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The effect of centrifugation at various g force levels on rheological properties of rat, dog, pig and human red blood cells

Ferenc Kiss[a], Eniko Toth[a], Kornel Miszti-Blasius[b] and Norbert Nemeth[a,*]
[a]*Department of Operative Techniques and Surgical Research, Institute of Surgery, Faculty of Medicine, University of Debrecen, Hungary*
[b]*Institute of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary*

Abstract. Laboratory investigations often require centrifugation of blood samples for various erythrocyte tests. Although there is a lack of data about the effect of centrifugation at various g force levels on erythrocyte rheological properties. We aimed to investigate the effect of a 10-minute centrifugation at 500, 1000 or 1500 g at 15°C of rat, dog, pig and human venous (K3-EDTA, 1.5 mg/ml) blood samples. Hematological parameters, erythrocyte deformability, cell membrane stability, osmotic gradient ektacytometry (osmoscan) and erythrocyte aggregation were determined. Hematological and erythrocyte deformability parameters showed interspecies differences, centrifugation caused no significant alterations. Cell membrane stability for human erythrocytes centrifuged at higher g level showed less decrease in deformability. Osmoscan O min parameter showed slight elevation in dog centrifuged aliquots. Erythrocyte aggregation parameters changed unexpectedly. Rat and dog erythrocyte aggregation indices significantly dropped in centrifuged aliquots. Pig erythrocyte aggregation indices increased significantly after centrifugation. Human erythrocyte aggregation was the most stable one among the investigated species. The used centrifugation protocols caused the largest alterations in erythrocyte aggregation in a controversial way among the investigated species. On the other hand, erythrocyte deformability parameters were stable, cell membrane stability and osmoscan data show minor shifts.

Keywords: Red blood cell deformability, red blood cell aggregation, comparative hemorheology, sample preparation, mechanical stress

1. Introduction

Routine laboratory work during sample preparation for various measurements often contains the centrifugation of blood samples. The investigation of red blood cells frequently requires several washing in order to prepare cell suspensions or hematocrit level settled to a standard value, for which the centrifugation of blood samples is used [1, 5, 12].

It is known that mechanical stress causes the injury of erythrocytes, which depends both on the magnitude of the mechanical force and the length of the exposure time [20]. The forces on red blood cells cause alterations in the cell membrane and after a point it leads to irreversible injury of the membrane and

*Corresponding author: Norbert Nemeth, MD, PhD, Department of Operative Techniques and Surgical Research, Institute of Surgery, Faculty of Medicine, University of Debrecen, H-4032 Debrecen, Nagyerdei krt. 98, Hungary. Tel./Fax: +36 52 416 915, E-mail: nemeth@med.unideb.hu.

the cytoskeleton, ending in cytolysis [15, 20]. During sample preparation, centrifugation sub-hemolytic injury to the erythrocytes will not be noticed, although several properties of red blood cell can be affected, which cause alterations in the later performed laboratory investigations. The rheological properties of erythrocytes (deformability and aggregation) are determined by cellular factors, aggregation also by plasmatic ones, which are partially based on the membrane properties and status of the erythrocytes [10, 24], therefore these parameters could be affected if centrifugation causes mechanical stress in the sub-hemolytic zone.

So far there is a lack of data in the literature whether centrifugation during sample preparation affects red blood cell deformability or aggregation, and if yes, at what magnitude. We hypothesized that centrifugation at various g force levels might cause deteriorations in the red blood cell rheological properties, which magnitude might be even different among the species (rat, dog, pig and human).

2. Materials and methods

2.1. Ethical approval

The animal experiment parts were approved and registered by the University of Debrecen Committee of Animal Research (permission Nr.: 19/2011/UD CAR), in accordance with national and EU regulations (the Hungarian Animal Protection Act (Law XVIII/1998) and the Edict 63/2010).

Human blood samples were obtained from volunteers under existing Clinical Ethical Committee approval (permission Nr.: DE OEC RKEB/IKEB 3625–2012).

2.2. Experimental animals, human samples

Eight healthy male Sprague-Dawley outbred rats (age: 6–8 months; bodyweight: 408 ± 59.7 g), 7 healthy female inbred beagle dogs (age: 17–19 months; bodyweight: 12.6 ± 1.3 kg) and 9 healthy female Hungahib pigs (age: 10–12 weeks; bodyweight: 16.2 ± 1.9 kg) were involved into the experiments.

Human blood samples were collected from 15 healthy volunteers (11 females and 4 males; age: 26–40 years).

2.3. Blood sampling and experimental protocol

Blood sampling occurred in the morning hours using 21 G BD Eclipse™ blood collection needle into 3 ml BD Vacutainer® tube containing 1.5 mg/ml K_3-EDTA as anticoagulant (Becton, Dickinson and Company, USA) [1].

Blood sampling in case of the rat was performed under general anaesthesia (60 mg/kg, i.p. Thiopenthal®) by direct cardiac puncture. After sampling the rats were sacrificed by exsanguination. From beagle dogs blood was obtained by cephalic vein puncture and from pigs under general anaesthesia (10 mg/kg, i.m. ketamin; 1 mg/kg, i.m. xylazin) by medial saphenous vein puncture.

Human blood samples were collected via puncturing the median cubital vein.

Each sample after gentle mixing was divided into 4 aliquots: native (base), '500 g', '1000 g' and '1500 g'. Aliquots were centrifuged for 10 minutes at 15°C at 500, 1000 or 1500 *g*, respectively (Hettich Universal 32 R centrifuge, Hettich Co., Germany). Besides centrifugation no other preparation or intervention was performed on the samples.

Laboratory measurements were completed within 2 hours, to avoid the effect of storage on red blood cell properties, especially the highly sensitive erythrocyte aggregation [21]. All aliquots were carefully and gently mixed before laboratory test measurements to provide homogenous samples.

2.4. Laboratory investigations

To check whether centrifugation causes changes in main red blood cell describing variables that have an effect on the erythrocyte-rheological properties quantitative and qualitative hematological parameters were determined by a Sysmex F-800 semi-automated microcell counter (TOA Medical Electronics Co., Ltd., Japan). The test requires approximately 70 μl of blood. Red blood cell count (RBC [T/l]), mean corpuscular volume (MCV [fl]) and mean corpuscular hemoglobin concentration (MCHC [g/dl]) were analyzed in this study. For analyzing the effect of centrifugation between the species relative values were calculated (changes in percentage versus own base value).

By a LoRRca MaxSis Osmoscan (Mechatronics B.V., The Netherlands) several erythrocyte-rheological properties were determined. Red blood cell deformability, red blood cell membrane stability and osmotic-gradient red blood cell deformability (osmoscan) tests were performed using the ektacytometer based on laser-diffraction. 360 kDa molecular weight polyvinylpyrrolidone (PVP 360, Sigma-Aldrich Corp., USA) was used to create high viscosity in isotonic phosphate-buffer solution (PVP-PBS, 33.4 mPa.s, 295–310 mOsmol/kg, pH 7.2) as a required medium for the ektacytometric tests for passing the applied shear stress to the erythrocytes to make their passive elongation possibly to the shear stress. In case of conventional red blood cell deformability measurement 10 μl blood was gently mixed in 1 ml PVP-PBS. The device determines the elongation index (EI) of red blood cells at known shear stress (SS, [Pa]) levels by the analysis of the laser-diffractogram. Higher EI values refer to higher deformation of the cells [12]. For the comparison of the EI-SS curves EI value quantified at 3 Pa and the maximal elongation index (EI_{max}) and the shear stress required for the half of the maximal elongation ($SS_{1/2}$, [Pa]) – calculated by the device's software based on the Lineweaver-Burk equation – and there ratio (EI_{max} / $SS_{1/2}$) were analyzed [1, 2, 12]. For analyzing the effect of centrifugation between the species relative values were calculated (changes in percentage versus own base value).

For red blood cell membrane stability measurement the instrument performs two conventional deformability tests from the same sample under the previously described way prior to and after a 5-minute, 100 Pa shearing stress to the erythrocytes. Sensitive, injured cells acquire more trauma due to this magnitude of applied stress causing their deformability to decrease [4, 15], so the 'after' deformability test determines lower erythrocyte deformation capacity. For data comparison the ratio of the after and before values was calculated from the same type of parameters that were used at the conventional red blood cell deformability test and for interspecies comparison relative values were calculated (changes in percentage versus own base value).

In case of the osmotic-gradient red blood cell deformability (osmoscan) test 250 μl blood is gently mixed in 5 ml PVP-PBS and the device measures EI at a constant shear stress (30 Pa), while the sample is continuously pumped into the measuring chamber where the osmolality is step-wise changing from 0–500 mOsmol/kg by precisely mixing of 0 and 500 mOsmol/kg PVP-distilled water, PVP-PBS solutions, respectively (33.4 mPa.s, pH 7.2). In the hypoosmotic range the instrument determines the osmolality point, where cells are swollen maximally, so it their critical hemolytic volume (O min, [mOsmol/kg]) and the elongation index related to this osmolality value is the EI min parameter. Also it measures the point, where the elongation of cells is the highest (O EI max, [mOsmol/kg], EI max), which refers to the optimal osmolality for the erythrocytes with the most favorable surface-volume ratio, and in the hyperosmotic

range the point, where the EI is half of the EI max (O hyper, [mOmol/kg], EI hyper). As red blood cell membrane acquires injury or just being sensitive against trauma the minimum values of the test increase and the maximum ones decrease, so the area of the elongation index – osmolality curve (Area, [au]) shrinks, narrows [9]. For analyzing the effect of centrifugation between the species relative values were calculated (changes in percentage versus own base value).

Red blood cell aggregation was tested by a Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany). The device is based on light transmission method and requires approximately 20 μl of blood. The test starts with the disaggregation of the sample at 600 s^{-1} for 10 seconds then suddenly the shear rate drops to zero (M mode) or to a low value (3 s^{-1}, M1 mode). The instrument determines the aggregation index by the light transmission intensity change through the sample during the first 5 or 10 seconds of the aggregation process (M 5 s, M 10 s, M1 5 s, M1 10 s). Higher index values refer to enhanced red blood cell aggregation.

2.5. Statistical analysis

Data are presented as means ± S.D. Intra-group comparison was performed using one way ANOVA with Bonferroni *post hoc* test or in case normality test failed Kruskal-Wallis one way ANOVA on Ranks with Dunn's *post hoc* test was used. For the comparison of the human base values to the investigated animal species' ones two-sample Student's *t* test or Wilcoxon Rank Sum test was used depending on the distribution of the data. $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Red blood cell describing hematological parameters

Table 1 shows the analyzed hematological parameters. Base values showed expected interspecies differences. Red blood cell count was significantly higher in all species versus human (human < pig < dog < rat) ($p < 0.001$). Mean corpuscular volume was significantly lower in all species compared to human (rat < pig < dog < human) ($p < 0.001$). Mean corpuscular hemoglobin concentration was significantly lower in the pig versus the human ($p = 0.002$). Rat and dog values were significantly higher compared to human ($p = 0.003$ and $p = 0.033$, respectively).

In case of the rat and dog centrifuged aliquots a slight decrease (up to 10%) was notable in red blood cell count compared to the base aliquots' value while the pig and human sample showed basically no changes. Centrifugation caused a slight cell swelling (about 2–5%) in the dog samples. Mean corpuscular hemoglobin concentration slightly increased in all centrifuged aliquots showing the largest (about 4–12%) increase in the human. The difference of the changes in the analyzed hematological due to centrifugation was not significant between the species versus human.

3.2. Red blood cell deformability

Figure 1 shows the elongation index – shear stress curves of all groups of the investigated species. Beside the expected interspecies differences, centrifugation did not alter the elongation index curves in any of the species. Table 2 shows the calculated parameters, in which interspecies differences can be nicely seen in the base values. EI at 3 Pa was significantly ($p < 0.001$) lower in the dog versus the

Table 1
Selected red blood cell describing quantitative and qualitative hematological parameters of rat, dog, pig and human samples. Data of centrifuged aliquots are presented as relative changes to the base values

Variable	Species	Base in absolute value	Relative change vs. base value [%]		
			500 g	1000 g	1500 g
RBC [T/l]	rat	$7.84 \pm 1.12^{\#}$	91.14 ± 13.05	95.04 ± 13.94	100.11 ± 11.41
	dog	$7.21 \pm 0.74^{\#}$	90.88 ± 6.41	95.39 ± 9.33	91.61 ± 11.45
	pig	$6.19 \pm 0.62^{\#}$	101.57 ± 9.1	100.99 ± 11.35	98.66 ± 6.31
	human	5.09 ± 0.6	105.98 ± 22.95	98.62 ± 22.49	102.61 ± 17.28
MCV [fl]	rat	$58.5 \pm 4.17^{\#}$	100.13 ± 4.2	102.49 ± 4.22	102.76 ± 3.62
	dog	$74 \pm 1.72^{\#}$	103.61 ± 3.46	105.19 ± 4.4	102.36 ± 2.19
	pig	$63.63 \pm 4.79^{\#}$	97.5 ± 6.35	98.98 ± 7.79	99.29 ± 7.53
	human	92.78 ± 8.13	100.57 ± 5.73	100.27 ± 2.7	102.15 ± 5.99
MCHC [g/dl]	rat	$27.54 \pm 3.45^{\#}$	106.68 ± 18.63	103.6 ± 20.4	104.05 ± 19.12
	dog	$28.51 \pm 1.36^{\#}$	104.64 ± 7.82	100.79 ± 9.9	104.71 ± 7.43
	pig	$23.61 \pm 1.23^{\#}$	102.59 ± 7.46	101.16 ± 9.77	102.99 ± 9.91
	human	26.24 ± 2.65	109.8 ± 24.69	111.83 ± 35.68	104.33 ± 11.81

means $\pm$ S.D., $^{\#}p < 0.05$ vs. human.

human. Rat and pig values were significantly higher ($p < 0.001$ and $p = 0.033$, respectively) compared to the human. EI_{max} values were significantly ($p < 0.001$) lower in the pig and higher in the dog versus the human. $SS_{1/2}$ was the highest in the dog and lowest in the pig. Both were statistically different compared to human values ($p < 0.001$ and $p = 0.002$, respectively). EI_{max} / $SS_{1/2}$ values were the lowest in the dog and highest in the rat. Both were statistically different compared to the human ($p < 0.001$ and $p = 0.047$, respectively).

In the centrifuged aliquots EI_{max} values were stable in all species, centrifugation did not cause any change in them. Elongation index value measured at 3 Pa were slightly decreased (about 3–4%) in rat and human sample due to centrifugation, which caused a slight increase (about 3–8%) of the $SS_{1/2}$ value in these two species. Centrifugation induced changes between the species and the human were not statistically significant in any of the red blood cell deformability parameters.

3.3. Red blood cell membrane stability

Figure 2 shows the after-before ratio of the elongation index – shear stress curves of the aliquots, which were determined before and after of the 5-minute 100 Pa shear stress and Table 3 the calculated, numerical results. Because of the irregular shape of the 'after' elongation index – shear stress curves under 0.95 Pa, the ratios are presented only above 0.95 Pa. Interesting interspecies differences can be noticed between the shapes of the curves. Red blood cells showed generally the largest decrease in their deformability due to the shear stress in the human. EI values decreased by 2.9–13% under 10 Pa and 18–34.6% above 10 Pa shear stress. Rat erythrocytes showed 10.6–37.6% decrease in the elongation index values determined under 10 Pa but at higher shear stress levels EI decreased only by 2.7–8.2% compared to the before 5-minute 100 Pa shear stress deformability values. Dog erythrocytes showed 22.5–35% higher EI values after the shearing at 0.95 Pa but above 1 Pa EI was generally 13.2–27.5% lower. Pig samples showed only a minor decrease compared to the other samples. After the shearing their erythrocytes' EI value measured

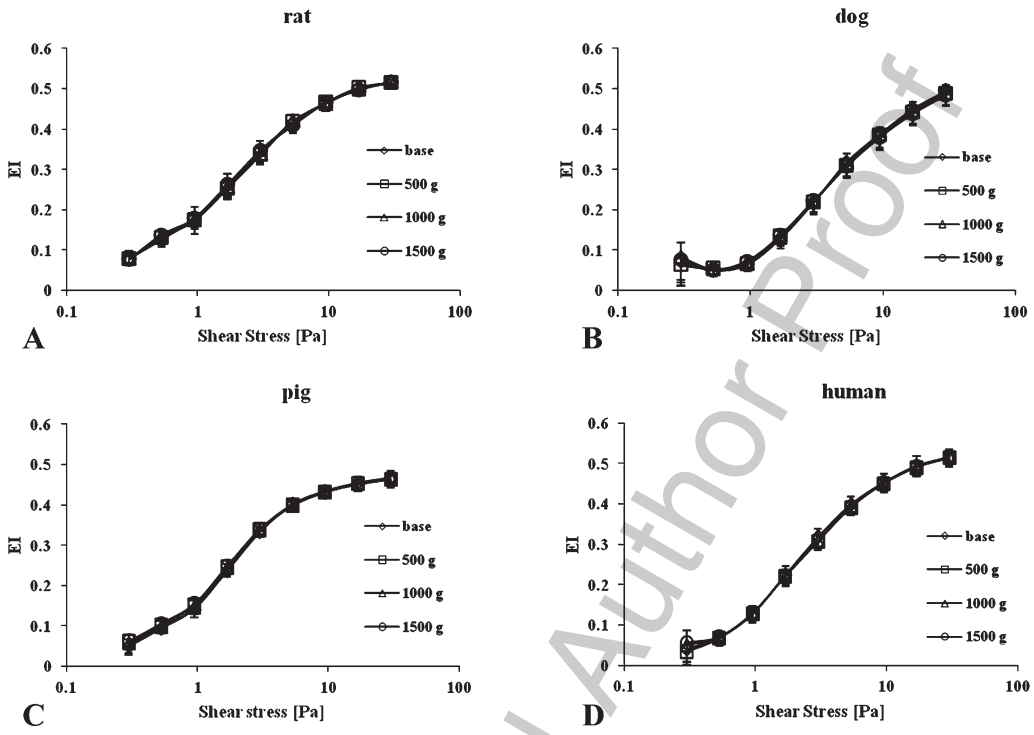

Fig. 1. Red blood cell elongation index (EI) – shear stress [Pa] curves of rat (A), dog (B), pig (C) and human (D) base and centrifuged aliquots.

under 10 Pa dropped only by 1.2–24% and at high shear stress levels it even showed increase up to 2.7%. In Table 3 the ratio of the calculated parameters after and before the mechanical stress numerically showed the interspecies differences in the base samples. The ratio of EI at 3 Pa was significantly higher in all investigated species versus human (rat: $p = 0.003$; dog: $p = 0.044$; pig: $p < 0.001$). The ratio of EI_{max} was significantly lower in dog compared to human ($p = 0.041$). The ratio of $SS_{1/2}$ values were significantly lower in rat and pig versus human ($p = 0.003$ and $p = 0.002$, respectively), while dog showed significantly higher values compared to human ($p = 0.019$). The ratio of EI_{max} / $SS_{1/2}$ values were significantly higher in all investigated species compared to human ones (rat: $p = 0.003$; dog: $p = 0.005$; pig: $p < 0.001$).

By looking at the before and after elongation index – shear stress curves (Fig. 2) there is not an obvious change that could be noticed due to the centrifugation in any of the investigated species except the human samples, where centrifugation at higher g level seemed to result less decrease in deformability due to the shearing stress. The calculated parameters (Table 3) showed some minor alterations, $SS_{1/2}$ after/before values increased up to 5% in the human and up to 8% in the rat. Centrifugation did not cause significant changes in the red blood cell membrane stability parameters between the species and the human.

3.4. Osmotic gradient ektacytometry

Table 4 shows the Osmoscan results, of which base values showed the largest interspecies differences in EI min, O min, O EI max and Area parameters. EI min values were significantly lower in the dog and pig versus the human ($p = 0.002$ and $p < 0.001$, respectively). O min was the lowest in the dog and the

Table 2
Red blood cell deformability describing parameters of rat, dog, pig and human samples. Data of centrifuged aliquots are presented as relative changes to the base values

Variable	Species	Base in absolute value	Relative change vs. base value [%]		
			500 g	1000 g	1500 g
EI at 3 Pa	rat	$0.346 \pm 0.025^{\#}$	96.94 ± 4.92	97.79 ± 3.04	99.89 ± 4.47
	dog	$0.218 \pm 0.019^{\#}$	100.81 ± 9.21	100.06 ± 8.22	101.81 ± 10.19
	pig	$0.331 \pm 0.014^{\#}$	101.91 ± 2.63	100.72 ± 2.04	101.99 ± 1.96
	human	0.319 ± 0.019	96.38 ± 5.62	96.97 ± 4.99	97.23 ± 5.77
EImax	rat	$0.541 \pm 0.017^{\#}$	99.48 ± 2.51	99.5 ± 2.17	100.67 ± 1.27
	dog	$0.51 \pm 0.017^{\#}$	101.3 ± 4.61	100.02 ± 6.38	99.08 ± 5.06
	pig	$0.47 \pm 0.021^{\#}$	99.04 ± 2.82	99.77 ± 1.38	99.13 ± 2.16
	human	0.538 ± 0.026	100.68 ± 3.44	100.21 ± 3.36	99.99 ± 3.19
SS ½ [Pa]	rat	2 ± 0.39	108.25 ± 14.37	105.56 ± 13.87	97.78 ± 12.28
	dog	$4.35 \pm 0.6^{\#}$	99.49 ± 10.66	98.78 ± 13.46	101.62 ± 14
	pig	$1.81 \pm 0.25^{\#}$	97.91 ± 8.19	99.71 ± 6.35	97.52 ± 7.67
	human	2.23 ± 0.47	101.08 ± 17.4	105.26 ± 14.86	103.64 ± 19.22
EImax / SS ½ [Pa^{-1}]	rat	$0.28 \pm 0.05^{\#}$	92.84 ± 13.66	95.8 ± 13.41	104.11 ± 12.3
	dog	$0.119 \pm 0.018^{\#}$	104.71 ± 14.45	104.98 ± 19.82	99.66 ± 17.14
	pig	0.263 ± 0.029	101.39 ± 8.05	100.34 ± 6.46	102.03 ± 7.33
	human	0.254 ± 0.068	101.45 ± 22.12	97.44 ± 18.54	98.98 ± 22.61

means $\pm$ S.D., $^{\#}p < 0.05$ vs. human.

highest in the pig samples both being statistically different to the human base values ($p < 0.001$). O EI max values were also the lowest in the dog and highest in the pig. Both species showed statistically difference to the human data ($p < 0.001$). Area values were significantly lower in all species versus the human data (rat: $p = 0.005$; dog: $p = 0.017$; pig: $p < 0.001$).

Centrifugation increased pig EI min values by about 8–12%, while O min and O EI max values slightly increased in dog centrifuged aliquots (about 4–7% and 3–6%, respectively). Changes in osmoscan data caused by the centrifugation were not significant between the species and the human parameters.

3.5. Red blood cell aggregation

Red blood cell aggregation index values also showed interspecies differences in the base samples (Fig. 3). Rat and pig samples had significantly lower aggregation index values at M 5 sec mode versus the human values ($p < 0.001$). Parameters at M1 5 sec mode were significantly lower in the rat ($p = 0.002$) and higher in the dog and pig versus the human ($p < 0.001$ and $p = 0.061$, respectively). At M 10 sec rat and pig samples indices were lower compared to the human, the difference was significant in case of the pig ($p = 0.077$ and $p < 0.001$, respectively). M1 10 sec indices were slightly lower in the rat and slightly higher in the dog and pig versus the human. In case of the pig the difference was statistically significant ($p = 0.014$).

Centrifugation caused various shifts in the aggregation indexes being different both in the magnitude and the direction in the investigated species, therefore values were analyzed as absolute values for easier interpretation (Fig. 3). Rat blood cells aggregation indices showed a continuous, even higher than 50%

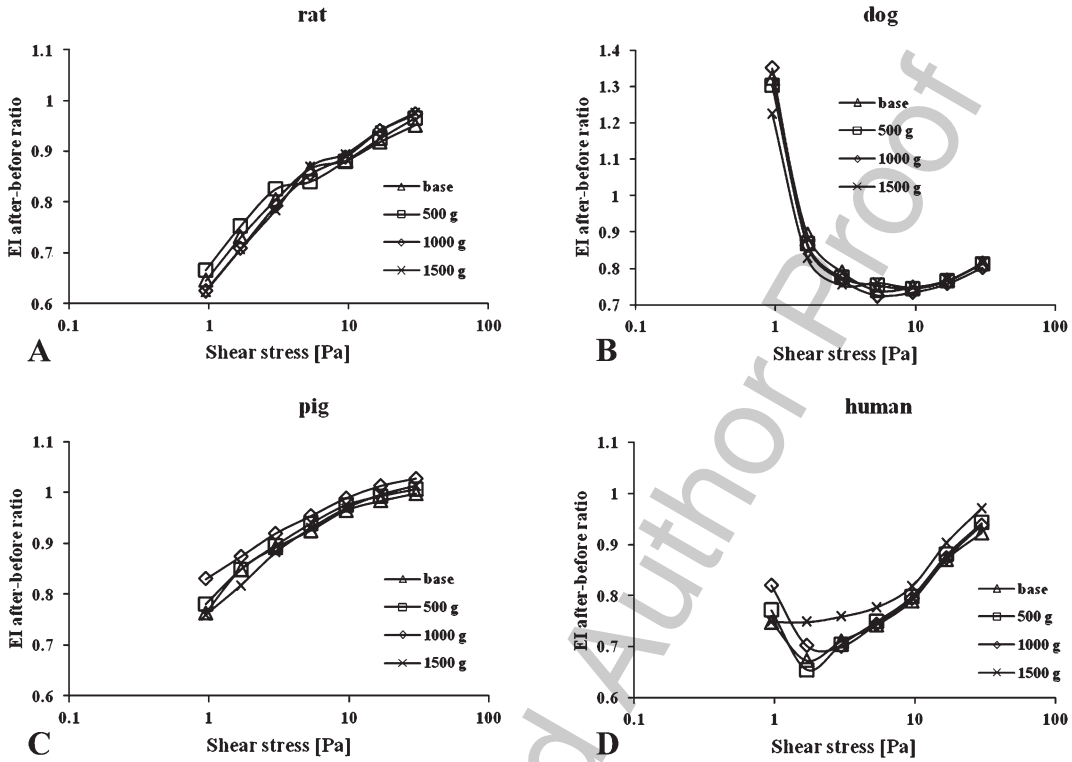

Fig. 2. Ratio of the 5-minute 100 Pa shear stress application after and before measured red blood cell elongation index (EI) – shear stress [Pa] curves of rat (A), dog (B), pig (C) and human (D) base and centrifuged aliquots.

drop due to higher g level centrifugation and almost every aliquots had significantly lower values compared to its base ($p < 0.001$).

Due to the centrifugation canine red blood cell aggregation index values also decreased but its magnitude was smaller than in the rat and it mostly affected the results determined at the 5th second of the aggregation process. All M1 5 s values were significantly lower compared to the base value ($p < 0.001$).

Porcine red blood cell aggregation index values showed increase in the centrifuged aliquots. When aggregation index was determined at stasis (M mode) the indices in all aliquots were about 2 times higher compared to the base values ($p < 0.001$).

Centrifuged human red blood cells showed a slice aggregation index increase at M mode but generally their aggregation index values were stable compared to the other species' ones.

4. Discussion

The aim of any laboratory test is to provide information about the actual state, condition of the investigated patient. Since many tests can only be carried out under *in vitro* conditions it is high of importance to use proven, standardized techniques to avoid or minimize any changes in the investigated parameters due to the *in vitro* conditions. For all this purpose guidelines for the clinical laboratory practice have been developed that are based on studies investigating the effect of various sampling techniques [8, 13],

Table 3
Red blood cell membrane stability parameters of rat, dog, pig and human samples. Data of centrifuged aliquots are presented as relative changes to the base values

Variable	Species	Base in absolute value	Relative change vs. base value [%]		
			500 g	1000 g	1500 g
EI at 3 Pa after/before	rat	$0.8 \pm 0.07^{\#}$	103.07 ± 8.96	98.92 ± 11.2	98.15 ± 9.93
	dog	$0.79 \pm 0.11^{\#}$	99.33 ± 16.58	98.4 ± 13.48	96.64 ± 11.14
	pig	$0.89 \pm 0.06^{\#}$	99.35 ± 5.25	102.47 ± 3.52	99.12 ± 10.57
	human	0.71 ± 0.06	99.94 ± 11.82	99.3 ± 11.84	104.36 ± 10.61
EI_{max} after/before	rat	0.9 ± 0.05	103.96 ± 4.21	103.57 ± 2.99	103.59 ± 3.52
	dog	$0.85 \pm 0.08^{\#}$	99.84 ± 7.62	102.49 ± 12.65	102.02 ± 8.02
	pig	0.98 ± 0.04	100.56 ± 3.25	103.45 ± 5.05	100.5 ± 7.38
	human	0.92 ± 0.11	102.82 ± 20.61	104.6 ± 20.24	103.73 ± 19.85
SS $^1/_2$ [Pa] after/before	rat	$1.89 \pm 0.29^{\#}$	98.63 ± 13.55	111 ± 32.57	115.13 ± 27.61
	dog	$1.81 \pm 0.52^{\#}$	82.92 ± 15.52	115.03 ± 28.81	102.27 ± 19.2
	pig	$1.43 \pm 0.21^{\#}$	103.16 ± 7.83	95.17 ± 6.82	108.26 ± 12.16
	human	3.16 ± 1.33	114.73 ± 50.32	121.27 ± 47.97	113.14 ± 41.46
EI_{max} / SS $^1/_2$ [Pa^{-1}] after/before	rat	$0.49 \pm 0.09^{\#}$	107.28 ± 16.32	100.24 ± 27.9	94.56 ± 22.82
	dog	$0.49 \pm 0.11^{\#}$	123.49 ± 23.58	93.07 ± 22.06	102.55 ± 19.46
	pig	$0.7 \pm 0.1^{\#}$	97.78 ± 4.92	109.42 ± 12.56	93.82 ± 12.55
	human	0.33 ± 0.11	106.48 ± 55.6	106.55 ± 62.5	99.66 ± 30.1

means $\pm$ S.D., $^{\#}p < 0.05$ vs. human.

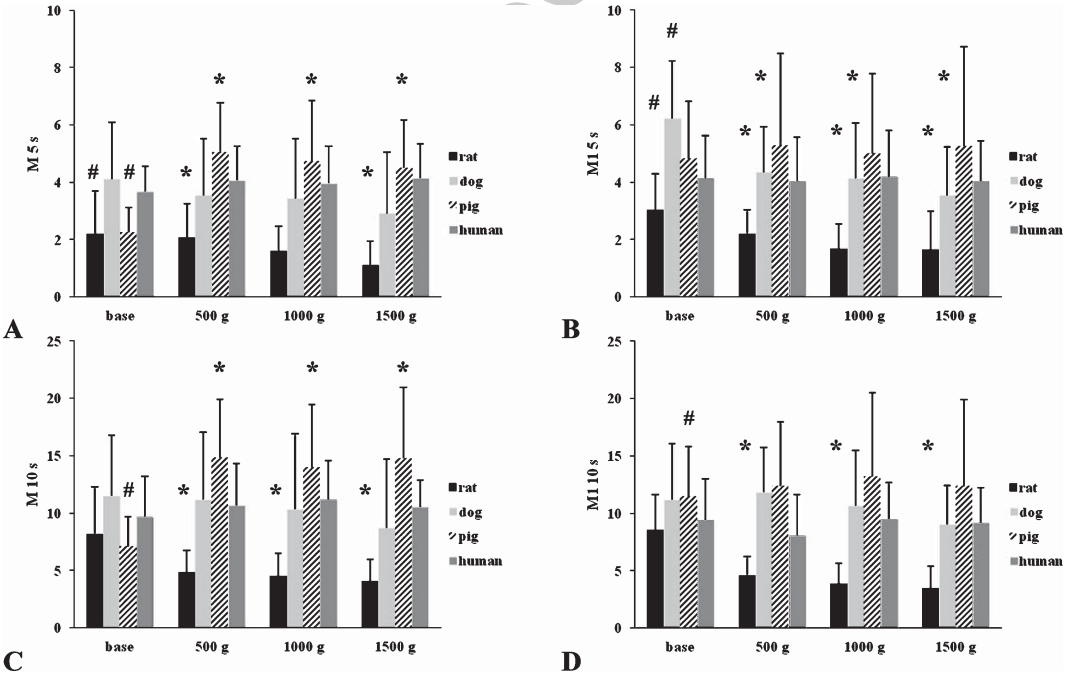

Fig. 3. Aggregation index M 5 s(A), M1 5 s (B), M 10 s (C) and M1 10 s(D) values of rat, dog, pig and human base and centrifuged aliquots. means $\pm$ S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. human.

Table 4
Red blood cell osmotic gradient ektacytometry (Osmoscan) parameters of rat, dog, pig and human samples. Data of centrifuged aliquots are presented as relative changes to the base values

Variable	Species	Base in absolute value	Relative change vs. base value [%]		
			500 g	1000 g	1500 g
EI min	rat	0.133 ± 0.01	99.9 ± 15.22	110.65 ± 8.28	98.33 ± 11.51
	dog	$0.107 \pm 0.02^{\#}$	101.46 ± 12.44	106.97 ± 16.24	103.36 ± 12.87
	pig	$0.094 \pm 0.01^{\#}$	112.15 ± 13.88	108.92 ± 14.51	108.57 ± 18.07
	human	0.134 ± 0.02	94.73 ± 15.42	101.62 ± 17.15	100.17 ± 15.17
EI max	rat	0.52 ± 0.01	99.81 ± 1.91	101.35 ± 1.53	100.69 ± 1.02
	dog	0.525 ± 0.01	98.47 ± 1.78	98.58 ± 1.49	98.16 ± 2.16
	pig	0.501 ± 0.01	100.41 ± 1.44	97.93 ± 3.84	99.85 ± 1.97
	human	0.516 ± 0.01	99.31 ± 2.53	100.27 ± 3.18	99.73 ± 2.67
EI hyper	rat	0.26 ± 0.003	99.85 ± 1.76	101.38 ± 1.5	100.61 ± 0.96
	dog	0.263 ± 0.005	98.44 ± 1.78	98.55 ± 1.53	98.05 ± 2.2
	pig	0.251 ± 0.007	100.41 ± 1.45	97.93 ± 3.75	99.77 ± 2
	human	0.258 ± 0.007	99.2 ± 2.68	100.24 ± 3.24	99.67 ± 2.73
O min [mOsmol/kg]	rat	150.86 ± 8.86	99.73 ± 1.26	100.16 ± 3.02	100.16 ± 3.83
	dog	$129 \pm 9.8^{\#}$	107.06 ± 5.95	108.09 ± 5.55	104.44 ± 7.45
	pig	$179.75 \pm 12.2^{\#}$	99.8 ± 3.69	100.17 ± 3.36	101.1 ± 4.85
	human	152.23 ± 5.18	98.44 ± 4.54	99.84 ± 3.2	99.96 ± 2.94
O EI max [mOsmol/kg]	rat	301.38 ± 28.71	98.53 ± 3.47	102.78 ± 8.23	104.58 ± 14.01
	dog	$261.29 \pm 12.94^{\#}$	106.64 ± 3.5	105.55 ± 5.04	103.86 ± 5.11
	pig	$348.25 \pm 22.02^{\#}$	101.57 ± 3.51	101.86 ± 4.34	102.31 ± 5.47
	human	303.07 ± 16	102.31 ± 6.46	99.67 ± 3.69	98.86 ± 5.77
O hyper [mOsmol/kg]	rat	444.38 ± 32.19	99.85 ± 1.31	100 ± 1.08	99.72 ± 1.82
	dog	410.67 ± 14.79	105 ± 4.87	105.13 ± 5.18	103.46 ± 5.15
	pig	474.63 ± 9.69	99.21 ± 2.15	100.09 ± 2.49	99.79 ± 2.65
	human	456.79 ± 12.15	99.81 ± 0.8	99.9 ± 1.68	100.17 ± 0.92
Area [au]	rat	$132.03 \pm 6.75^{\#}$	101.1 ± 2.47	96.88 ± 5.52	100.38 ± 5.38
	dog	$134.05 \pm 3.9^{\#}$	103.52 ± 4.79	102.35 ± 4.93	101.13 ± 4.55
	pig	$120.38 \pm 5.81^{\#}$	99.88 ± 4.45	98.64 ± 7.22	97.77 ± 6.91
	human	139.72 ± 4.49	98.65 ± 2.26	99.79 ± 2.45	100.23 ± 2.64

means $\pm$ S.D., $^{\#}p < 0.05$ vs. human.

preferred anticoagulant types for different blood tests [6, 25, 26], sample preparation [11, 28], sample handling [14, 27] and storage time (*in vitro* aging) [19, 23].

In experimental research all the above mentioned circumstances should be standardized, sometimes species specific way to be able to provide extrapolabe data for the clinical practice. For this the colorful interspecies differences have to be revealed and described and even many times laboratory methods also have to be adjusted, fine tuned species specific ways [12, 29]. Furthermore sample handing and preparation also needed to be investigated for the laboratory/experimental animal species used in research because interspecies differences (e.g.: rat and dog erythrocytes are sensitive for environmental changes) can cause that sample handling or preparation may affect laboratory parameters in a different way at a different magnitude that can be completely colorful among the species [3, 21, 29]. One of these sample preparation

step is centrifugation that is a daily used task in laboratory work to separate cells by type or age, or to prepare washed red blood cells, which can be suspended in various media for different investigations.

Therefore in this project we focused on the hemorheological effect of centrifugation at various g force levels in rat, dog, pig and human blood samples. Our main findings besides the expected interspecies differences were the followings: (1) Out the quantitative and qualitative hematological parameters, the investigated red blood cell describing factors (RBC, MCV, MCHC) were stable and 10-minute centrifugation at 500, 1000 or 1500 *g* at 15°C did not cause significant changes in them versus their base values. (2) Red blood cell deformability as one of the rheological properties of erythrocytes was stable against the investigated centrifugation protocols, it did not show significant changes in any of the examined species. (3) Red blood cell membrane stability test, which serve information about the mechanical stress induced injury-susceptibility of the erythrocyte membrane did not show significant alterations in the centrifuged aliquots versus their base values, although human erythrocytes seemed to show less decrease in deformability due to the applied shearing stress in aliquots centrifuged at higher g level. (4) Osmoscan test results only show differences in the dog, out of which is the most important that O min value increased in the centrifuged aliquots, meaning that the majority of red blood cells burst at a less lower osmolality value in the hypoosmolal range. (5) Red blood cell aggregation, as the other erythrocyte-rheological parameter due to the centrifugation altered differently in the investigated species both by the direction and the magnitude of the change.

It is known that similarly to other biological parameters hemorheological properties show interspecies, gender differences and also sampling technique, sample handling, preparation and measurement technical conditions have an effect on them at a very colorful manner [3, 18, 21, 22, 29].

Kaperonis and Chien (1989) investigated high speed centrifugation caused water loss on red blood cells. They found that the minimal force for water loss vary depending on erythrocyte age and health. Water loss caused increase in MCHC, as the major determinant of cell inner viscosity results decrease in deformability and higher susceptibility for shear stress induced cell damage [17]. In our findings centrifugation at 1000 or 1500 g slightly increased MCHC only in human samples without having any effect on MCV. Red blood cell deformability parameters were stable in all species, although erythrocyte cell membrane stability test showed alterations in human samples. The results suggest that human red blood cells centrifuged at higher g force suffered less injury in the deformability capacity by the 5-minute 100 Pa shear stress application. The background of this phenomenon needs to be methodologically more widely and precisely investigated to reveal and understand the so far bit controversial results.

In case of centrifugation not only the shear stress is increasing by higher g levels but the mechanical injury of erythrocyte also may occur by the increasing pressure leading to higher force level on cells as they directly contact or pass by each other. Yasuda et al. (2001) investigated the effect of shear and pressure on red blood cell hemolysis, and they found that beside shear stress, pressure is an important factor determining the hemolysis rate. They measured 4 times higher hemolysis under 600 mmHg versus 0 mmHg pressure at constant 1500 s^{-1} shear rate [30]. Higher pressure can cause cell membrane and cytoskeleton injury at a higher magnitude that could lead to hemolysis. Probably the most notable change what we could detect by the osmoscan test was that dog erythrocytes showed increased susceptibility for osmotic injury in the hypoosmolal range when they were centrifuged at a higher g force. As osmotic gradient ektacytometry provides information about cell membrane and cytoskeleton intactness and strength about osmotic stress [9], our finding might signals that dog erythrocyte are more susceptible to cell membrane and cytoskeletal injury due to centrifugation than the other investigated species.

Our findings about red blood cell aggregation changes due to centrifugation showed large diversity among the investigated species. Rat erythrocytes showed the largest drop in aggregation index values

in the centrifuged aliquots but dog red blood cell aggregation indices also decreased. From a previous experiment, where rat and dog blood samples red blood cell rheological properties were investigated under various *in vitro* conditions (storage time and temperature) we found that aggregation property was the most sensitive to *in vitro* environment conditions, especially in case of the rat [21]. However, pig red blood cells showed a large increase in the aggregation parameters and human samples seemed to be the most stable one at this investigation protocol using light-transmission method. The cause of these colorful changes between the aggregation profiles of the investigated species is not revealed so far. Red blood cell aggregation determining cellular factors like deformability and morphology (MCV) were stable in all species. The third cellular factor, the glycocalyx structure could not be investigated in this study. However, membrane and glycocalyx injuries may also lead to a change in the surface charge of the erythrocytes that would cause shift in the electrostatic repulsion force between the cells. Furthermore any change in the conformation of fibrinogen, as a plasmatic determining factor for aggregation, may cause alteration in the fibrinogen non-covalent binding capacity to erythrocytes and glycocalyx penetration capacity, which would affect red blood cell aggregation forces, as well [24].

Butler et al. (1992) investigated red blood cell susceptibility for mechanical stress induced hemolysis, and found that plasma and plasma components provide protection for erythrocytes against hemolysis compared to other suspending media (e.g.: isotonic tris-buffered saline) [7]. Kameneva et al. (2003) also found similar results on bovine red blood cells. In their experiments bovine erythrocytes were suspended in various media and similar to plasma, polyethylene glycol (20 kDa, 2% solution) had significant erythrocyte protective function against shear stress induced hemolysis compared to phosphate buffered saline or Dextran 40 solution [16]. Probably, behind their findings can be the protective role of macromolecules. Maybe as they cannot penetrate the glycocalyx layer of red blood cells, they can reduce erythrocyte membrane injuries due to cell-to-cell contact interactions, help erythrocytes to keep up their negative surface charge and maybe other plasma components can help to stabilize the cell membrane properties. Of course for investigating these questions and theories further experiments with wider and more membrane specific investigating methods are required.

5. Conclusion

Centrifugation at 500, 1000 or 1500 *g* force levels for 10 minutes at 15°C caused significant and controversial alterations in erythrocyte aggregation- determined by light-transmission method. It dropped in rat and dog samples, increased in the pig and was relatively stable in the human. On the other hand, erythrocyte deformability parameters were stable, cell membrane stability and osmoscan data show minor shifts. Therefore in red blood cell aggregation studies, if it is possible centrifugation should be avoided during sample preparation (mainly in case of rat, dog and pig blood samples), or at least the diverse effect of centrifugation on erythrocyte aggregation properties should be taken into consideration, when evaluating data.

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