. Differences in EI values at shear stress of 3 Pa showed the highest difference ($p<0.001$). EI_{max} increased, supposedly because of the altered slope of EI-SS curves. The increase in $SS_{½}$ was smaller in all PVP solutions than in rats, however, reached the significance level compared to the state before treatment ($p<0.001$).

Standardized difference values were much lower than in rats. Below shear stress of 2 Pa the values were the highest in the 30 mPa.s viscosity media, then at higher shear stress values data of all three medias were close to each other.

The standardized difference values referred that the rat red blood cells are much more sensitive against heat treatment than the beagle dog red blood cells.

The Rheoscan-D200 slit-flow ektacytometer is a modern hemorheological laboratory instrument for red blood cell deformability measurement, of which sensitivity can differ regarding race and gender differences.

The comparative investigation of ektacytometric measurement sensitivity was followed by the demonstrability of deformability impairment degree due to the heat treatment of red blood cells.

In both species red blood cell deformability values showed significant differences also in the comparison of native blood samples and red blood cell-PBS suspensions. Supposedly, the much lower hematocrit values of the red blood cell-PBS suspensions are in the background. Native blood samples had at least four- or fivefold higher mean hematocrit values (rats: 44.56%; dogs: 54.58%) than the red blood cell-PBS suspensions, which were prepared to 10% hematocrit value.

As it was expected, both in rats and dogs all sample showed significant decrease in the elongation index values after heat treatment. However, in rats the changes were more expressed.

In case of dog blood samples there was not as much expressed difference compared to the states before and after heat treatment as it was observed in rat blood samples. The shape of elongation index - shear stress curves remained regular and also the standardized difference values were much lower than in rats. Similarly to the rat blood samples, the highest standardized difference values in the low shear stress zone were insurable with the use of the 30 mPa.s viscosity PVP solution.

4.4. The utilisability of results in the field of experimental surgical research

There is no hemorheological measurement-technical guideline relative to animal experiments and hemorheological experimental surgical research so far, which would be similar to ones for human laboratory use.

The comparative investigations of the laboratory animal races' hemorheological and especially the micro-rheological parameters (red blood cell deformability, red blood cell aggregation) can supply data for better understanding the race and gender differences as well as for the establishment of blood sampling, sample handling, sample storage and sample preparation standards, considering the sensitivity of the actual measuring insturment.

Significant hemorheological inter-species and gender differences were found in CD rats and beagle dogs. The differences were mostly expressed in red blood cell deformability and aggregation. Considering the gender differences is important for planning studies to form homogenous, unisex experimental groups, since the gender differences may reach or even mask the degree of real differences between experimental groups.

The knowing of the significant hemorheological race and gender differences, what were found in the CD rat and beagle dog blood samples can serve the planning of experimental groups. It is recommended to form homogenous, unisex experimental groups or directly investigating the actual pathophysiological process by genders, since the results gained from different case-numbered, mixed-gender groups can mask or distort the real differences between the experimental groups.

The comparative data relative to blood sample handling and storage can increase the laboratory measurement security. The results obtained from inadequately handled or too lately tested samples can differ from the real values in a large degree. It is especially true for rat red blood cell aggregation values. Our comparative date can be useful to evaluate the effect of transport between laboratories, which can supply a standing-ground for the analysis of results.

Regarding the demonstrability of changes in ektacytometry the 30 mPa.s viscosity PVP solution is recommended to use. In case of using different viscosity solutions the fact must be taken under consideration that the results obtained from various viscosity suspending medias are not comparable to each other.

According to our results it can be concluded in reference to the blood samples of the investigated laboratory animal races that the changes originating from inadequate sample handling, sample preparation and measurement-technical circumstances as well as the race and gender differences can reach or even mask the degree of the investigated pathophysiological changes in the experimental surgical models. Therefore the comparative data can supply a standing-ground for research planning, hemorheological measurement execution and result analysis.

5. SUMMARY OF MAJOR RESULTS AND CONCLUSIONS

1. We pointed out that in CD rats females have better red blood cell deformability values while in beagle dogs males do. In contrast, red blood cell aggregation is enhanced in females in case of CD rats and in males in case of beagle dogs. This relation is controversial to the human data, since it is known in the literature that males have enhanced red blood cell aggregation with „worse" red blood cell deformability values.
2. We were the first describing that during sample storage the changes of micro-rheological parameters (red blood cell deformability and red blood cell aggregation) in CD rat and beagle dog blood samples show differences between the two laboratory animal races. Red blood cell deformability measurements are stable within 4-6 hours by storing at room temperature but the red blood cell aggregation of rat blood samples has to be possibly determined already within an hour.
3. We were the first who described in rat blood samples during sample storage the early, within-2-hour developing, 40-60% aggregation index decline and its past-24-hour paradox elevation. The phenomenon was neither detectable in beagle dog blood samples and it is nor known in human comparative investigations encountered in the literature.
4. We demonstrated that the differences in the red blood cell deformability values of Sprague-Dawley rat and beagle dog blood samples can be detected in a different degree when using 15, 20 and 30 mPa.s viscosity polyvinyl-pyrrolidone (PVP) solutions for the slit-flow ektacytometric measurements. Race and gender differences were obtained in the highest degree by using the 30 mPa.s viscosity solution.

5. We were the first noticing that the red blood cells of Sprague-Dawley rats and beagle dogs have different degree sensitivity against heat treatment (48 °C, 9 minutes). The differences were detected the most sensitively when the 30 mPa.s viscosity PVP solution was used.

According to our hopes, these results can conduce to the better understanding of race and gender hemorheological differences as well as to the establishment of blood sampling, sample handling, sample storage, sample preparation standards and to the development of the experimental animal hemorheological measurement-methodological guideline.

In extenso publications serving base for the thesis

1. Németh N, Baskurt OK, Meiselman HJ, **Kiss F**, Uyuklu M, Hevér T, Sajtos E, Kenyeres P, Tóth K, Furka I, Mikó I. Storage of laboratory animal blood samples causes hemorheological alterations: Inter-species differences and the effects of duration and temperature. *Korea-Aust Rheol J* 2009. **21.** 127-133. **IF: 0,965**
2. Németh N, **Kiss F,** Furka I, Mikó I. Gender differences of blood rheological parameters in laboratory animals. *Clin Hemorheol Microcirc* 2010. **45.** 263-272. **IF: 2,838**
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4. **Kiss F,** Sajtos E, Mátyás L, Magyar Zs, Furka I, Mikó I, Németh N. Testing red blood cell deformability of laboratory animals by slit-flow ektacytometry in various viscosity media: inter-species and gender differences. *Korea-Aust Rheol J* 2010. **22.** 113-118. **IF: 0,948**

In extenso publications connecting to the issue of the thesis

5. Németh N, Gulyás A, Bálint A, Pető K, Bráth E, **Kiss F**, Furka I, Baskurt OK, Mikó I. Measurement of erythrocyte deformability and methodological adaptation for small-animal microsurgical models. *Microsurgery* 2006. **26.** 33-37. **IF: 0,882**
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Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Kiss, F.**, Sajtos, E., Mátyás, L., Magyar, Z., Furka, I., Mikó, I., Németh, N.: Testing red blood cell deformability of laboratory animals by slit-flow ektacytometry in various viscosity media: Inter-species and gender differences.
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