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

In vitro and *in vivo* micro-rheological comparative studies: bilhaemia and anticoagulants

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1. INTRODUCTION

Hemorheology is the study of the flow properties of blood's cellular and plasmatic components. It covers various factors including blood viscosity, red blood cell deformability and aggregability, and interactions between blood cells and plasma. These properties are key to maintaining proper blood circulation and ensuring adequate oxygenation of tissues. Abnormal hemorheological properties can lead to impaired blood flow, which may be a risk factor for thrombotic events and other cardiovascular complications.

Certain diseases, such as embolism, stroke, and thrombosis, affect hemorheological parameters. Early recognition and treatment of these conditions are very important. For individuals with any of the predisposing factors, prevention is the primary consideration, for which several disease-specific therapies have been developed. The range of drugs used is constantly expanding, so it is possible to select the potentially most appropriate agent. However, as with most drugs, anticoagulants and antiplatelet agents can affect hemorheological variables. In addition to cardiovascular diseases, other pathological conditions such as tumor lesions, polycystic ovary syndrome, hypertension, chronic liver diseases (CLD) such as cirrhosis or non-alcoholic fatty liver disease (NAFLD), may alter micro-rheological parameters of blood flow. In addition to the more common liver diseases and injuries, hemorheological parameters may also be altered in rare pathologies such as bilhaemia, due to the role of the liver in the synthesis of proteins involved in blood coagulation and regulation.

The interaction between bilhaemia and blood clotting may have important clinical implications in patients with liver disease or bile duct obstruction. Blood coagulation disorders in these patients may take the form of thrombotic events or hemorrhagic complications, depending on the balance between procoagulant and anticoagulant factors. Thrombotic complications, such as portal vein thrombosis, may occur due to a procoagulant state induced by bilhaemia. In contrast, hemorrhagic complications may result from the combined effects of reduced clotting factor production, vitamin K deficiency, and platelet dysfunction.

Currently, there is little literature on the effects of bilhaemia and antiplatelet and anticoagulant drugs on hemorheological parameters. Our study aimed to investigate the changes that occur during bilhaemia in rat and pig models and to explore the effects of a potentially new anticoagulant preparation and compare them with those caused by unfractionated heparin.

2. AIMS OF THE STUDY

1. We aimed to develop rat and pig animal models that would allow for a comprehensive study of bilhaemia.
2. To study the direct effects of bilhaemia on conventional and osmotic gradient deformability of red blood cells, red blood cell aggregation, and hemodynamic parameters in the developed animal models.
3. We aimed to investigate the dynamics of these changes and the differences between species.
4. Our aim was to develop a protocol for a porcine model that would allow the testing of dual antiplatelet and anticoagulant (APAC) at escalating doses.
5. The protocol developed was to investigate the effects of APAC on micro-rheological parameters, including red blood cell deformability and aggregation.
6. We then aimed to compare different doses of APAC with different doses of unfractionated heparin (UFH) and draw conclusions for both agents.

3. MATERIALS AND METHODS

The experiments were carried following the European Union Directive (Directive 2010/63/EU) and National Regulations (Act XXVIII of 1998 on the protection and sparing of animals) and with the approval of the University of Debrecen Committee of Animal Welfare (registration Nr.: 17/2022/UDCAW and 3/2021/UDCAW).

3.1. Effect of bile on hemodynamic and micro-rheological parameters in bilhaemia

3.1.1. Experimental animals and sampling protocol

Six healthy male Wistar (Crl:WI) rats (bodyweight: 458.3 ± 24.5 g) were used at 12–14 months of age in this study. The rats were kept in standard cages (Eurostandard IV, Tecniplast, Buguggiate, Italy) at a temperature of 22 ± 2 °C, humidity of 55% ± 10%, lighting on a 12–12 h light/dark cycle, and with free access to water and standard rat food.

Also, we included six female Hypor pigs (12–13-week-old, bodyweight: 20.8 ± 1.7 kg) in this study. Ventilation (15–20× air change/hour) and heating (central and underfloor heating) of the enclosures were provided, in a temperature range of 22–26 °C, according to the weight of the animals. Extreme and sudden wide fluctuations in humidity were avoided during the housing of the animals. The animals were supplied with a feed mix appropriate for their species, and water was provided by a self-watering system.

3.1.1.1. Experimental protocol in rats

Under general anesthesia with 100 mg/kg of ketamine i.p. (CP-ketamine hydrochloride 10%, Produlab Pharma BV, Raamsdonksveer, The Netherlands), 10 mg/kg of xylazine i.p. (CP-xylazine hydrochloride, 2%; Produlab Pharma BV, Raamsdonksveer, The Netherlands), a 26 G cannula was inserted into the lateral tail vein for blood sampling, fluid therapy, and bile administration. The right common carotid artery was prepared and cannulated (O.D. 0.965 mm, Polyethylene Tubing Clay Adams, 427411, BD IntramedicTM, Sollentuna, Sweden). After fixation with a central ligature, the cannula was connected to an invasive hemodynamic monitoring system (Hemosys monitor system LD-01, Experimetria Ltd., Budapest, Hungary). For bile collection, median laparotomy was performed, and the ductus choledochus was prepared, opened by microsurgical techniques, and cannulated (Micro-Renathane®, MRE-025 type. 0.25 mm outer diameter, × 0.12 mm inner diameter; Braintree Sceintific Inc., Braintree, MA, USA). A 1 mL syringe was connected to the cannula with a 27 G needle. Bile was collected for 1 h, providing an extracted bile volume of approximately 250 µL.

3.1.1.2. Experimental protocol in pigs

The following anesthesia protocol was used: for pre-medication, i.m. 1–2 mg/kg of azaperone (Stresnil, Elanco GmbH, Cuxhaven, Germany); for induction of anesthesia, i.m. 2 mg/kg of xylazine (CP-xylazine hydrochloride, 2%) and 20 mg/kg of ketamine (CP-ketamine hydrochloride 10%); for maintenance of permanent anesthesia, i.v. 1 mg/kg of xylazine and 10 mg/kg of ketamine, supplemented with i.v. 2 mg/kg of diazepam (Diazepeks 5 mg/ml, AS Grindeks, Riga, Latvia). The animals were intubated, and external jugular vein cannulation (Certofix Trio, 7F, B.Braun Trading Ltd., Budapest, Hungary) was performed unilaterally for blood sampling, fluid therapy, and bile administration. The left common carotid artery was also cannulated (Certofix Trio, 7F) and connected to an invasive hemodynamic system (Hemosys monitor system LD-01). Bile was obtained by direct puncture of the gallbladder via upper median laparotomy. For this, 23 G needles and a syringe with a volume of 5 mL were used, and 5 mL of bile was collected.

3.1.1.3. Blood sampling protocol

Before surgery and bile administration, venous blood (for *in vitro* studies, 1.5 mL, for *in vivo* studies, 0.5 mL of sodium EDTA 1.8 mg/mL) was collected from the cannulated veins. Baseline measurements were performed, and the effects of bile *in vitro* were investigated after adding 1 μL or 5 μL of bile to 500 μL of blood. In the *in vivo* studies, blood samples were taken 5 min after i.v. bolus injection of 200 μL/kg of bile.

3.1.2. Laboratory measurements

3.1.2.1. Hematological parameters

Red blood cell count (RBC [T/L]), white blood cell count (WBC [G/L]), hemoglobin concentration (Hgb [g/dL]), hematocrit (Hct [%]), mean corpuscular volume (MCV [fL]), mean corpuscular hemoglobin (MCH [pg]), mean corpuscular hemoglobin concentration (MCHC [g/dL]), and platelet count (Plt [G/L]) were measured using a Sysmex K-4500 microcell counter (TOA Medical Electronics Co., Ltd., Kobe, Japan).

3.1.2.2. Red blood cell conventional and osmotic gradient deformability measurements

A LoRRca Maxsis Osmoscan ektacytometer (RR Mechatronics International B.V., Zwaag, The Netherlands) was used to determine the deformability of red blood cells. The blood samples were sheared, and laser diffraction techniques were used to measure the elongation of the cells. The so-called elongation index (EI) was determined as a function of shear stress (SS,

Pa; range: 0.3 to 30 Pa). To perform the traditional deformability test, 2 mL of a polyvinylpyrrolidone (PVP)–PBS solution (PVP: 360 kDa, Sigma-Aldrich Co., St. Louis, MO, USA; PVP-PBS solution viscosity = 30.5 mPas, osmolality = 303 mOsmol/kg, pH = 7.5) was carefully mixed with 10 µL of sample (whole blood or blood–bile suspension). Every measurement was performed at 37 °C. Comparative data from the EI–SS curves were calculated, i.e., EI values at 3 Pa, and the Lineweaver–Burk equation ($1/EI = SS_{1/2}/EI_{max} \times 1/SS + 1/EI_{max}$) was used for the parameterization of individual EI–SS curves, providing the maximal elongation index (EI_{max}) and the shear stress at half EI_{max} ($SS_{1/2}$, Pa). Lowe EI or EI_{max} and high $SS_{1/2}$ values represent impaired red blood cell deformability.

Measurements of osmotic gradient deformability (osmoscan) were performed with 250 µL of sample and 5 mL of isotonic PVP-PBS (see above). As the device mixed low-osmolarity (0 mOsm/kg) and high-osmolarity (500 mOsm/kg) PVP solutions with the sample, the osmolality of the suspension varied. In this module, the determination of EI was carried out at constant shear stress (30 Pa). As the osmolality of the blood sample was steadily increased, the sample was aspirated into this PVP solution, and the elongation index was continually recorded. The outcome was a recognizable EI–osmolality (O) curve with multiple noteworthy spots, such as, in a low-osmolality range, the minimal elongation index (EI min) and the associated osmolality value (O min), as well as the maximal elongation index (EI max, note that it is not the same as EI_{max} in the Lineweaver–Burk equation, see above) and the associated O (EI max) value, and in the higher osmolality range, EI hyper (half of the maximal elongation index in the high-osmolarity environment) and O hyper. The area under each unique EI–O curve was calculated. Additional parameters, such as ΔEI (absolute difference between maximal and minimal EI values), ΔO (absolute difference between osmolality values at maximal and minimal EI), and ratio values, such as EI max/EI min (rEI), O (EI max)/O min (rO), ΔEI/ΔO, and rEI/rO, were also calculated.

3.1.2.3. Determination of red blood cell aggregation

A Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Roetgen, Germany) was used to determine the aggregation index values of the blood samples. The technique is based on the light transmittance photometric method. The test requires 20 µL of blood. After disaggregation by a controlled shearing system (shear rate: 600 s^{-1}), light transmission was tested for 5 or 10 s at stasis (M values, shear rate: 0 s^{-1}) or at a low shear (M1 values, shear rate: 3 s^{-1}). The measurements were carried out at room temperature (20–25 °C). High index values (M 5 s, M1 5 s, M 10 s, M1 10 s) represent enhanced RBC aggregation.

3.1.3. Statistical analysis

To estimate the necessary sample number (sample size) for the experiment, Mead's resource equation method was used. For statistical analyses, SigmaStat Software 3.1.1.0. was used (Systat Software Inc., San Jose, CA, USA). For general data presentation, means ± S.D. (standard deviation) are shown. After testing the normality of the data distribution by the Kolmogorov–Smirnov test, differences between the doses were analyzed by t-test or the Mann–Whitney rank sum test, and one-way ANOVA or Kruskal–Wallis's test was used based on the results of the normality test. A p-value of < 0.05 was considered statistically significant.

3.2. Effect of dual antiplatelet and anticoagulant (APAC) agents on red blood cell deformability and aggregation in a porcine model

The following research was a collaborative study with the Helsinki University Central Hospital (Prof. Dr. Riitta Lassila and Dr. Annukka Jouppila) and the Division of Clinical Laboratory Science, University of Debrecen (Dr. Zsuzsa Bagoly).

3.2.1. Experimental animals and sampling protocol

3.2.1.1. Hemorheological effects of APAC

Venesection (external jugular veins) was performed under general anesthesia (pre-medication: 2 mg/kg azaperone (Stresnil, i.m.; induction of anesthesia: 2 mg/kg xylazine (CP-Xylazine-hydrochloride, 2%; Produlab Pharma BV, The Netherlands), i.m., 20 mg/kg ketamine (CP-Ketamine hydrochloride 10%, Produlab Pharma BV, The Netherlands), maintenance of anesthesia: 0.9 mg/kg xylazine and 8 mg/kg ketamine. Blood was drawn (ethical approval registered by University of Debrecen Committee of Animal Welfare, Nr.: 3/2021/UDCAW) from the right external jugular vein of female Hypor pigs ($n = 5$/group; bodyweight: 23.6 ± 1.58 kg) to the vacutainer tubes (BD Vacutainer® tubes, 1.8 mg/ml K_3-EDTA; Becton, Dickinson and Company, USA), and the vehicle (1 mL of 137 mM NaCl and 10 mM Na_2HPO_4, pH: 7.5) and test substance (APAC; 7.84 mg/ml; Aplagon Ltd/Cadila Pharmaceuticals Ltd.) was group-specifically injected intravenously (i.v.) via the left external jugular vein. After cannulation, a continuous fluid replacement was provided on both sides (right and left external jugular vein) during the study with physiological saline (1080.4 ± 45.9 ml, "Baxter" Sodium Chloride 0.9%, pH = 4.5-7, osmolarity: 308 mOsm/l, Baxter Hungary Kft.). For urine drainage, a suprapubic cystostomy catheter was inserted. A tube was placed in the trachea to assist ventilation (WATO EX-20Vet, Shenzhen Mindray Animal Medical Technology Co., LTD., China). $PaCO_2$ was set to 35–45 mmHg, and PaO_2 was set at 100–130 mmHg.

At the beginning of the study, the vehicle (1 mL of 137 mM NaCl and 10 mM Na_2HPO_4, pH: 7.5) was introduced for both groups, followed by blood sampling 5 min later. Subsequently, APAC was administered at escalating doses of 0.25 mg/kg, 0.5 mg/kg, and 0.75 mg/kg, i.v. The current estimated clinical IV bolus dosing is compatible with the doses of 0.25–0.5 mg/kg (approx. 45–90 IU/kg of heparin), whereas 0.75 mg/kg is over the maximal allowance based on the toxicology program. The Control group received only the vehicle. The volume of the formulations containing the active substance was 4 mL, of which 3.77 ± 0.004 mL was the volume of vehicle administered to the control animals for the 0.25 mg/kg dose 3.57 ± 0.04 mL, for the 0.5 mg/kg and 3.36 ± 0.06 mL for the 0.75 mg/kg dose. For both groups, blood was collected 15 min after the first administration. At the end of the study, 140 IU/kg protamine sulphate (1400 anti-heparin IU/ml, lot F2084FI1, Leo Pharma, Denmark) was administered, and blood was taken again 15 min later. The blood samples were collected also for a cooperative coagulation biomarker study.

3.2.1.2. Comparison of APAC with UFH

The anesthesia for the experimental animals was the same as previously described. Blood was drawn from the right external jugular vein of female Hypor pigs ($n = 5$/group; bodyweight: 23.3 ± 1.06 kg) to the vacutainer tubes (BD Vacutainer® tubes, 1.8 mg/ml K_3-EDTA; Becton, Dickinson and Company, USA), and the vehicle (1 mL of 137 mM NaCl and 10 mM Na_2HPO_4, pH: 7.5) and APAC or UFH (Heparibene Na 25000 IU, Ratiopharm Arzneimittel Vertriebs GmbH.) were injected i.v. through the left vein. The effects of preparations at 0.5 mg/kg (the maximal clinical APAC dose, approx. 90 IU/kg of heparin i.v.) and 0.75 mg/kg were compared.

The vehicle was administered first, followed by the low dose, and then escalated with the higher bolus dose of APAC or UFH. The first blood sample was obtained 5 min after the vehicle was administered and was taken as a baseline; the blood was collected again 15 min after the test substances were administered.

3.2.2. Laboratory measurements

3.2.2.1. Hematological variables

The qualitative and quantitative hematological variables were measured by ADVIA 120 hematology automate (Siemens Healthcare Gmbh, Germany) and Sysmex K-4500 microcell counter (TOA Medical Electronics Co., Ltd., Kobe, Japan). In this study, red blood cell count (RBC [T/L]), white blood cell count (WBC [G/L]), hemoglobin concentration (Hgb [g/dl]),

hematocrit (Hct [%]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin (MCH [pg]), mean corpuscular hemoglobin concentration (MCHC [g/dl]), and platelet count (Plt [G/L]) were assessed.

3.2.2.2. Red blood cell deformability and membrane (mechanical) stability measurements

The red blood cell conventional deformability measurements were performed with the LoRRca MaxSis Osmoscan ektacytometer described in section 3.1.2.2 using the following (PVP)-PBS solution: PVP: 360 kDa, Sigma-Aldrich Co. USA; PVP-PBS solution viscosity = 30.4 mPas, osmolality = 302 mOsmol/kg, pH = 7.2.

For red blood cell membrane (mechanical) stability measurements, sample preparation was performed as described for conventional deformability measurements. A conventional deformability measurement was performed, and the cells were then exposed to mechanical stress at a shear stress of 100 Pa for 300 seconds, followed by a re-measurement of deformability. The evaluation was performed by comparing the ratios of deformability values measured before and after mechanical stress (before/after ratio).

3.2.2.3. Determination of red blood cell aggregation

Using a LoRRca MaxSis Osmoscan ektacytometer (Mechatronics BV, The Netherlands), the RBC aggregation was examined. The laser backscattering method was used to run the device. The Couette-system rotates the blood sample to be disaggregated; the rotor then quickly stops, allowing the variations in the light's intensity reflection from the blood sample to be detected. Aggregation index (AI [%]) was the parameter that was analyzed. The test needs one milliliter of whole blood.

3.2.3. Statistical analysis

SigmaStat Software 3.1.1.0 (Systat Software Inc., San Jose, CA, USA) was used to carry out the statistical analyses. Data are expressed as mean ± S.D. (standard deviation). The number of cases was estimated using the statistical program G*power. Differences between groups were analyzed by t-test or the Mann–Whitney rank-sum test, differences between each blood sampling by paired t-test or Wilcoxon rank sum test, and the repeated measures ANOVA or Friedman's test was used based on the results of the Kolmogorov–Smirnov normality test. A p-value of < 0.05 was considered statistically significant.

4. RESULTS

4.1. Effect of bile on hemodynamic and micro-rheological parameters in bilhaemia

4.1.1. Hemodynamic variables

After administration of a single dose of bile bolus, mean arterial pressure (MAP) and heart rate (HR) began to decrease rapidly. Different times were required for the two species to decrease and then normalize. In rats the values normalized within a short time, whereas in pigs the recovery was much slower.

4.1.2. Hematological parameters

For rats, a slight increase was observed in the examined parameters, except for the white blood cell count, which was significant for hemoglobin ($p = 0.002$ vs. 1 μL of bile; $p = 0.015$ vs. 5 μL of bile; $p = 0.048$ vs. *in vivo* 200 μL/kg of bile) and hematocrit values ($p < 0.001$ vs. 1 μL of bile; $p = 0.006$ vs. 5 μL of bile; $p < 0.001$ vs. *in vivo* 200 μL/kg of bile), compared to intact whole blood. In contrast, for pigs, a slight decrease or stagnation was observed for most parameters, compared to intact blood.

4.1.3. Red blood cell deformability

When the elongation index–shear stress curves were evaluated, different variation was observed between species. For rats, after both *in vitro* and *in vivo* bile administration, a decrease and then an increase in the elongation index values were observed in the low shear stress range (<3 Pa) and normalization in the high shear stress range (>3 Pa), compared to intact whole blood. For pigs, a decrease in the elongation index values was observed following the *in vitro* administration of 1 μL of bile, whereas an increase in the EI values at low shear stress (<3 Pa) and a decrease at high shear stress (>3 Pa) were observed following the *in vitro* administration of 5 μL and the *in vivo* administration of 200 μL/kg of bile, respectively, compared to intact whole blood.

4.1.4. Red blood cell aggregation

For the rat samples, comparing the two *in vitro* and *in vivo* doses, *in vitro*, 5 μL of bile significantly decreased the aggregation index values compared to that of intact whole blood ($p < 0.001$), and 1 μL of bile (M 5 s, M1 5 s, and M 10 s: $p < 0.001$; M1 10 s: $p = 0.017$) affected all four parameters. Following the administration of 200 μL/kg of bile *in vivo*, significant differences in the aggregation index values were observed between the groups when measured under static conditions after 5 s (M 5 s) of disaggregation ($p = 0.002$ vs. base conditions, $p =$

0.003 vs. 1 µL of bile). The values of the other three parameters (M1 5 s, M 10 s, M1 10 s) were extremely low.

For pigs, no significant changes in the aggregation index values were observed when comparing intact whole blood with samples after *in vitro* or *in vivo* treatment with bile, in contrast to the significant differences observed in rats.

4.2. Effect of dual antiplatelet and anticoagulant (APAC) agents on red blood cell deformability and aggregation in a porcine model

4.2.1. Hemorheological effects of APAC

4.2.1.1. Hematological variables

Overall, the complete blood count (CBC) did not change significantly despite some minor variation. All values remained in the biologically normal range throughout the experiment. Slight changes in hematological variables were observed after each dose, but in some cases these changes were statistically significant (Hct: $p = 0.008$, Plt: $p = 0.031$), but in all cases the values were biologically within the normal range.

4.2.1.2. Red blood cell deformability

For the EI-SS curves, the elongation index values at 3 Pa, maximal EI and the SS belonging to the half maximal EI were compared, based on Lineweaver-Burk equation: $1/EI = SS_{1/2}/EI_{max} \times 1/SS + 1/EI_{max}$. The EI values at the SS of 3 Pa did not change significantly in any of the groups.

Comparing the data obtained by parameterizing the curves, it was observed that at a higher shear stress (30 Pa), the maximal EI values impaired in the treated group. 75 min after the 0.5 mg/kg dose, these decreases reached significance compared to the Control group ($p = 0.024$). Some parameters were significantly reduced in the APAC group 15 min after administration of protamine sulphate (EI_{max}: $p = 0.032$ vs. Control group; EI_{max}: $p = 0.047$, $SS_{1/2}$: $p = 0.026$, $EI_{max}/SS_{1/2}$: $p = 0.034$ vs. 5 min after vehicle).

4.2.1.3. Red blood cell membrane (mechanical) stability

A slight decrease in elongation index values at 3 Pa was observed in the APAC group compared to the values measured after administration of the vehicle. A significant difference was measured between the APAC and Control groups 75 min after administration of the 0.25 mg/kg dose ($p=0.043$). In contrast, an increase in maximum elongation index values was observed after both doses of the test drug compared to baseline. For the other parameters, a

slight decrease was observed for $SS_{1/2}$ and a slight increase for $EI_{max}/SS_{1/2}$, with no significant change after either dose.

4.2.1.4. Red blood cell aggregation

In the RBC aggregation based on light reflection, differences in the aggregation index values were not observed between the groups when measured under static conditions after disaggregation. A slight increase in the aggregation index was observed in the APAC group after administration of each dose compared to the Control group, but this slight increase was not significant.

4.2.2. Comparison of APAC with UFH

4.2.2.1. Hematological parameters

The slight hemoconcentration seen in the APAC group was also observed in the UFH group after both doses. A relative increase in platelet count was detected in the UFH group (0.5 mg/kg $p = 0.01$ vs. baseline). In contrast, a relative decrease of platelet count was found in the APAC group after both doses ($p < 0.001$ vs. baseline). Although some hematological values were significantly altered, they were all biologically within the normal laboratory range for the species.

4.2.2.2. Red blood cell deformability

Comparing the EI-SS curves, at low shear stress (< 3 Pa) both agents caused similar changes, with the values increasing. Conversely, at higher shear stress, a slight deterioration was observed in the APAC group, while an improvement was still observed in the UFH group.

Dosage of 0.75 mg/kg UFH caused a significant increase ($p < 0.001$ vs. baseline) in elongation index values measured at a shear stress of 3 Pa. The deterioration observed in the APAC group at the higher shear stress in the curves was also present the deterioration in the maximum elongation index (EI_{max}) values. Both doses significantly decreased the EI_{max} (0.5 mg/kg $p = 0.024$; 0.75 mg/kg $p = 0.023$ vs. baseline), indicating a deterioration in the RBC elongation at higher shear stress. The impairment caused by APAC at the high heparin 0.75 mg/kg was also significant compared to the same dose of UFH (approx. 135 IU/kg) ($p = 0.009$).

4.2.2.3. Red blood cell membrane (mechanical) stability

When comparing the ratios of the values measured before and after mechanical stress, no significant changes were observed between the different doses or between the groups. Slight

changes were observed, but none of the preparations tested caused changes in the membrane or composition of the cells to such an extent that they were unable to withstand mechanical stress.

4.2.2.4. Red blood cell aggregation

A dose of 0.5 mg/kg of APAC and UFH slightly increased the rate of aggregation compared to baseline. This enhancement was not striking for the 0.75 mg/kg dose. Similar changes were observed for both agents at the doses tested, which were not significant for either agent or dose.

5. DISCUSSION

5.1. Effect of bile on hemodynamic and micro-rheological parameters in bilhaemia

In our study, we found that the mean arterial pressure decreased in both species after intravenous bile administration. In rats, all hematological parameters, except white blood cell count, showed an increase *in vitro* and *in vivo*, while in pigs, a slight increase or stagnation was observed. No significant changes in red blood cells conventional and osmotic gradient deformability were observed. A significant decrease in red blood cell aggregation was observed in rat samples after the *in vitro* administration of 5 μL of bile, with an unmeasurable decrease in aggregation after *in vivo* bile administration. No significant difference in the aggregation index values was observed in pigs.

The calculation of the bilhaemia dosage was based on the *in vitro* dose of 1 μL. Accordingly, 1 μL of bile was added to 500 μL whole blood, resulting in a bile–blood suspension of 0.2%. The average blood volume in rats is 54–70 mL/kg, while that in pigs is 56–69 mL/kg. So, the circulating blood volume in rats ranges from 25 to 32 mL, and that in pigs from 1165 to 1435 mL. Following the administration of 200 μL/kg, a bile-to-blood ratio of approximately 0.2–0.3% was achieved *in vivo*.

Bile acids affect blood pressure and heart rate. Previous studies demonstrated that heart disease can result from aberrant bile acid metabolism. Heart rate variability, stress response sensitivity, and QT interval lengthening are linked to conditions such as primary biliary cholangitis and intrahepatic cholestasis of pregnancy, which can raise the risk of cardiovascular complications. Furthermore, cirrhotic cardiomyopathy, a frequent consequence of liver cirrhosis marked by extended QT intervals and systolic or diastolic failure, might be linked to bile acids' impact on cardiovascular function.

Though there is a great deal of disagreement, three theories have been put up to explain how bile acids could cause bradycardia: (1) by creating a monolayer on the surface of the cell membrane to cause mechanical interference; (2) by lowering the sluggish inward calcium current, preventing the membrane from conducting action potentials; (3) by functioning as an antimuscarinic antagonist, since physostigmine can intensify or counteract their effects.

Moreover, gallbladder inflammation can trigger nerve reflexes that lower blood pressure and heart rate and possibly cause cardiac ischemia or arrest. Heart rate and arterial blood pressure can be impacted by reflex coronary vasoconstriction, which is brought on by the connection between gallbladder distension and variations in coronary blood flow.

It was demonstrated that bile acids cause a process known as eryptosis, i.e., cell death of erythrocytes. The process by which bile acids cause red blood cells to absorb more calcium is mediated by the activation of cation channels. This raises cytosolic Ca^{2+} activity and ultimately causes eryptosis, the suicidal death of erythrocytes marked by the exposure of phosphatidylserine on the cell surface. It was demonstrated that bile salt-associated hemolysis is partially calcium-mediated. Moreover, the bile acid concentrations needed to induce eryptosis are greater than those needed to cause hemolysis.

Exercise was also shown to influence alterations in human bile and red blood cell lipids. Moreover, increased red blood cell distribution width was linked to pregnancy-related intrahepatic cholestasis, a disorder that impairs the bile flow.

Results indicate that the red blood cell uptake of calcium is influenced by bile acid concentration. Human erythrocytes were demonstrated to absorb calcium *in vitro* when exposed to bile salts, and the amount of calcium absorbed depends on bile salts' concentration. Bile salts cause hemolysis at high doses by co-micellizing the lipid components of the cell membrane. However, through as-yet-undefined mechanisms, bile salts are also linked to hemolysis at lower concentrations. Calcium uptake is stimulated 4- to 25-fold when bile salts are present, and the amount of calcium absorbed depends on the bile salt concentration. The degree of this increase was attributed to ATP depletion or exposure to trifluoperazine, both of which reduce red blood cell calcium pump activity. Furthermore, calcium increases the hemolytic activity of bile salts; this effect is greatest in a buffer containing 100 mM KCl/50 mM of NaCl. In conclusion, the concentration of bile salts determines the amount of calcium uptake in red blood cells, which is influenced by the concentration of bile acids.

Bile acids can also stimulate the intake of sodium and/or the export of intracellular potassium into erythrocytes, which can result in lysis. Moreover, ceramide and Ca^{2+} entry have a similar impact to that of bile acids in stimulating suicidal cell death.

There are notable variations in the composition of bile acids among rats, pigs, and humans when their bile chemistry is compared. Studies showed that bile samples from rats and pigs have significant quantitative differences in the amounts of various bile acids, including taurocholic acid (TCA) and glycocholic acid (GCA), with changes of more than 400% in certain instances. Furthermore, research demonstrated that there are significant differences in the composition of plasma, urine, and bile acids, as well as their metabolites, between various species, with minipigs, rats, and mice displaying the most divergent bile acid profiles in comparison to humans. Additionally, a possible connection between the composition of bile acids and the activity of calcium ionophores in the intestine was suggested by an investigation

conducted on pigs concerning the relationship between bile acids reported intestinal fluid secretory activity and their properties.

A variety of factors may be involved in the variations in bile acid composition found in different animals. These variables include variations in the enterohepatic circulation of bile acids, intestinal absorption, and liver metabolism. Furthermore, various species differ in the expression and activity of bile acid transporters as well as of the enzymes involved in the synthesis and metabolism of bile acids. Calcium transport and ionophore activity are two physiological and pharmacological processes that may be affected by species-specific variations in bile acid composition. In conclusion, a variety of complex and multifactorial variables, including variations in intestinal absorption, enterohepatic circulation, liver metabolism, and the expression and activity of bile acid transporters and enzymes, contribute to interspecies differences in bile acid composition and thus to differences in hemorheology and microcirculation due to bilhaemia.

Limitations of our study include the fact that only two animal species were studied. We only tested two doses *in vitro* and used a short incubation period in our *in vivo* experiments, so that only acute changes could be detected. Another limitation of the present research is that there is anatomical difference between swine and rats regarding the presence or absence of the gallbladder. Pigs have, like humans, a well-defined gallbladder, but in rats, this anatomical structure is missing. This fact influenced the method of bile sampling: from rats bile was collected from the cannulated ductus choledochus, while from pigs it was obtained directly from the cholecyst.

5.2. Effect of dual antiplatelet and anticoagulant (APAC) agents on red blood cell deformability and aggregation in a porcine model

The increased risk of bleeding complications associated with the combination of antithrombotic agents with different mechanisms of action, have raised interest in the development of cardiovascular therapies with dual antiplatelet and anticoagulant (APAC) activity. Using naturally produced heparin proteoglycans as a framework for biosynthetic alternatives, the protein conjugated UFH chains offer a promising route. The micro-hemorheological properties of APAC did not differ from UFH, and these data do not refer to specific safety issues for this novel naturally occurring HEP-PG mimetic.

Our *in vivo* study showed that dual APAC in escalated bolus doses maintained the CBC relatively stable, but after protamine neutralization, some decrease of platelet count occurred. The dosing scheme in boluses 0.25 mg/kg to 0.5 mg/kg (approximately 45–90 IU/kg of heparin)

was clinically relevant but the highest dose of 0.75 mg/kg exceeds the maximal dose allowance. We have unpublished data on the administration of APAC in healthy volunteers, which does not impact the CBC values (data in file).

Both APAC and UFH enhanced RBC aggregation. At low shear stress, an improvement in red blood cell deformability was observed with both treatments, but at higher shear stress, red blood cell deformability deteriorated, which may have contributed to the increased RBC aggregation. Compared to heparin, APAC caused similar but minor microrheological changes that were not physiologically significant *in vivo*. Although some hematological variables were altered, they were retained within biologically normal laboratory range for the species. Heparin is widely used in clinics for the treatment of thromboembolism and as an anticoagulant, for example during extracorporeal circulation. Initial hyper-aggregation of RBC is observed in most patients with cardiovascular disease. The treatment with heparin, i.e., UFH or APAC in these patients could potentially affect blood rheology.

Previous studies have shown that APAC was reno-protective in acute ischemic kidney injury, in contrast to UFH. We also have similar preliminary experimental data in myocardial infarction (submitted) and stroke. APAC as a heparin proteoglycan mimetic inhibits coagulation initiated by the intrinsic coagulation pathway and thrombin, yet it differs from conventional heparins in inhibiting platelet aggregation and procoagulant activity. Collagen-induced platelet aggregation is unaffected or even enhanced by UFH, whereas APAC attenuates platelet aggregation and deposition on collagen surfaces in a dose-dependent manner. Platelet-induced thrombosis is prevented by APAC under high shear rates, whereas in the presence of UFH, these vessels are occluded. In a collagen-coated AV shunt model, local administration of APAC reduced both platelet and fibrin deposition. PET scans have demonstrated that APAC targets and binds with a longer retention time to the injury site compared to UFH.

Patients with effective acetylsalicylic acid (ASA) inhibition have been reported to have lower plasma fibrinogen level and red blood cell aggregation values compared to the ineffective acetylsalicylic acid medication (ASA resistance). The fractal dimension of erythrocytes decreases with increasing aspirin concentration, so the rate of aggregation decreases. At the same time, increasing aspirin concentration and shear force increase the deformation index of red blood cells, i.e., their elongation capacity improves. ASA together with APAC enhances the platelet aggregation inhibition potential, albeit APAC is not directly impacting the thromboxane pathway.

In our study, we found that RBC deformability improved with low shear stress, but the erythrocyte elongation index moderately decreased under higher shear stress. Still, increasing the dose of UFH or APAC did not affect this effect.

Heparin-induced red blood cell aggregation remains a complex and somewhat enigmatic phenomenon within the realm of medical science, posing challenges in terms of its therapeutic implications. While heparin, a commonly used anticoagulant, is generally considered beneficial for its antithrombotic properties, its apparent role in stimulating RBC aggregation raises intriguing questions and potential concerns. RBC aggregation is influenced by many factors such as hematocrit, free radicals, and fibrinogen levels. The hemoconcentration measured with hematocrit here in pigs was small, although mathematically significant, not biologically so. Previous studies have found that a significant increase in RBC aggregation occurs at higher hematocrit levels, which also shows interspecific differences.

One notable indicator of this phenomenon is the elevation in the erythrocyte sedimentation rate (ESR), a parameter frequently used to gauge inflammation. The increase in ESR and low shear blood viscosity are often associated with increased RBC aggregation, and this observation is especially important for patients receiving heparin therapy. Underlying health conditions that already influence RBC aggregation, i.e. sickle cell anemia or diabetes, could potentially have effects from heparin-induced RBC aggregation.

Moreover, the promotion of RBC aggregation by heparin can have broader implications in the realm of microcirculation. The microcirculatory system, consisting of tiny blood vessels and capillaries, plays a crucial role in tissue oxygenation and nutrient delivery. However, the alterations were numerically significant, but its biological effects are not clear. It is still not known where the ‘point’ is where micro-rheological changes turn to microcirculatory deterioration.

Heparin’s impact on blood viscosity and erythrocyte sedimentation rate has been the subject of extensive research, consistently revealing its influence on the aggregation of RBCs across various concentrations. The data consistently indicates a noteworthy trend of increasing blood viscosity and ESR levels, aligning with a concomitant rise in RBC aggregation. This phenomenon, while intriguing, raises concerns regarding the potential adverse effects of heparin on blood rheology.

An illustrative example of heparin’s influence is the observation that, in all examined donor blood samples, the average ESR surged by approximately 75% when the heparin concentration reached 100 U/ml. Such significant changes in ESR are indicative of an alteration in RBC behavior, particularly their tendency to aggregate. These findings collectively point

towards a negative effect of heparin on RBC aggregation, a factor of importance in understanding its clinical implications. The decrease of platelet count in the samples might be related to a ‘relative’ *in vitro* decrease, supposedly related to the role of heparin in altering the red blood cell surface properties.

Limitations of the study include the relatively low case number and the few selected dosages for comparison. The experiments were performed in juvenile pigs with healthy vasculature, and we plan to investigate changes in pigs with atherosclerosis in the future. The experiment was a short-term study with repeated bolus dosing, and we were targeted to detect acute alterations of hematologic and hemorheological variables.

6. MAIN FINDINGS AND CONCLUSION

1. We have developed rat and pig animal models that provide the opportunity to study bilhaemia on a large scale.
2. No changes in parameters describing red blood cell deformability were observed after bile administration. There was a significant difference in aggregation index values between the two species studied. The red blood cells of rats were more sensitive after both *in vitro* and *in vivo* bile administration. In both species, *in vivo* bile caused a decrease in mean arterial pressure and heart rate.
3. The magnitude and dynamics of the acute hemodynamic effects of bile differed between the two species studied. In rats the values normalized within a short time, whereas in pigs the recovery was much slower.
4. A protocol was developed to allow the testing of dual antiplatelet and anticoagulant (APAC) in escalating doses. Our study is the first to investigate the micro-rheological correlates of APAC administration. Considering the data, we conclude that UFH and APAC have similar micro-rheological properties.
5. Our *in vivo* study showed that APAC at escalated bolus doses did not significantly affect the total blood count, but after neutralization with protamine sulphate, the platelet count was slightly reduced.
6. APAC enhances red blood cell aggregation. At low shear stress, an improvement in red blood cell deformability was observed, but at higher shear stress, red blood cell deformability deteriorated, which may have contributed to the increased red blood cell aggregation.
7. Compared to heparin, APAC caused similar but smaller micro-rheological changes that were not physiologically significant *in vivo*. Although some changes in hematological parameters were observed, they were still within the biologically normal laboratory range for the species.

7. PUBLICATIONS

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List of publications related to the dissertation

1. **Mátrai, Á. A.**, Varga, Á., Fazekas, L., Baráth, B., Nellamkuzhi, N. J., Nghi, T. B., Németh, N., Deák, Á.: Effect of Bile on Hemodynamics and Blood Micro-Rheological Parameters in Experimental Models of Bilhemia.
   Metabolites. 14 (4), 1-13, 2024.
   DOI: http://dx.doi.org/10.3390/metabo14040211
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2. **Mátrai, Á. A.**, Varga, Á., Baráth, B., Ványolos, E., Orbán-Kálmándi, R. A., Lóczi, L., Bagoly, Z., Jouppila, A., Lassila, R., Németh, N., Deák, Á.: Heparin-like effect of a dual antiplatelet and anticoagulant (APAC) agent on red blood cell deformability and aggregation in an experimental model.
   J Thromb Thrombolysis. [Epub ahead of print], 2024.
   DOI: http://dx.doi.org/10.1007/s11239-024-03040-8
   IF: 2.3 (2023)

List of other publications

3. Varga, Á., **Mátrai, Á. A.**, Fazekas, L., Al-Khafaji, M. Q., Ványolos, E., Deák, Á., Szentkereszty, Z., Pető, K., Németh, N.: Changes in microcirculation of small intestine end-to-end anastomoses in an experimental model.
   Microvasc. Res. 156, 1-8, 2024.
   DOI: http://dx.doi.org/10.1016/j.mvr.2024.104731
   IF: 2.9 (2023)

4. Varga, Á., **Mátrai, Á. A.**, Baráth, B., Fazekas, L., Brasil, F. S., Mehta, A., Ványolos, E., Deák, Á., Lesznyák, T., Pető, K., Németh, N.: Local and Systemic Micro-Rheological Changes during Intestinal Anastomosis Operation: a Metabolic Dependence in an Experimental Model.
   Metabolites. 14 (8), 2-15, 2024.
   DOI: http://dx.doi.org/10.3390/metabo14080458
   IF: 3.4 (2023)

5. Al-Smadi, M. W., Fazekas, L., Varga, Á., **Mátrai, Á. A.**, Aslan, S., Beqain, A., Al-Khafaji, M. Q. M., Baráth, B., Novák, L., Németh, N.: Minor micro-rheological alterations in the presence of an artificial saphenous arteriovenous shunt, as an arteriovenous malformation model in the rat.
Clin. Hemorheol. Microcirc. 87 (1), 27-37, 2024.
DOI: http://dx.doi.org/10.3233/CH-231825
IF: 2.1 (2023)

6. Bánhegyi, R. J., Veréb, B., **Mátrai, Á. A.**, Rácz, B., Beke, S.: Daganatos betegek glykaemiás és tápláltsági állapotának vizsgálata.
Orv. hetil. 164 (23), 900-910, 2023.
DOI: http://dx.doi.org/10.1556/650.2023.32796
IF: 0.8

7. 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: 4.1

8. 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: 6

9. 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

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10. **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.
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