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

Examining the dependence of red blood cell aggregation on hematocrit, and its changes during polycystic ovary syndrome

by Barbara Bedőcs-Baráth (MSc)

Supervisor: Ádám Deák (PhD)

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MEDICAL SCIENCES

Debrecen, 2023.

Examining the dependence of red blood cell aggregation on hematocrit, and its changes during polycystic ovary syndrome

by Barbara Bedőcs-Baráth, MSc

Supervisor: Ádám Deák, PhD

Doctoral School of Clinical Medicine, University of Debrecen

Head of the Defense Committee:

Prof. Zoltán Szekanecz, PhD, DSc

Reviewers:

Prof. Lajos Bogár, PhD, DSc

Prof. Róbert Póka, PhD, DSc

Members of the Defense Committee:

Dr. Éva Katona, PhD

Dr. György Nádasy, CSc

The PhD Defense takes place in the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

15th of January, 2024., 13:00

1. INTRODUCTION

Hemorheology, as Alfred Levin Copley, an American hematologist defined it in 1951: *"The summary designation of the flow theory of the cellular and plasmatic components of the blood in macro- and microscopic dimensions, as well as the rheology of the vessel wall in contact with the blood."*. In recent decades, hemorheological research has become more and more widespread, so we can get information about the effect of more and more pathological processes on blood flow, especially on red blood cells, thanks to the wide range of measurement methods that can be used, such as different measurement methods based on light transmission and light reflectance, to ektacytometry, microscopic and filtration techniques.

Hemorheological parameters such as whole blood and plasma viscosity, red blood cell deformability, and aggregation are influenced by many factors. These influencing factors are already well known, but we do not yet fully understand the effect of the parameters on each other. Previous studies have shown that the relationship between hematocrit and whole blood viscosity is not linear, nor is the aggregation-hematocrit relationship. They also described that the hematocrit-viscosity relationship, the mechanical trauma caused by centrifugation, as well as aggregation, deformability, and cell membrane stability also show interspecies differences.

During many pathological processes, we experience changes in hemorheological parameters. Mainly in diseases affecting the cardiovascular system, such as high blood pressure, and myocardial infarction, but also in the case of metabolic changes, such as type II diabetes mellitus or insulin resistance. Polycystic ovary syndrome (PCOS) is a very common disease among women of reproductive age. Although cardiovascular and metabolic changes are common in PCOS, and hemorheological parameters also show gender differences, the number of scientific publications describing hemorheological changes occurring in polycystic ovary syndrome is not significant.

One part of our studies was aimed at exploring the differences in the aggregation-hematocrit relationship between species (human, rat, dog, pig), while in the other part, we wanted to investigate the hemorheological effects of polycystic ovary syndrome in a rat model.

2. AIMS OF THE STUDY

1. We aimed to compare the aggregation of each species (human, rat, dog, and pig) measured at physiological hematocrit.

2. Examining the issue further, our goal was to compare aggregation parameters (static and kinetic) that can be determined using different measurement methods using blood samples set to different hematocrit values and to explore their differences between species.

3. The question arose as to which parameters could be useful for determining the "optimal" hematocrit range for aggregation, and whether these values show diversity between species.

4. Our goal was to set up a PCOS rat model that can be used for hemorheological studies.

5. Carrying out a comprehensive hematological and hemorheological examination of the adjusted model, specifically examining the effect of PCOS on red blood cell aggregation.

3. MATERIALS AND METHODS

3.1. Determination of red blood cell aggregation in red blood cell-autologous plasma suspensions adjusted to different hematocrit values, concerning four species

3.1.1. Experimental animals and human blood sampling

The animal experiment was registered by the University of Debrecen Committee of Animal Welfare (UDCAW), taking into account the EU Directive on animal experiments (EU Directive 63/2010) and the Hungarian Animal Protection Act (Law XXVIII of 1998 "On the Protection and Welfare of Animals") (permission registration numbers: 13/2014/UDCAW; 7/2014/UDCAW; 24/2016/UDCAW). The human blood samples were taken from volunteers, and our measurements were performed with the permission of the Regional Research Ethics Committee of the University of Debrecen (permission registration number: DE-RKEB 3189-2010).

Blood samples were taken from 6 male Wistar rats (body weight: 353±80 g; age: 6 months; anesthesia: 60 mg/kg thiopental [Tiobarbital Braun 1g; B. Braun, Melsungen, Germany], intraperitoneally; sampling site: vena cava caudalis); 6 male Beagle dogs (body weight: 18.05±2.05 kg; age: 3 years; anesthesia: 10 mg/kg ketamine [CP-Ketamine hydrochloride 10%, Produlab Pharma BV, The Netherlands] + 1 mg/kg xylazine [CP-Xylazine hydrochloride, 2%; Produlab Pharma BV, The Netherlands] + 0.25 mg/kg diazepam (Seduxen, 0.5%, Richter Gedeon Nyrt., intramuscularly; sampling site: vena cephalica); and 7 Hungahib-39 female pigs (body weight: 15 .26±1.02 kg; age: 3 motnhs; anesthesia: 15 mg/kg ketamine + 1 mg/kg xylazine, intramuscularly; sampling site: saphenous vein) into blood collection vacuum tubes containing K3-EDTA (1.8 mg/mL; BD Vacutainer ® Becton, Dickinson and Company, USA).

The human blood specimens were provided by 7 healthy male volunteers (average age: 29±6 years). Sample collection was performed by punction of the median cubital vein, using a needle with a diameter of 21G, into vacuum tubes containing K3-EDTA.

Laboratory measurements were performed within 4 hours.

3.1.2. Sample preparation

Samples were centrifuged (3000 rpm, 10 min), the plasma was set aside, and the "buffy coat" was removed. Afterward, the red blood cell suspension was diluted 1:1 with phosphate-buffered saline (PBS) solution. It was centrifuged again (3000 rpm, 10 min), then the supernatant was removed and this washing step was repeated once again.

We determined the hematocrit value of the red blood cell suspension (Sysmex F-800 and Sysmex K-4500, TOA Medical Electronics Corp. Ltd., Japan) and prepared our 20, 40, and 60% red blood cell-autologous plasma suspensions.

3.1.3. Determination of Red Blood Cell aggregation

3.1.3.1. Determination of red blood cell aggregation based on the light transmission

Among the measuring methods based on light transmission, we used the Myrenne MA-1 aggregometer (Myrenne GmbH, Germany). For one measurement, 20 uL of anticoagulated blood is required, which is applied to a glass lens polished at an angle of 2°. After closing the lid of the instrument, a glass plate spreads the sample over the glass lens, then generates a 500 s^{-1} shear stress with the rotation of the lens, which disaggregates any aggregates that may be present in the sample. Then the device can define four aggregation index parameters, these parameters are M 5sec, M1 5sec, M 10sec, and M1 10sec. In the case of M 5 sec and M 10sec, after the disaggregation, the lens stops completely (stasis) and measures the intensity of the transmitted light in the 5th and 10th seconds of the aggregation. In the case of M1 5sec and

M1 10sec parameters, the movement of the lens does not stop completely, it just slows down (3 s^{-1}). The intensity of the transmitted light is determined by the infrared detector located under the lens. The light source is in the lid of the device, behind the glass plate. The device is not temperature controlled, the measurements were carried out at room temperature (22-25 °C). In the case of each parameter, 4 measurements were made, and we worked with the average of them.

3.1.3.2.Determination of red blood cell aggregation based on the light reflection

We performed our measurements based on light reflectance with the LoRRca MaxSis Osmoscan ektacytometer (Mechatronics BV, The Netherlands). Unlike the Myrenne MA-1 aggregometer, 700-1000 uL of anticoagulated whole blood is required for the measurement. The device measures in a Couette system, which consists of a static (bob) and a rotatable cylinder (cup). The blood sample is pipetted into the about 0.3 mm gap between the two cylinders. The first phase of the measurement is also disaggregation, which is achieved with the help of a speed gradient (600 s^{-1}) by rotating the outer cylinder, and then the program records the changes in the intensity of the reflected light for 120 seconds. Both the light source (670 nm red laser) and the detector are built into the bob.

The LoRRca provides information not only on the early, fast phase of aggregation (rouleaux formation, 5-15 seconds) but also on the entire aggregation process. The device calculates several parameters from the obtained data during the measurements, these are the aggregation index (AI, [%]), the amplitude (Amp, [au]), and the syllectogram half-life (t1/2, [s]).

3.1.4. Statistical analysis

Statistical analyzes were performed using the SigmaStat program (Systat Software Inc., San Jose, California, USA). Depending on the distribution of the data, we used Student's t-test, Mann-Whitney, and ANOVA tests. Values of $p<0.05$ were considered statistically significant.

3.2. Effect of polycystic ovary syndrome on micro-rheological parameters

3.2.1. Experimental animals, treatment, and sampling protocol

Owning the necessary permissions (permission registration number: 17/2019/UDCAW), 18 Wistar, 3 months old, female rats were included in the research. The animals were divided randomly into two groups (n=9/group), namely the control group (body weight: 291.6±20.6g) and the PCOS group (body weight: 264.5±25.1g). The control group did not get any treatment. The PCOS group, received 4 mg/animal of estradiol valerate (Estradiol valerate, Y0000046, Sigma-Aldrich, St. Louis, Missouri, USA) solved in sesame oil (0.5 mL; Sesame oil, S3547, Sigma- Aldrich, St. Louis, Missouri, USA), by subcutaneous injection. Blood specimens were taken before the treatment and monthly thereafter, by puncture of the lateral tail vein, using a 26G cannula (Becton, Dickinson and Company, USA), into tubes containing K3-EDTA, under anesthesia, with a mixture of ketamine-xylazine-atropine (100 mg/kg, CP-Ketamine hydrochloride 10%, Produlab Pharma BV, The Netherlands; 10 mg/kg, CP-Xylazine hydrochloride, 2%; Produlab Pharma BV, The Netherlands 0.05 mg/kg, atropinum sulfuricum 0.1%, Egis Pharmaceuticals PLC, Budapest, Hungary).

3.2.2. Determination of the estrous cycle

The estrous cycle of the animals was checked by microscopic investigation of vaginal smears. Before the start of the study, and then monthly, on 5 consecutive days, samples were taken from the animals using the lavage technique, with 250-500 uL of distilled water and a

filtered pipette tip. The smears were dried overnight at room temperature and then stained according to a modified Giemsa protocol. The smears were fixed in methanol (Methanol Technical, 20903, VWR International, USA) for half a minute, then stained with Giemsa (PanReac AppliChem Giemsa's Azur-Eosin-Methylene Blue solution, 251338, ITW Reagents) for 1 minute, washed with distilled water and overnight dried at room temperature. The evaluation was done with a light microscope (Nikon Eclipse E200, Nikon Europe BV, The Netherlands).

3.2.3. Laboratory measurements

3.2.3.1. Determination of body weight and blood sugar level

The animals' body weight and blood glucose levels were checked weekly (AccuCheck Active blood sugar meter, Roche Diabetes Care GmbH, Germany). On the 120th day of the study, an oral glucose tolerance test (OGTT) was performed, as follows: after fasting for 12 hours, 2 g/kg glucose was solved in 1.5 ml of distilled water and fed to the animals using the gavage technique. Blood glucose levels (mmol/L) were measured at 6 different times (before and 15, 30, 60, 90, and 120 minutes after glucose administration).

3.2.3.2. Determination of hematological parameters

Hematological parameters were measured with the Sysmex K-4500 microcell counter. The device determines the white blood cell count (Fvs, [$x10^9$/L]), red blood cell count (Vvs, [$x10^{12}$/L]), and platelet count (Thr, [$x10^9$/L]) based on the aperture-impedance, while hemoglobin is measured by spectrophotometry (Hb, [g/dL]). From these measured data, calculates the hematocrit (Hct, [%]), mean cell volume (MCV, [fL]), mean cell hemoglobin (MCH, [pg]), and mean cell hemoglobin concentration (MCHC, [g /dL]) values. 80 uL of anticoagulated blood is required for the measurements.

3.2.3.3. Determination of hemorheological parameters

The red blood cell aggregation measurements were performed with the Myrenne MA-1 aggregometer described in 3.1.3.1. chapter.

Red blood cell deformability was measured using the LoRRca MaxSis Osmoscan ektacytometer. 10 uL of whole blood is suspended in 2 mL of PVP solution (Polyvinyl-pyrrolidone: 360 kDa Sigma-Aldrich, St. Louis, Missouri, USA, pH=7.0-7.2; viscosity= 25.2-28.0 mPas; osmolality: 294-295 mOsm/kg). The suspension is pipetted into the Couette system described in chapter 3.1.3.2., and then a shear stress of 0.3-30 Pa is applied to the sample by rotating the cup. During the measurement, the device illuminates the sample with a 670 nm laser, and the resulting diffraction patterns are recorded by a CCD camera. From the width (W) and length (L) of the diffraction images, the software calculates the elongation index (EI), which is a dimensionless value describing the deformability, using the formula (L-W) / (L+W). During the deformability measurement, the obtained EI values are plotted as a function of the applied shear stress (SS, [Pa]). By the parametrization of the obtained curve, several other parameters can be determined, such as the maximum elongation index (EI_{max}), the shear stress corresponding to half of the EI_{max} ($SS_{1/2}$, [Pa]), and the ratio of these two parameters ($EI_{max}/SS_{1/2}$). For parameterization, we use the Linewaver-Burke equation, which is $1/EI= SS_{1/2}/ EI_{max} \times 1/ SS+1/ EI_{max}$.

3.2.3.4. Hormone level mesurements

To confirm the development of PCOS, hormone measurements were performed using the ELISA (enzyme-linked immunosorbent assay) method (LH, FSH, testosterone, IBL International GmbH, Germany). We used a solid-phase sandwich ELISA to determine the LH and FSH levels, where anti-LH and anti-FSH antibodies are found on the surface of the 96-well microplate. Samples were incubated with antibodies conjugated with horseradish peroxidase

enzyme for 30 min, then unbound conjugates were washed off and substrate was added to the reaction. Testosterone measurements were performed using the competitive ELISA method. Samples with an unknown concentration of testosterone and a specific amount of enzyme-labeled antigen "competed" for the binding sites of the antibodies on the surface of the wells. After one hour of incubation, the plate was washed to stop the reaction, and the substrate was added. At the end of both methods, the color intensity was measured using an ELISA microplate reader (Tecan Infinite M200, Tecan Trading AG, Switzerland), and then the hormone concentration of each sample was calculated using our standard curve.

3.2.4. Histological examination

At the end of the experiment, a histological examination was performed on the ovaries. The animals were euthanized after administration of a triple dose of anesthetic (300 mg/kg body weight, ketamine hydrochloride 10%; 30 mg/kg body weight, xylazine hydrochloride). The ovaries were fixed in 10% neutral formaldehyde (Leica Biosystems Inc., Leider Lane Buffalo Grove, IL, USA) and then embedded in paraffin. 6 μm sections were prepared and stained with hematoxylin-eosin staining (Sigma-Aldrich, St. Louis, Missouri, USA). The number of follicles, corpora lutea, and cystic follicles in the ovaries of each rat was determined under 10x magnification.

3.2.5. Statistical analysis

Statistical analyzes were performed using the SigmaStat program (Systat Software Inc., San Jose, California, USA). Depending on the distribution of the data, paired t-test, and Wilcoxon test were used within groups, and Student's t-test and Mann-Whitney test were used for comparison between groups. Values of $p<0.05$ were considered statistically significant.

4. RESULTS

4.1. Determination of red blood cell aggregation in red blood cell-autologous plasma suspensions adjusted to different hematocrit values, concerning four species

4.1.1. Hematological parameters

It can be concluded that the adjustment of the hematocrit values of the prepared suspensions was successful. Red blood cell size and density did not change significantly, except for pig samples at 40% hematocrit, where MCH, and 60% hematocrit, where MCHC changed.

4.1.2. Red Blood Cell aggregation

4.1.2.1. Determination of red blood cell aggregation based on the light transmission

Among the four index parameters determined with the Myrenne MA-1 aggregometer, we focused on the M 5s parameter measured at 5 seconds in stasis, as we saw the most significant changes in this parameter. The M 5s parameter showed significant interspecies differences for native hematocrit (human vs. rat: $p < 0.0001$; human vs. pig: $p < 0.0001$; dog vs. rat: $p = 0.005$; dog vs. pig : $p = 0.0164$). The differences were also significant in red blood cell-autologous plasma suspensions at 20% hematocrit (human vs. pig: $p = 0.0187$), at 40% hematocrit (human vs. pig: $p = 0.0006$; human vs. rat: $p < 0.0001$; pig vs. rat: $p = 0.0251$; dog vs. rat: $p < 0.0001$) and in suspensions adjusted to 60% hematocrit (human vs. rat: $p < 0.0001$; human vs. dog: $p = 0.0002$; rat vs. pig: $p = 0.02$).

The aggregation index values were plotted as a function of the hematocrit, and the hematocrit values corresponding to the peak of each index parameter were obtained by deriving the curves with the highest regression coefficient fitted. This turned out to be 43.95% for humans, 42.48% for dogs, and 47.63% for pigs. In the case of rat blood samples, we could only calculate the maximum point with the M1 10s, which was at a hematocrit of 44.44%.

4.1.2.2. Determination of red blood cell aggregation based on the light reflection

The aggregation index (AI [%)] measured by the reflectometric method showed significant differences between each species, native hematocrit (human vs. dog: $p = 0.0351$; pig vs. rat: $p = 0.0207$; pig vs. dog $p < 0.0001$), and in autologous red blood cell plasma suspensions at 20% hematocrit (human vs. dog: $p = 0.0006$; rat vs. dog: $p < 0.0001$; dog vs. pig: $p = 0.0274$), at 40% hematocrit (human vs. pig: $p = 0.0039$); human vs rat: $p < 0.0001$; pig vs. rat: $p = 0.0081$; pig vs. dog; $p = 0.0371$; dog vs. rat: $p < 0.0001$), and for the 60% suspensions (human vs. pig: $p = 0.0002$; human vs. rat: $p < 0.0001$; dog vs. rat: $p = 0.0032$). Only in the case of rat data, we could calculate the hematocrit value corresponding to the maximum of aggregation: 43.3% ($y = –0.0835x^2 + 7.2315×–70.481$). For the other species, the curves did not give a maximum point for this parameter.

Amplitude (amp [au]) of blood samples with native hematocrit was significantly different between species (human vs. rat: $p <0.0001$; human vs. dog: $p <0.0001$; human vs. pig: $p = 0.0096$; dog vs. rat $p <0.0001$; vs. pig $p <0.0001$; rat vs. pig $p = 0.0423$). The values of the rat, dog, and pig red blood cell - autologous plasma suspensions at 20% hematocrit differed significantly from the human samples ($p <0.0001$, $p = 0.0024$, and $p <0.0001$). In the case of 40% suspensions, we saw strong significant differences in all possible combinations ($p <0.0001$). 60% suspensions were significantly different between human and dog ($p < 0.0001$), human and rat ($p = 0.0101$), rat and dog ($p < 0.0001$), and rat and pig ($p = 0 .0001$).

In the case of the total intensity change, the hematocrit value corresponding to the previously mentioned peak was calculated for each species: 40.86% in humans ($y = –0.0287x^2 + 2.2881 × – 23.641$), 39.37% in rats ($y = –0.0027x^2 + – 0.212$). 0.0183), 44.29% in dogs ($y = –0.0233x^2 + 2.064 × – 25.884$) and 52.8% in pigs ($y = –0.0056x^2 + 0.5921×– 4.9259$).

In the values of the syllectogram half-life ($t_{1/2}$ [s]) of the native hematocrit samples, we also showed significant differences between the investigated species (human vs. dog: $p = 0.0025$; dog vs. rat: $p = 0.004$; dog vs. pig: $p < 0.0001$). At 20% hematocrit, we found a significant difference between rat and human ($p = 0.035$), rat and dog ($p = 0.0002$), and rat vs. pig ($p = 0.0001$); In our 40% samples, human vs. pig ($p < 0.0001$), human vs rat ($p < 0.0001$), rat vs dog ($p = 0.0015$) and dog vs pig ($p = 0.0268$); At 60% hematocrit, human vs. pig ($p = 0.0001$), human vs. dog ($p < 0.0001$), human vs. rat ($p < 0.0001$), rat vs. dog ($p = 0.001$) and dog vs. pig ($p = 0.0275$) for comparisons. In human, dog, and pig samples, t1/2 increased with the hematocrit value, and the aggregation process slowed down. For dog and pig sample data, the maximum points of the fitted curves were identified as previously described: 54.21% for dogs ($y = –0.0051x^2 + 0.5529 \times – 6.5992$) and 58.5% for pigs ($y = –0, 0004x^2 + x\ 0.0004x^2 +$ 0.0. 1.43). In the rat samples, we were able to count a minimum point, which is 46.15% ($y = 0.0234x^2 – 2.1889x + 51.847$).

4.2. Effect of polycystic ovary syndrome on micro-rheological parameters

4.2.1. Determination of the estrous cycle

Examining the vaginal smears, we found that the estrous cycle of the PCOS group stopped from the 90th day of the experiment in the pro-oestrus (77.78%) and met-oestrus (22.22%) phases and did not normalize, while the estrous cycle of the controls remained normal during the experiment.

4.2.2. Laboratory measurements

4.2.2.1. Body weight and blood sugar level

When examining the relative values, the body weight of the control animals increased significantly compared to the baseline data ($p=0.017$) for the 4th month. In the PCOS group, we found an increase in absolute (120th day vs. base $p=0.003$) and relative (120th day vs. base $p=0.03$) values.

We did not observe any changes in the animals' behavior. Their fur was tidy, their eyes were clear and no porphyrinic discoloration was visible. We did not see any difference in food and water consumption between the two groups.

The blood sugar level in the PCOS group increased continuously, and by the 4th month, it was significantly higher than the initial value ($p=0.021$).

During the OGTT, the blood sugar level of both the control group and the PCOS group showed a significant increase 15 minutes after the administration of glucose compared to the baseline data. In the PCOS group, the blood sugar level increased until the 30th minute, after which we observed a sharp decrease. The values of the control group decreased after the peak measured at 15 minutes. In the case of both groups, we obtained significantly higher results at all times than in the baseline measurements, however, there was no statistically significant difference between the two groups.

4.2.2.2. Hematological parameters

In the PCOS group, red blood cell count (day 120 vs. baseline $p=0.015$, day 120 vs. control $p<0.001$), hematocrit (day 120 vs. baseline $p=0.004$, day 120 vs. control $p=0.004$), and hemoglobin values (day 60 vs. baseline $p<0.001$, day 60 vs. control $p<0.001$, day 120 vs. control $p<0.001$) showed a significant decrease compared to baseline values and the control group. Furthermore, in the PCOS group, the number of white blood cells decreased significantly (day

120 vs. baseline p=0.03, day 90 vs. control p=0.041), and the platelet count (day 120 vs. baseline p<0.001) and MCV (day 90 vs. control p=0.006, day 30 vs. baseline p=0.015, vs. control p= 0.017) significantly increased. In the MCH values (day 90 vs. base p<0.001, vs. control p=0.006, day 120 vs. base p=0.002; day 30 vs. control p=0.02), a significant increase was observed in the PCOS group after the initial decrease. There was a continuous increase in MCHC values (day 60 vs. baseline p<0.001, day 90 vs. baseline p=0.003, day 120 vs. baseline p=0.001).

4.2.2.3. Hemorheological parameters

The elongation index (EI 3Pa) values measured at 3 Pa increased on the 60th (vs. baseline p=0.047) and 90th day (vs. baseline p<0.001) in the PCOS group. In the case of EI_{max}, we observed a significant increase in the PCOS group (day 120 vs. baseline p=0.035). $SS_{1/2}$ values were significantly reduced on day 90 (vs. base p<0.001, vs. control p<0.001) and day 120 (vs. base p<0.001). On day 90 (vs. base p<0.001) and day 120 (vs. base p<0.001) the $EI_{max}/SS_{1/2}$ ratio was significantly increased.

In the M 5s (120th day vs. base p<0.001, 120th day vs. control p=0.004) and M1 5s index parameters (120th day vs. base p<0.001, 120th day vs. control p=0.015) a significant, continuous increase was observed in the PCOS group. There was also an increase in the parameters M 10s (120th day vs. base p=0.005) and M1 10s (120th day vs. base p<0.001) in the first two months, but after that, the values of these parameters decreased, and the two there was no longer a statistically significant difference between the groups.

4.2.2.4. Hormone levels

In FSH levels no significant difference was found in either group, either compared to their baseline values or each other's values. LH values increased significantly in the PCOS

group on day 120 compared to baseline values (p=0.036). Testosterone levels increased in both groups.

4.2.2.5. Histological examinations

In the case of the PCOS group, the ovary and uterus showed macroscopic abnormalities: large ovaries with cystic follicles visible even through the surface layer, and the uterus was enlarged and edematous.

The histological examination of the ovaries showed polycystic lesions in the PCOS group, while the control group had normal ovarian structures. In the PCOS group, there were many cystic follicles, the number of corpora lutea decreased, and the number of healthy follicles decreased significantly. More primary, secondary, and antral follicles were seen in the ovaries of the control group.

5. DISCUSSION

5.1. Determination of red blood cell aggregation in red blood cell-autologous plasma suspensions adjusted to different hematocrit values, concerning four species

The exact process and mechanism of red blood cell aggregation are not yet fully understood, but the influencing factors at the plasmatic and cellular levels are well known. Plasma proteins such as fibrinogen, immunoglobulins, or transferrin can be classified as the main influencing factors. Fibrinogen is considered the most important plasma protein during red blood cell aggregation, it is a positive acute phase protein, so its level increases during inflammation or cardiovascular diseases, which explains the increase in aggregation in inflammatory processes. The degree of aggregation is determined by the forces of aggregation (supporting aggregation) and disaggregation (acting against aggregation). The latter include shear stress, the surface charge of red blood cells, and membrane properties such as glycocalyx composition and degree of rigidity. The shape, size, and last but not least the number of cells (hematocrit) play a significant role in the process.

Among the hemorheological parameters, aggregation shows the greatest interspecies diversity. There are many factors behind this. These include the differences in shape and size of the red blood cells of different species, the characteristics of fibrinogen binding, the hemodynamic and vascular geometric properties characteristic of the given animal species, their nutrition, lifestyle, and the differences in the glycocalyx composition of the red blood cells. We also find differences in the normal fibrinogen level of each species (human: 150-300 mg/dl, CD rat: 128-153 mg/dl; beagle dog: 177-229 mg/dL; pig: 213-273 mg/dl). These differences can also cause the diversity of aggregation since the level of fibrinogen affects the degree of aggregation. Windberger et al. (2003) established "reference ranges" for the values of the aggregation index measured by the Myrenne aggregometer in stasis, examining blood samples of different species adjusted to a hematocrit value of 40%. It is 0.3–1.2 in rats; in dogs 2.7–4;

for pigs 2.2-4.6. The average of the aggregation index values obtained for our 40% suspensions fell within these intervals in all cases.

Nutrition also plays an important role in the values of hemorheological parameters. High cholesterol not only has a direct effect on the cardiovascular system but can also increase whole blood viscosity as an indirect effect. Viscosity has an exponential relationship with the hematocrit value. The hematocrit/viscosity ratio varies between species, this ratio increases with hematocrit, but after an "optimal" hematocrit value (which is the hematocrit value of maximum oxygen carrying capacity) there is a decrease. As a result of the increased viscosity of whole blood, the hematocrit/viscosity ratio decreases, thereby decreasing the blood's ability to carry oxygen. In samples with native hematocrit, the „optimal hematocrit" value is higher in rats than in dog samples. This "optimal" value depends on the applied shear stress. At the most frequently applied shear stress (90 s–1), the maximum of hematocrit/viscosity ratio is at 36.5% Hct in rats and 39.7% in dogs.

Previous studies have found that hematocrit significantly affects aggregation, but this relationship is not linear. In the M 5 s index parameter obtained with the Myrenne MA-1 aggregometer, we observed a decrease in the tested samples with a hematocrit of 20% compared to whole blood for all species. A kind of "peak" was observed in the human, dog, and pig data, followed by a decrease in the aggregation index. Examining human samples, Deng et al. (1994) found this peak around 40-42% hematocrit, in our study this calculated peak value for the M5s index parameter in humans, dogs, and pigs was nearly 40% hematocrit. In our rat samples, this peak was observed only at the M1 10 s parameter. This suggests that rat aggregation takes place in the first 5-10 seconds of the process.

The aforementioned is also confirmed by the aggregation index values measured with the LoRRca device, as this parameter represents the change in light intensity occurring in the first 10 seconds of the aggregation process, and only rat blood showed the "peak" (43.3%) for

this parameter. In suspensions with a hematocrit of 20%, the values of the dog samples were significantly higher, in 40-60% the results of the rat and human samples were significantly different from the other species.

During measurements based on light reflectance, the human, dog, and pig samples did not show the above-mentioned "peak". In the case of these, the aggregation indices of the 20% suspensions were higher than those of the 40% samples. In addition, t1/2 increased continuously, and the aggregation process slowed down as the hematocrit increased. The largest increase was found in human samples, from which it can be concluded that the aggregation ability of human red blood cells is the most sensitive to changes in hematocrit among the examined species.

The "optimal" hematocrit values described by us are not the same as the optimal value of the Hct/viscosity ratio. There is not only a difference in meaning, since the optimum of the Hct/viscosity ratio is the point of the curve where the highest possible hematocrit value is combined with the lowest possible viscosity value at a given speed gradient, while the peak described in our study indicates the peak of aggregation; but there are also value differences. The previously mentioned Hct values for maximum aggregation in rats and dogs are higher for all parameters than the optimal values of the Hct/viscosity ratio described in previous studies. Increased aggregation promotes the formation of plasma skimming in the capillaries, which means that the number of red blood cells in the vessel lumen decreases. As a result, the blood supply to the tissues decreases, thereby decreasing their oxygen delivery. This may be the reason why the maximum oxygen transport capacity Hct value is lower than the aggregation maximum Hct value. This suggests that adequate blood supply to the tissues can only be ensured if the red blood cell aggregation does not reach the maximum level. Excessive aggregation can impair oxygenation.

Previous studies have shown that the $t_{1/2}$ of rats is five times lower than that of pigs and ten times lower than that of dogs. In our study, the $t_{1/2}$ values of the native hematocrit samples of dogs were also significantly higher than in the case of human, rat, and pig blood samples (dog > rat > pig > human). In the case of a normal, native hematocrit, dog red blood cells aggregate the slowest, and human blood cells the fastest. The average of the values measured with native hematocrit of dogs is significantly higher than that of other species, and the AI values of dogs are the lowest. This phenomenon can be explained by the non-linear relationship between hematocrit and aggregation.

The acceleration of the aggregation process in our rat samples was observed in the middle Hct range. The syllectogram half-life values decreased, as described by Deng et al. (1994). At the same time, the AI belonging to the minimum of $t_{1/2}$ was the highest, so a significant part of the rat's red blood cell aggregation takes place in the first 10 seconds of the process. The peculiarities of the different measurement methods (methods based on light transmission and light reflectance, static-kinetic parameters) can explain these differences.

Regarding the amplitude, Deng et al. found the highest values in 40% samples. Our study clearly shows that the greatest change in light intensity is in the 40-50% Hct range, however, this varies from species to species. The lowest values were measured in rats, followed by pig, human, and dog samples. It follows that rat blood aggregates the least, as previous comparative studies have shown. In the case of whole blood, the lowest Amp values were found in rats, which was also confirmed by our study.

The limiting factors of our study are the small number of cases and methodological features (e.g. sample preparation, measurement methods, instrument specifications). However, these data can have additional informational value, and this allows us to better explore the hemorheological differences between species and the aggregation properties of red blood cells with varying hematocrit values.

5.2. Effect of polycystic ovary syndrome on micro-rheological parameters

The etiology of polycystic ovary syndrome (PCOS) is not yet fully understood, but it is well-known that PCOS is one of the most common endocrinological diseases of fertile women. PCOS has many effects on blood circulation, it increases plasma viscosity, which is a macro-rheological parameter, thereby affecting micro-rheological parameters. In addition, PCOS is characterized by increased testosterone, luteinizing hormone (LH), fasting insulin levels, and higher triglyceride and fibrinogen concentrations. Fibrinogen level shows a positive correlation with plasma viscosity. Increased plasma viscosity increases whole blood viscosity and can impair blood flow. Simmonds et al described increased red blood cell aggregation, whole blood and plasma viscosity, decreased hematocrit, and unchanged red blood cell deformability in women with PCOS compared with age-matched healthy controls.

The main therapeutic goals are the restoration of the regularity of the menstrual cycle, the treatment of infertility, and the stabilization of carbohydrate balance. Infertility treatments include progesterone therapy and clomiphene citrate. In the case of insulin resistance and type II diabetes, metformin therapy is most often used. In recent years, various inositol derivatives, which play a key role in insulin signaling processes as second messengers, have become common drugs in the treatment of insulin resistance. In addition, different inositol isoforms affect ovulation and thus may help in the treatment of infertility.

There are conflicting data on the effects of estradiol valerate (EV) on glucose metabolism in rats. The studies reported elevated fasting blood sugar levels or found no difference between the treated and control groups. Dănăsea et al. described elevated fasting glucose levels, but no difference was detected during an oral glucose tolerance test. In our experiment, we experienced a continuously rising fasting blood sugar level in the PCOS group,

however, no significant difference was found between the two groups during the OGTT measurements.

To induce PCOS, the animals received a single dose of 4 mg/animal of estradiol valerate in 0.5 mL of sesame oil, in the form of a subcutaneous injection. Estradiol valerate is a long-acting form of estrogen that causes gonadotropin-releasing hormone (GnRH) dysregulation in the hypothalamic-pituitary axis, resulting in insufficient release and/or storage of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the EV-induced model, the effect on the testosterone level is dose-dependent, while the testosterone level increases with the use of 2 mg EV, the free testosterone level mostly decreases with the administration of 4 mg EV, although elevated testosterone levels have been reported with the latter dose as well. In our study, the testosterone level was elevated in both groups, but there was no significant difference between the time points within the group, nor between the control and PCOS groups. We also found conflicting data regarding the effect of EV treatment on LH levels. Several studies have reported increased LH levels in the EV-induced rat model, while others have found decreased LH levels. In estrogen-induced models, FSH levels were generally reduced or no changes were found. During our study, LH levels increased in the PCOS group, while no difference was found in FSH levels in either group.

As a result of EV treatment, the estrous cycle of rats is disrupted and stops and amenorrhea occurs in animals. The human menstrual cycle has two phases: the follicular phase and the luteal phase. The follicular phase begins on the first day of menstruation and lasts until ovulation (1-14 days); the luteal phase begins after ovulation and ends 14 days after (15-28 days). In contrast, the sexual cycle of rats has four phases: pro-estrous, estrous, met-estrous, and di-estrous. In the different phases, different cell types predominate in the vaginal smears, and based on this, it can be determined which phase the examined animal is in at a given moment. Lara et al. and Ramadoss et al. found that the estrous cycle of rats stopped in the di-

oestrus phase as a result of EV treatment. In our study, we found that the oestrus cycle of the PCOS group stopped in the pro-estrous (77.78%) and met-estrous (22.22%) phases. The cycles of the control animals remained normal until the end of the experiment. Since the Lee-Boot effect (the cycle of female rodents stops or slows down without the presence of males) can be ruled out, acyclicity can be attributed to hormone treatment.

Women's fertility declines with age, which can be attributed to changes in the functioning of the ovaries, a decrease in the quantity and quality of oocytes, and hormonal changes. This so-called ovarian aging has also been observed in rats, with Anzalone et al reporting that middle-aged rats have fewer follicles than young animals. In the model we used, the number of cystic follicles and primary follicles increased in the ovaries, and no new corpora lutea were found, instead regressive old corpora lutea were located in the ovaries. In our study, we found many cystic follicles in the PCOS group, and the number of corpora lutea and healthy follicles decreased. Kang et al. (2015) used continuous light to induce PCOS in their model, they found enlarged ovaries and observed large cysts visible to the naked eye on the surface of the ovaries. Mirabolghasemi et al. (2017) examined the changes in uterine tissue and found that the thickness of the lumen epithelium and uterine wall increased in the PCOS group. Macroscopically, the uterus became enlarged and edematous, the ovaries were enlarged, and cystic follicles appeared through the superficial layer.

In our experiment, we found a decrease in the number of red blood cells, hemoglobin, and hematocrit levels, as well as an increase in the aggregation indices in the PCOS group, which was already visible in the first month of the research. In the PCOS group, a significant increase in EI was observed for the EI and EImax values measured at 3 Pa, this change may have occurred due to the effect of the large amount of estrogen administered. In an in vitro experiment, Farber et al. (2018) showed that exogenous estrogen increases the deformability of red blood cells, while no difference was found in red blood cell aggregation, however, Brun et

al. (2021) described that estradiol reduces deformability and increases eryptosis. We observed a significant decrease in the number of white blood cells in the PCOS group, while the values of the controls also decreased, but not to the same extent. The aging of the animals may also play a role in these changes. The number of white blood cells and the elongation index of female rats decrease with advancing age, while the parameters of the red blood cell aggregation index show a slight increase.

During our study, we detected PCOS-specific changes in metabolic parameters (increase in body weight and elevated fasting blood sugar levels), in the sexual cycle (acyclicity), and in histological characteristics (absence of mature follicles, the appearance of cysts). The hormone treatment caused significant changes in the hematological (red blood cell count, hematocrit, hemoglobin levels) and hemorheological parameters during the examined period. We have described for the first time the increase in red blood cell aggregation and deformability during PCOS induced by estradiol-valerate treatment in rats. Our results draw attention to the importance of micro-rheological tests in further PCOS research.

6. MAIN FINDINGS AND CONCLUSIONS

1. We found that the relationship between red blood cell aggregation and hematocrit varies by species. Based on our results, it can be said that the aggregation ability of human red blood cells is the most sensitive to changes in hematocrit among the examined species.

2. The smaller degree of aggregation observed in the case of lower or higher hematocrit is not shown in all parameters examined. The most striking differences could be detected with the light transmission method in stasis (M5s mode) and the amplitude parameter determined by the light-reflectance principle.

3. For the first time, we determined the differences between species (human, rat, dog, pig) of the haematocrit values belonging to the aggregatio maximum, both for static and dynamic aggregation parameters.

4. During tests in the animal model of polycystic ovary syndrome (PCOS), we observed characteristic symptoms and changes described in human patients, such as weight gain, elevated fasting blood sugar, interrupted estrous cycle, as well as the absence of mature follicles, and the appearance of large cysts.

5. During the examined period, we saw significant changes in hematological (decrease in red blood cell count, hematocrit, and hemoglobin values) and hemorheological parameters. We have described for the first time that red blood cell aggregation and deformability are increased during estradiol-valerate-induced PCOS in rats.

7. PUBLICATIONS

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/199/2023.PL
Subject: PhD Publication List

Candidate: Barbara Bedőcs-Baráth
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Baráth, B.**, Varga, Á., Mátrai, Á. A., Pocsai, K., Németh, N., Deák, Á.: Estradiol Valerate Affects Hematological and Hemorheological Parameters in Rats.
   Metabolites. 12 (7), 602, 2022.
   DOI: http://dx.doi.org/10.3390/metabo12070602
   IF: 5.581 (2021)

2. **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.
   Clin. Hemorheol. Microcirc. 78 (2), 187-198, 2021.
   DOI: http://dx.doi.org/10.3233/CH-211109
   IF: 2.411

List of other publications

3. Varga, Á., Mátrai, Á. A., **Baráth, B.**, Deák, Á., Horváth, L., Németh, N.: Interspecies Diversity of Osmotic Gradient Deformability of Red Blood Cells in Human and Seven Vertebrate Animal Species.
   Cells. 11 (8), 1-15, 2022.
   DOI: http://dx.doi.org/10.3390/cells11081351
   IF: 7.666 (2021)

4. Gál, K., Veres, K., Halmi, S., Bozoki-Beke, K., Fekete, K., Homoki, J., Gálné Remenyik, J., **Baráth, B.**, Varga, Á., Németh, N., Soltész, P.: The effect of rheopheresis treatment on the cytokine profile in diabetic foot syndrome with hyperviscosity in the aspect of clinical changes: a preliminary study.
   Clin. Hemorheol. Microcirc. 80 (2), 117-125, 2022.
   DOI: http://dx.doi.org/10.3233/CH-211188
   IF: 2.411 (2021)

5. Szántó, S., Módy, T., Némethné Gyurcsik, Z., Babják, L. B., Somogyi, V., **Baráth, B.**, Varga, Á., Mátrai, Á. A., Németh, N.: Alterations of Selected Hemorheological and Metabolic Parameters Induced by Physical Activity in Untrained Men and Sportsmen.
Metabolites. 11, 1-12, 2021.
DOI: http://dx.doi.org/10.3390/metabo11120870
IF: 5.581

6. Körei, C., Szabó, B., Varga, Á., **Baráth, B.**, Deák, Á., Ványolos, E., Hargitai, Z., Kovács, I., Németh, N., Pető, K.: Hematological, Micro-Rheological, and Metabolic Changes Modulated by Local Ischemic Pre- and Post-Conditioning in Rat Limb Ischemia-Reperfusion.
Metabolites. 11 (11), 776, 2021.
DOI: http://dx.doi.org/10.3390/metabo11110776
IF: 5.581

7. Mátrai, Á. A., Varga, G., Tánczos, B., **Baráth, B.**, Varga, Á., Horváth, L., Bereczky, Z., Deák, Á., Németh, N.: In vitro effects of temperature on red blood cell deformability and membrane stability in human and various vertebrate species.
Clin. Hemorheol. Microcirc. 78 (3), 291-300, 2021.
DOI: https://doi.org/10.3233/CH-211118
IF: 2.411

8. Szabó, B., Tánczos, B., Varga, Á., **Baráth, B.**, Ghanem, S., Rezsabek, Z., Al-Smadi, M. W., Németh, N.: Micro-rheological changes of red blood cells in the presence of an arterio-venous fistula or a loop-shaped venous graft in the rat.
Front. Physiol. 11, 1-12, 2020.
DOI: http://dx.doi.org/10.3389/fphys.2020.616528
IF: 4.566

9. Ghanem, S., Lesznyák, T., Fazekas, L., Tánczos, B., **Baráth, B.**, Nasser, M., Horváth, L., Bidiga, L., Szabó, B., Deák, Á., Pető, K., Németh, N.: Microrheology, microcirculation and structural compensatory mechanisms of a chronic kidney disease rat model: a preliminary study.
Clin. Hemorheol. Microcirc. 75 (1), 47-56, 2020.
DOI: http://dx.doi.org/10.3233/CH-190763
IF: 2.375

10. Varga, G., Ghanem, S., Szabó, B., Nagy, K., Pál, N., Tánczos, B., Somogyi, V., **Baráth, B.**, Deák, Á., Matolay, O., Bidiga, L., Pető, K., Németh, N.: Which remote ischemic preconditioning protocol is favorable in renal ischemia-reperfusion injury in the rat?
Clin. Hemorheol. Microcirc. 76 (3), 439-451, 2020.
DOI: http://dx.doi.org/10.3233/CH-200916
IF: 2.375

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

11. Varga, G., Ghanem, S., Szabó, B., Nagy, K., Pál, N., Tánczos, B., Somogyi, V., **Baráth, B.**, Deák, Á., Pető, K., Németh, N.: Renal ischemia-reperfusion-induced metabolic and micro-rheological alterations and their modulation by remote organ ischemic preconditioning protocols in the rat.
Clin. Hemorheol. Microcirc. 71 (2), 225-236, 2019.
DOI: http://dx.doi.org/10.3233/CH-189414
IF: 1.741

Total IF of journals (all publications): 42,699
Total IF of journals (publications related to the dissertation): 7,992

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

01 June, 2023

DEBRECENI EGYETEM
Egyetemi és Nemzeti Könyvtár

8. ACKNOWLEDGEMENT

I am indebted to many people for making this dissertation possible.

First of all, I would like to thank my supervisor, assistant professor ***Dr. Ádám Deák***, who already helped, supported, and ensured a good atmosphere during my SRS work here during my bachelor's and master's studies. Later, before and during my PhD studies, I could turn to him at any time, he was always open to new ideas, so my dissertation could be based on research that I could carry out with all my heart and soul.

I would like to express my thanks to ***Prof. Dr. Norbert Németh***, head of the department, who made me fall in love with hemorheology years ago, started me as an SRS student, and supported me throughout the journey that led to this thesis.

I would like to thank ***Prof. Dr. Irén Mikó*** and ***Prof. Dr. István Furka*** (1935-2021) for being able to learn from them.

I would like to thank ***Dr. Viktória Somogyi*** for her great help and sincere friendship, who introduced me to the mysteries of laboratory measurements.

I would like to thank ***Dr. Bence Tánczos, Ádám Varga,*** and ***Ádám Mátrai*** for their support, help, friendship, and the many hours spent in the lab, without them I would not have been able to get to this point.

A huge thank you goes to all former and current colleagues of the Department of Operative Techniques and Surgical Research, who directly or indirectly helped me during my PhD studies.

I would like to thank ***Gizella Haramura***, the senior laboratory analyst of the Division of Clinical Laboratory Science, for her professional help with the ELISA measurements.

I am grateful to ***Dr. Krisztina Deák-Pocsai*** for her help in carrying out the histological examinations.

Special thanks go to my family members, my siblings, but most of all ***My Parents***, who have always stood shoulder to shoulder with me and supported me during my studies so far.

I thank my best friend, ***Andrea Csemer***, for her perseverance, and for always listening to me and reassuring me when needed.

My Husband deserves a huge thank you, he has been my biggest support in recent years, who can calm me down and convince me that I can't give up with just one word.

Last but not least, I would like to thank all those who doubted me and did not believe that I would succeed, they were my biggest motivators.