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ORIGINAL ARTICLE

Interpretation of osmotic gradient ektacytometry (osmoscan) data: A comparative study for methodological standards

[AQ1] NORBERT NEMETH[1], FERENC KISS[1] & KORNEL MISZTI-BLASIUS[2]

[AQ2] [1]*Department of Operative Techniques and Surgical Research, Institute of Surgery and* [2]*Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary*

Abstract

Osmotic gradient ektacytometry (measuring elongation index in the function of osmolality at a constant shear stress) is a sensitive method to analyze red blood cell (RBC) deformability and investigating the optimal osmolality range for the cells in normal or pathophysiological cellular and micro-environmental conditions. However, the methodological conditions are different, since the results are influenced by the applied shear stress (SS). In this study we investigated rat, dog, pig and human blood samples at SS of 1, 2, 3, 5, 10, 20 and 30 Pa. To describe the range being related to the cell deformability, we introduced new calculated parameters obtained from the raw data of the elongation index (EI)-osmolality (O) curves. Our results showed that: (1) Osmoscan data tested at 20 or 30 Pa do not differ significantly from each other; (2) Under SS of 20 Pa the EImax, the O (EImax), the EI min and the area under curve nearly linearly decrease in the function of SS with different slope in rat, dog, pig and human blood; (3) Measurements under 3 Pa SS become unstable; (4) The differences between minimal and maximal EI and the belonging osmolality values, and their ratios, as new calculated parameters (ΔEI, ΔO, ΔEI/ΔO, EImax/EImin and O (EImax)/Omin) can be suitable for further analysis of the osmoscan curves together with other hemorheological parameters describing RBC deformability; and (5) Decreased erythrocyte deformability (by rigidifying with glutaraldehyde) can be reflected well with the following, calculated osmoscan parameters: ΔO, rO, rEI/rO and ΔEI/ΔO.

Key Words: *Osmotic gradient ektacytometry, osmoscan, standardization, inter-species differences, comparative investigation*

Introduction

Red blood cell deformability is an essential passive cellular ability mostly in passing through the micro-capillaries, but in the large vessels it also has importance (e.g. decreasing blood viscosity at higher shear rates). Numerous pathophysiological processes may cause impairment in the deformability of erythrocytes, since its determinants include cell surface/volume ratio, cell membrane viscosity, intracellular viscosity (hemoglobin [Hgb] content) and morphological factors [1,2]. Deformability can be investigated well by ektacytometry, among other methods, that determines the magnitude of cell elongation against applied shear stress at known levels [3,4].

Red blood cell deformability is also influenced by the micro-environmental pH and osmolality level [5–10]. Latter manifests deformability changes mostly by cell volume alterations, when cells are shrinking or swelling depending on the magnitude and direction of the osmolality changes compared to the optimal value. The so-called osmotic gradient ektacytometry has been developed and introduced in the early 1980s, which method determines the red blood cell deformability (elongation) at constant shear stress but at gradually changing osmolality [3,11], producing characteristic curves (Figure 1). The maximum point of the osmoscan curve represents the osmolality, at which value the cells have the highest possible elongation index (at given shear stress), the best possible deformability. In their paper, Clark and co-workers presented several results of various hematological disorders, as well as *in vitro* examinations showing various morphologies of those osmoscan curves [11].

For these tests precision ektacytometer is required that has been developed and improved a lot in the

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

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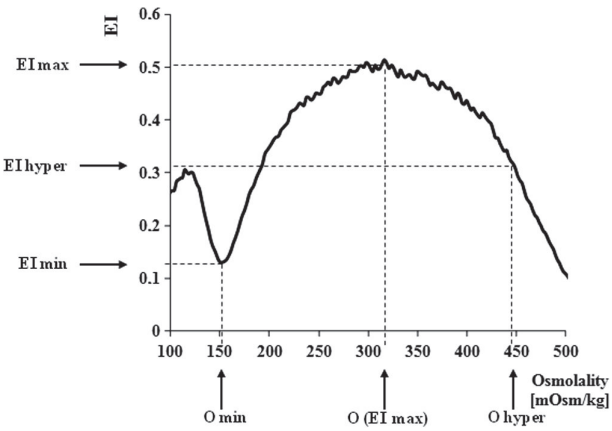

Figure 1. Representative elongation index (EI)-osmolality (O [mOsm/kg]) curve (osmoscan curve) with the parameters determined by the measurement method.

1990s, and couple of years ago the osmoscan function has also been integrated into the latest rotational ektacytometers [4].

Osmotic gradient ektacytometry is a very sensitive method. In hematological diseases, in which the red blood cell morphology, the structure and the cell surface/volume ratio are altered, this method can be informative [11–18]. Among others, Deuel and co-workers analyzed osmotic gradient ektacytometry results in hyperchromatous red blood cell subpopulation [19]. Ballas and Smith reported characteristic osmoscan data in sickle cell anemia [20]. Also in stomacytotosis [21–23], but above all, the most characteristic changes could be found in hereditary elliptocytosis and spherocytosis, in which the osmotic gradient ektacytometry has a diagnostic value [14,24–27].

However, the methodological conditions are different, since the results are influenced by the applied shear stress. Most of the data had been obtained from measurements using shear stress of 20 or 30 Pa, but without any justification. Are those data comparable? It is not known whether osmoscan results at 1, 2, 3, 5, 10, 20 or 30 Pa shear stresses differ and of which magnitude. How comparable are the obtained results of higher shear stress to the *in vivo* relations, where shear stress over 10 Pa is a rarity? Information about this kind of optimalization or comparative analysis hardly could be found in the literature [28,29].

How can osmoscan curves' morphological changes be translated and interpreted? Which parameters of the curves are informative in definitive conditions? What kind of further parameters can be calculated from the raw data that may reflect well the deterioration of red blood cell deformability or interspecies differences?

We planned our study according to these questions, in which not only a methodological comparison was the goal, but presentation of new index parameters for interpretation of osmoscan results in human blood samples and of laboratory/experimental animal species.

Materials and methods

Comparative analysis of osmoscan measurements at various shear stress conditions

The study was performed with permission (permission No: 19/2001 DE MAB), in accordance with the national and EU regulations (Act XXVIII of 1998, Edict 63/2010). After overnight fasting, in the morning hours blood samples were collected from nine CD outbred rats (male, bodyweight: 371.9 ± 28.3 g) by puncturing the lateral tail vein, from six beagle dogs (male and female, bodyweight: 11.2 ± 1 kg) by puncturing the cephalic vein, and via puncturing the medial saphenous vein of 15 juvenile Hungahib pigs (female, bodyweight: 18.2 ± 2.7 kg), using closed blood sampling system with 22 G needles (Vacutainer®, Becton Dickinson Diagnostic-Preanalytical Systems, UK; anticoagulant: K_3-EDTA, 1.5 mg/mL), except for rats. In rats samplings were completed using 25 G needles and syringes containing the anticoagulant.

We also examined blood samples of 15 volunteer healthy adult men and women (aged 26–40 years) with Clinical Ethical Committee approval (permission No: DE OEC RKEB/IKEB 3625-2012). The blood samples were collected (in the morning hours, after overnight fasting) by puncturing an antecubital vein. The anticoagulant was the same (K_3-EDTA, 1.5 mg/mL).

Osmoscan investigation of rigidified red blood cells

As a sub-trial of the study, samples of five volunteers (from the study group above) were centrifuged (800 *g*, 10 min, 15°C), the cells were washed in isotonic phosphate buffered saline (PBS) and red blood cell-PBS suspensions at 40% hematocrit (v/v) were prepared and treated with 0.001% or 0.005% glutaraldehyde (GA). The aim was to determine the deformability deterioration of GA-rigidified red blood cells by the GA treatment [30–32], and analyze whether the deformability impairment is manifested in osmoscan results, searching for the most sensitive parameters (classical or newly calculated ones) describing the differences.

Laboratory techniques

The general quantitative and qualitative hematological parameters were determined by a Sysmex F-800 microcell counter (TOA Medical Electronics Co. Ltd, Japan). Red blood cell deformability was tested by a LoRRca MaxSis Osmoscan device (Mechatronics BV, The Netherlands). Blood sample of 5 µL was

taken into 1 mL of isotonic polyvinyl-pyrrolidone solution (360 kDa PVP in normal phosphate buffered saline; viscosity = 27 mPas, osmolality = 290–300 mOsm/kg; pH ~ 7.3) and mixed gently. At constant temperature of 37°C, the elongation index (EI) values were determined in the function of shear stress (SS) in a range of 0.3–30 Pa, based upon the laser diffraction pattern changes. The EI is equal to (L −W) / (L +W), where L is the length and W is the width of the diffractogram [3,4]. EI increases with red blood cell deformability. For the comparison of individual EI-SS curves Lineweaver-Burk analyses were performed, calculating the maximal elongation index (EI_{max}) and the shear stress values at half EI_{max} ($SS_{1/2}$ [Pa]), according to the following formula: $1/EI = SS_{1/2}/EI_{max} \times 1/SS + 1/EI_{max}$. Furthermore, $EI_{max}/SS_{1/2}$ ratio was also calculated [33].

For the osmoscan test 250 µL of blood was gently mixed in 5 mL PVP solution. During the measurement the device generates a constant shear stress at a given value (can be set before the test), while the osmolality is continuously and gradually rising from 0–500 mOsmol/kg (data can be recorded over this, depending on the high-osmolality PVP solution) and the sample is also continuously aspirated into the measuring chamber. So, at a constant shear stress EI values are measured along the osmolality (Figure 1). The parameters given by the device are the followings: Minimal elongation index values measured at low-osmotic environment (EI min), maximal elongation index values at the given shear stress (EI max; please note that it is not the EI_{max} calculated by the Lineweaver-Burk equation above), half of the maximal elongation index values at high-osmotic environment (EI hyper), osmolality at minimal EI (Omin), osmolality at maximal EI (O [EImax]), osmolality at EI hyper (O hyper) and the area under the individual elongation index-osmolality curves (Area).

When osmoscan test was performed at low shear stress values, due to the unstable shape of the elongation index curve at constant shear stress along elevating osmolality the software could not mark correctly the EI min and O min point values. Therefore in these cases each osmoscan test was individually evaluated and analyzed for setting the correct parameter points using the raw data recorded by the software.

Newly calculated osmoscan parameters

Using the standard parameters of the elongation index-osmolality raw data (see above), the following new parameters were calculated:

- ΔEI = the difference between maximal and minimal EI values;
- ΔO = the difference between osmolality values at maximal and minimal EI
- ΔEI/ΔO;
- EI max/EI min = ratio of maximal and minimal EI values (rEI);
- (EImax)/O min = ratio of osmolality values at maximal and minimal EI (rO)
- rEI/rO.

Statistical analysis

Data are presented as means ± standard deviation (SD). Differences within the groups (e.g. osmoscan values tested at 1, 2, 3, 5, 10, 20 and 30 Pa of the same species' samples) were evaluated by one-way ANOVA (Bonferroni's or Dunn's method), while the inter-group analysis (between species) was carried out using Student's *t*-test or Mann-Whitney rank sum test, depending on the normality of data distribution. Values of native blood samples compared to 40% Hct RBC-PBS suspension and GA treated samples were compared using paired *t*-test or Wilcoxon test, according to the data distribution.

For the comparison of the sensitivity of the measured parameters to detect the existing differences, the standardized difference value was calculated [32,34]. It is the difference of the mean values of groups (e.g. native vs. treated samples) divided by the square root of the sum of their standard deviations' squares:

$$(mean_x - mean_y)/\sqrt{\sum(SD^2{}_x; SD^2{}_y)}.$$

Results

Comparative analysis of osmoscan measurements at various shear stress conditions

Table I shows red blood cells' quantitative and qualitative parameters of the examined animal species and the human. The EI-osmolality curves were proved to be the most regular and stable at 20 and 30 Pa in the examined animals species and the human. Figure 2 shows series of representative osmoscan curves of rat, canine, porcine and human blood samples. At 10 Pa the maximum (the highest) point of the curve started to gently shift to left and downwards and in the hyperosmotic region often undulation occurred. At lower shear stress the phenomenon was more prominent and gradual in its magnitude: Shifting to left and down, and with irregular and flattening hyperosmotic plot part. The tendency seemed to be consequent until about 2–3 Pa. At 1 Pa the curves were extremely irregular –practically not evaluable – in most of the samples. But not that in case of rat blood, where the osmoscan curves seemed to be the most stable in their morphology even at low shear stresses, including 1 Pa, too. These alterations were well detectable by analyzing the numerical data of the individual osmoscan curves (Table II). Only the raw data at 1 and 2 Pa shear stresses had to be re-analyzed skipping the irregular

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Table I. Comparative erythrocyte-related hematological values in rat, dog, pig and human blood samples.

Variable	Rat ($n=9$)	Dog ($n=6$)	Pig ($n=15$)	Human ($n=15$)
RBC [$\times 10^{12}$/L]	$7.13 \pm 0.53^{*\#}$	$6.9 \pm 0.35^{*\#}$	$5.84 \pm 0.65^{*}$	4.99 ± 0.68
Hct [%]	$48.49 \pm 2.11^{\#}$	$52.57 \pm 3.64^{*\#}$	$37.86 \pm 4.1^{*}$	47.38 ± 7.63
Hgb [g/L]	$129.6 \pm 3.7^{\#}$	$136.9 \pm 12^{\#}$	$91.2 \pm 6.4^{*}$	125.1 ± 12.7
MCV [fL]	$68.22 \pm 5.22^{*+}$	$76.05 \pm 1.69^{*\#}$	$65.08 \pm 6.55^{*}$	94.63 ± 6.98
MCH [pg]	$18.23 \pm 1.22^{*\#}$	$19.82 \pm 1.39^{*\#}$	$15.73 \pm 1.47^{*}$	24.88 ± 1.78
MCHC [g/L]	$267.8 \pm 11.4^{\#}$	$260.5 \pm 18.1^{\#}$	$242.6 \pm 20.1^{*}$	263.9 ± 26.5
RDW-CV%	$12.83 \pm 0.62^{\#}$	$13.79 \pm 0.6^{\#}$	$18.07 \pm 1.31^{*}$	13.58 ± 0.6

$^{*}p<0.05$ vs. human; $\#p<0.05$ vs. pig; $^{+}p<0.05$ vs. dog. RBC, red blood cells; Hct, hematocrit; Hgb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; RDW-CV, Red cell distribution width-corpuscular volume.

and unstable data segments. In all other cases data were used as the device calculated.

The maximal elongation index (EI max) values determined at 20 and 30 Pa shear stresses showed the lowest values in the canine and porcine blood samples, while higher values were obtained in rat blood. The highest values were found in human blood. These interspecies differences seemed to be equalized by decreasing the shear stress applied for the measurements.

Osmolality at the EI max values showed regularly the highest values in the porcine blood. The values were lower in human, in canine and in rat blood, in this sequence. EI max values of the curves obtained at 20 and 30 Pa did not show mentionable differences, however, the decreasing were obvious under 20 Pa. In case of O (EI max) values differed even between the 20 and 30 Pa tests. The decrease of O (EI max) in the function of the applied shear stress (1–30 Pa) was almost gradually linear (rat $R^2=0.907$, dog $R^2=0.955$, pig $R^2=0.979$, human $R^2=0.986$).

The parameters of hyperosmotic phase directly determined by the maximal EI values, since its half

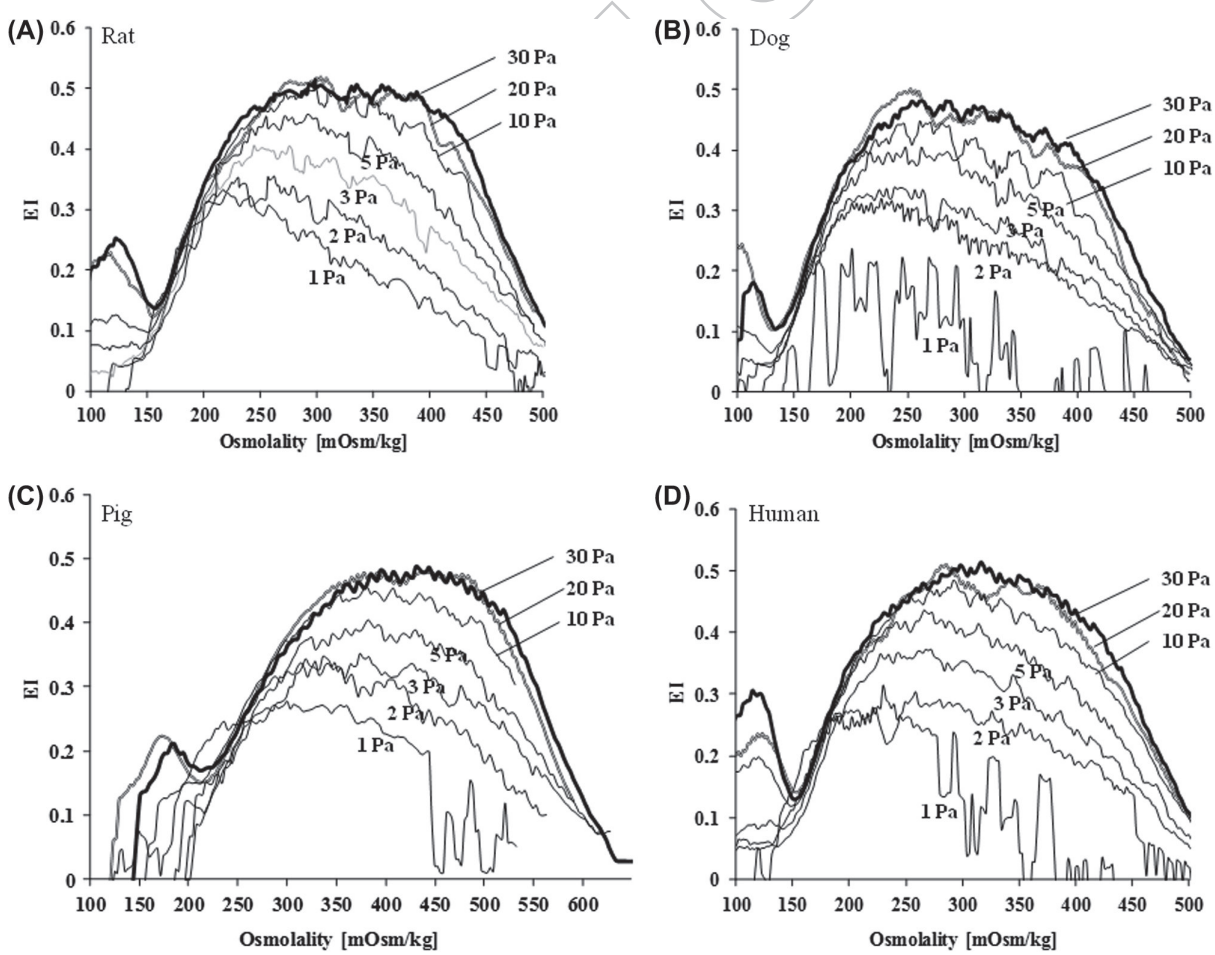

Figure 2. Representative osmoscan curves of rat (A), dog (B), pig (C) and human (D) blood samples tested at various shear stresses (1, 2, 3, 5, 10, 20 and 30 Pa).

Table II. Osmotic gradient ektacytometry data of rat, dog, pig and human blood samples tested at shear stresses of 1, 2, 3, 5, 10, 20 and 30 Pa.

		Shear stress						
Variable	Species	1 Pa	2 Pa	3 Pa	5 Pa	10 Pa	20 Pa	30 Pa
EI min	rat	− 0.061 ± 0.08	0.022 ± 0.03	0.051 ± 0.02	0.070 ± 0.01*	0.102 ± 0.004	0.125 ± 0.007	0.128 ± 0.01
	dog	− 0.162 ± 0.05	− 0.036 ± 0.09	− 0.039 ± 0.08	0.043 ± 0.003*#	0.076 ± 0.009#	0.102 ± 0.007	0.106 ± 0.008#
	pig	− 0.003 ± 0.09	0.005 ± 0.06	0.041 ± 0.06	0.085 ± 0.03*	0.114 ± 0.01	0.145 ± 0.02	0.141 ± 0.02
	human	0.013 ± 0.06	0.021 ± 0.06	0.056 ± 0.01	0.085 ± 0.01*	0.125 ± 0.02	0.124 ± 0.01	0.137 ± 0.01
EI max	rat	0.309 ± 0.01*#	0.362 ± 0.01*#	0.397 ± 0 01*	0.446 ± 0.009*	0.477 ± 0.01	0.508 ± 0.01	0.497 ± 0.02
	dog	0.253 ± 0.02*	0.322 ± 0.02*	0.343 ± 0.01*	0.401 ± 0.01*	0.438 ± 0.01#	0.484 ± 0.02	0.482 ± 0.01#
	pig	0.262 ± 0.04*	0.323 ± 0.02*	0 358 ± 0.01*	0.404 ± 0.01*	0.443 ± 0.009#	0.468 ±.0 008	0.472 ± 0.03
	human	0.273 ± 0.01*	0.314 ± 0.01*	0.370 ± 0.01*	0.430 ± 0.01*	0.472 ± 0.02	0.513 ± 0.01	0.515 ± 0.01
EI hyper	rat	0.154 ± 0.008*	0.181 ± 0.01*	0.199 ± 0.005*	0.223 ± 0.004	0.251 ± 0.03	0.254 ± 0.005	0.248 ± 0.01
	dog	0.126 ± 0.01*	0.161 ± 0.01*	0.171 ± 0.006*	0.201 ± 0.006	0.219 ± 0.007#	0.242 ± 0.008#	0.241 ± 0.005#
	pig	0.131 ± 0.02*	0.162 ± 0.01*	0.179 ± 0.008*	0.202 ± 0.007	0.221 ± 0.004#	0.234 ± 0.004#	0.239 ± 0.007#
	human	0.136 ± 0.008*	0.157 ± 0.007*	0.184 ± 0 007*	0.214 ± 0.007	0.236 ± 0.01	0.256 ± 0.008	0.258 ± 0.007
O min [mOsm/kg]	rat	140 ± 12.1	136 ± 8	136.5 ± 10.5	147.8 ± 4.9	152.7 ± 7.1	157.8 ± 3.1	156.2 ± 6.3
	dog	125.6 ± 16.6	113.6 ± 11.9	110.1 ± 7.6#	117.5 ± 9.8#	124.1 ± 7.7#	127.1 ± 14.2	129.3 ± 11.2
	pig	181.7 ± 16.2#	188.5 ± 19.6#	194.4 ± 14.5#	195.1 ± 19#	203.1 ± 8.8#	205.3 ± 11.4#	204.5 ± 14.3#
	human	125.2 ± 10.8	124.2 ± 9	132.9 ± 9.2	140.1 ± 7.7	146.6 ± 5.5	149 ± 5.4	153.2 ± 5
O (EI max) [mOsm/kg]	rat	235.2 ± 19.9*	261.6 ± 19.8	274 ± 23.7	286.7 ± 6.5	303.8 ± 15.9	302 ± 17.9	307.3 ± 28.6
	dog	198.3 ± 12.3*	220 ± 9.6*	226.4 ± 15.7*	231.6 ± 26.7	244.5 ± 18.7	247 ± 11.2	265.3 ± 29.6
	pig	299 ± 27.7*#	333.9 ± 25.5*#	344.5 ± 19.4*#	361.7 ± 31.1#	380.7 ± 24.8#	401.2 ± 28.9#	409.7 ± 29.3#
	human	200.2 ± 16.1*	226.7 ± 16.3*	242.6 ± 15.1*	255.7 ± 14.4*	270.7 ± 14.6	298.1 ± 13.8	305.2 ± 15.9
O hyper [mOsm/kg]	rat	366.6 ± 45.6*#	419.5 ± 18.2	432.4 ± 6.4	437.1 ± 9	444.7 ± 6	453.6 ± 6.3	461.4 ± 8.7
	dog	226.5 ± 30.1*	394.5 ± 20.8	416.3 ± 11.9	411 ± 17.2	431.5 ± 14.9	436.3 ± 7.6	444.5 ± 13.3
	pig	417.7 ± 88.7*#	518.8 ± 33.1#	543.5 ± 24#	552.4 ± 17.1#	556.5 ± 20.5#	563.7 ± 13.6#	574 ± 12.9#
	human	229.9 ± 66.7*	405.6 ± 40.3	428.9 ± 9.7	438.1 ± 9.7	453.2 ± 13.9	450 ± 12.3	455.4 ± 14.3
Area	rat	49.98 ± 14.14*	74.9 ± 5.21*	88.53 ± 5.76*	99.41 ± 5.24*	112.28 ± 8.19*	126.7 ± 6.68	127.15 ± 8.88
	dog	21.3 ± 9.75*#	69.4 ± 5.18*	77.85 ± 5.41*	94.9 ± 2.85*	109.36 ± 3.36*	123.95 ± 9.35	126.91 ± 6.18
	pig	45.86 ± 15.28*	55.73 ± 6.54*#	65.13 ± 7.9*#	76.38 ± 7.95*#	85.94 ± 6.98#	91.88 ± 7.44#	99.39 ± 12.94#
	human	50.7 ± 11.1*	68.47 ± 6.94*	85.14 ± 5.22*	103.49 ± 4.9*	121.11 ± 5.18*	133.12 ± 6.94	137.82 ± 5.93

*$p < 0.05$ vs. values at 30 Pa; # $p < 0.05$ vs. human.

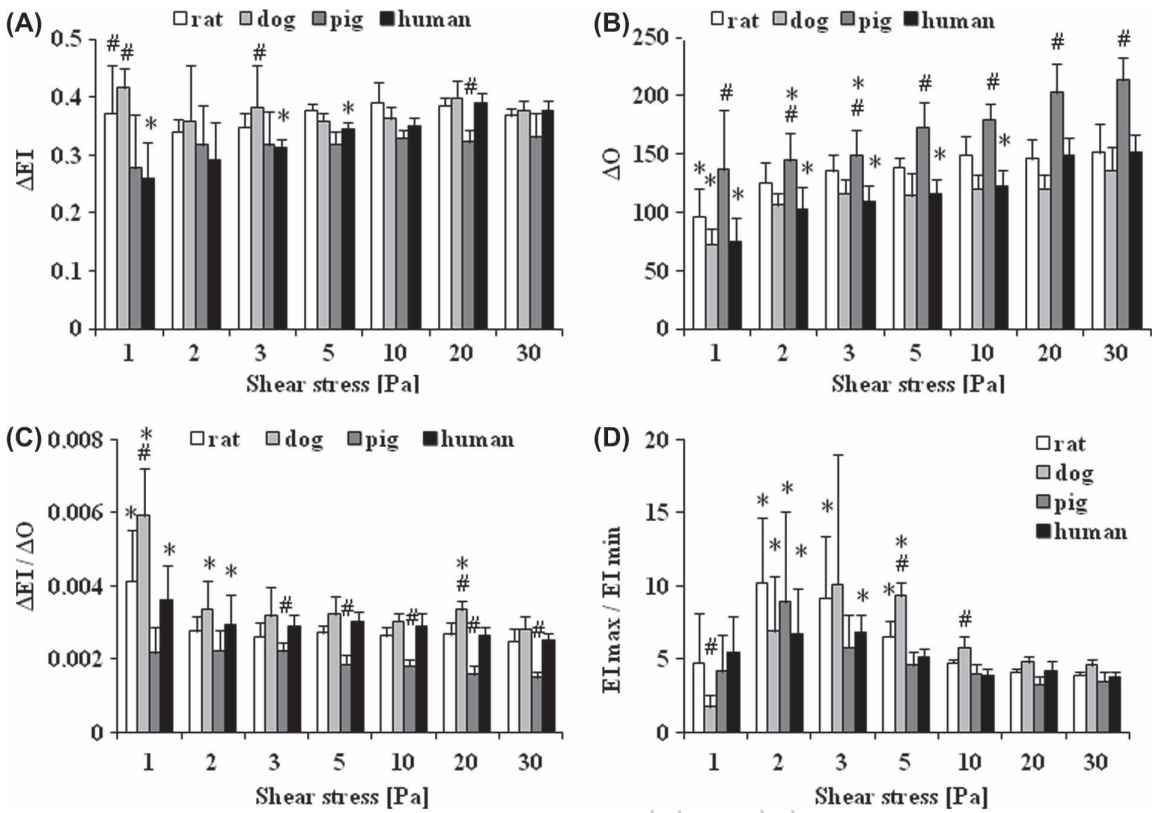

Figure 3. ΔEI (A), ΔO (B), ΔEI/ΔO (C) and EI max / EI min (D) values calculated from the original osmoscan raw data of rat, dog, pig and human blood samples tested at various shear stresses (1, 2, 3, 5, 10, 20 and 30 Pa). Means ± SD, *$p < 0.05$ vs. values at 30 Pa; #$p < 0.05$ vs. human.

gives the EI hyper and so the osmolality value belonging to this point (O hyper). The data obviously showed the previously described relations. In the porcine blood it was again shifted to right, toward higher osmolality values.

The minimal EI and the belonging osmolality represent the point, below which most of the cells rupture if the osmolality decreased further. When the osmoscan curve morphology was regular in a sample, this point was obvious, but under shear stress of 3–5 Pa this part of the curve became irregular, and so the EI min and O min value calculation was considered unstable.

By decreasing the applied shear stress for the measurements, the area under the EI-osmolality curves (Area) continuously shrank. At 10 Pa and below it the values were significantly lower compared to the 30 Pa data in all the species. The existing inter-species differences were well-traceable until 2 Pa: the highest Area values were found in human and rat blood, lower in the canine and the lowest ones in porcine blood.

Figure 3 and 4 show the alterations of our new calculated parameters. The ΔEI values –as the difference of EI max and EI min – were nearly similar in rat, canine and human blood tested at 20 or 30

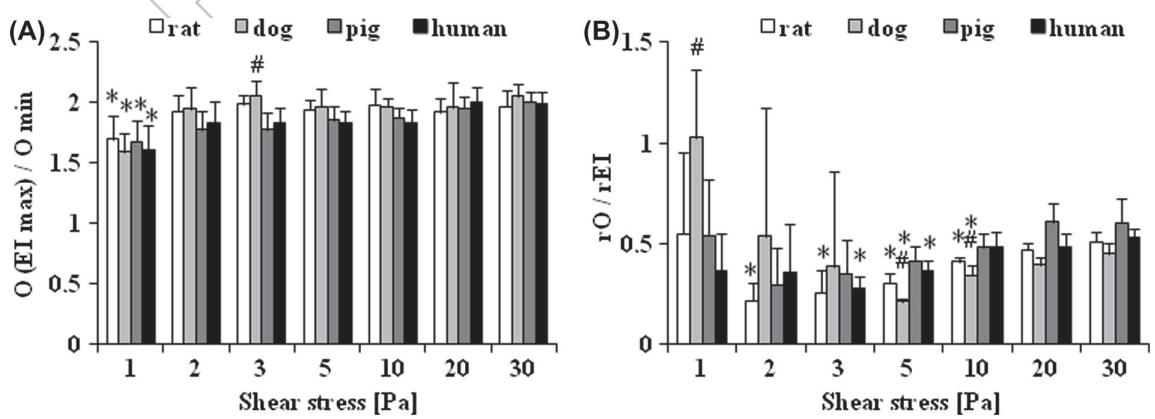

Figure 4. The O (EI max) / O min (A) and rO / rEI (B) values calculated from the original osmoscan raw data of rat, dog, pig and human blood samples tested at various shear stresses (1, 2, 3, 5, 10, 20 and 30 Pa). Means ± SD, *$p < 0.05$ vs. values at 30 Pa; #$p < 0.05$ vs. human.

[AQ5]

Table III. Hematological and red blood cell deformability values of native human blood samples ($n = 5$) and the suspensions of 40% Hct without or with 0.001% and 0.005% glutaraldehyde (GA).

Variable	Native	40% RBC-PBS	40% RBC-PBS + 0.001% GA	40% RBC-PBS + 0.005% GA
RBC [$\times 10^{12}$/L]	4.19 ± 0.27	4.33 ± 0.27	4.34 ± 0.29	4.43 ± 0.26
Hct [%]	38.7 ± 1.86	40.25 ± 1.51	39.67 ± 1.49	40.8 ± 2.24
Hgb [g/L]	110.3 ± 4.9	115.5 ± 5.1	115.2 ± 3.7	115.3 ± 3.6
MCV [fL]	92.4 ± 3.56	92.71 ± 4.93	91.7 ± 4.89	92 ± 3.85
MCH [pg]	26.37 ± 1.58	26.67 ± 1.79	26.64 ± 1.67	26.05 ± 1.26
MCHC [g/L]	285.3 ± 8.4	288 ± 10.7	290.4 ± 10	283.1 ± 8.7
RDW-CV%	14.1 ± 0.68	13.91 ± 0.68	14.08 ± 0.91	13.61 ± 0.85
EI at 3 Pa	0.248 ± 0.01	0.240 ± 0.01	0.103 ± 0.05*#	0.021 ± 0.03*#
EI_{max}	0.526 ± 0.02	0.511 ± 0.01	0.392 ± 0.07*#	0.278 ± 0.09*#
$SS_{1/2}$ [Pa]	3.53 ± 0.61	3.51 ± 0.51	8.22 ± 2.62*#	10.41 ± 1.38*#
EI_{max} / $SS_{1/2}$ [Pa^{-1}]	0.153 ± 0.02	0.147 ± 0.02	0.053 ± 0.02*#	0.027 ± 0.01*#

RBC-PBS, Red blood cells-phosphate buffered saline *$p < 0.05$ vs. native; #$p < 0.05$ vs. 40% RBC-PBS susp. Hct, hematocrit; Hgb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; RDW-CV, Red cell distribution width-corpuscular volume.

Pa, while porcine data were lower, because the EI max was definitely lower and EI min was higher in pigs. By decreasing the shear stress the inter-species difference became more prominent, and the human values also decreased (Figure 3A). The decreasing tendency was more obvious in case of the ΔO, reflecting the difference of O (EI max) and O min values. The highest values were of the porcine blood, always resulted by the width of the original osmoscan curves (Figure 3B). The ratio of these two parameters (ΔEI/ΔO) was therefore the lowest in the porcine blood. Interestingly, the rat and human values were very similar to each other, and the other inter-species differences became magnified by decreasing the shear stress values (Figure 3C).

The ratio of maximal and minimal EI values (EI max / EI min, or rEI) and ratio of osmolality values at maximal and minimal EI (O (EImax) / O min, or rO) and their relation (rEI/rO) showed the largest differences below 5 Pa. While rO values rather decreased gradually by decreasing shear stress (Figure 4A), the rEI values had the highest values between 2 and 5 Pa, drawing a 'peak' (Figure 3D). The rEI/rO decreased until 3 Pa, and increased again at 1 Pa, probably caused by the irregularity of the curve (Figure 4B).

Osmoscan investigation of rigidified red blood cells

Compared to the native blood samples, red blood cells' parameters did not change considerably during the preparation of 40% RBC-PBS suspension (Table III). However, it was interesting to see that elongation index values at shear stresses higher than 5 Pa were significantly lower versus the native blood. Although neither the quantitative nor the qualitative hematological parameters changed by the 0.001% or 0.005% GA treatments, as it was expected, the deformability values gradually decreased, showing significant difference compared to native samples and the untreated RBC-PBS suspension (Figure 5A, Table III). By osmoscan tests it was obviously

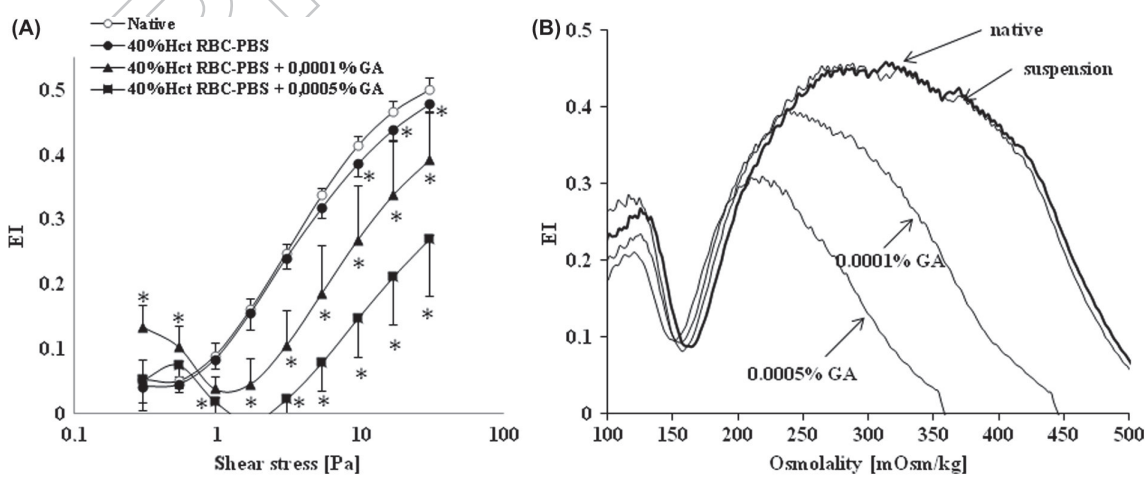

Figure 5. Elongation index (EI) – shear stress (SS [Pa]) curves (A) and representative osmoscan plots (B) of native human blood samples ($n = 5$) and the suspensions of 40% Hct without or with 0.001% and 0.005% glutaraldehyde (GA). A: means ± SD, in logarithmic scale, *$p < 0.05$ vs. native sample.

demonstrated too, that together with the impairing red blood cell deformability both the EI max and O (EI max) decreased, the area shrank, and the whole osmoscan curves shifted to left and downwards (Figure 5B).

The GA thus caused significant impairment in red blood cell deformability that was associated with obvious osmoscan data alterations. Through investigation whether which parameter is sensitive enough to reflect the deformability worsening (power of the variable), we calculated the standardized difference values. The highest standardized difference value is associated with the most sensitive parameter showing the existing difference. On Figure 6 we summarized standardized difference values against native sample. The deformability impairment (e.g. by 0.005% GA treatment) was reflected with the highest sensitivity by the EI at 3 Pa (6.19), the $SS_{1/2}$ (4.53), and the EI_{max} / $SS_{1/2}$ (4.24) parameters. In sequence, high standardized difference values (>2) were found in the osmoscan data mostly in the new calculated parameters: ΔO (3.57) > rO (3.37) > rEI/rO (3.19) > $\Delta EI/\Delta O$ (2.54) > Area (2.43) > EI max (2.26) > ΔEI (2.02). These findings suggest that the new calculated parameters of the osmoscan curves can be sensitive enough to detect impairment of red blood cell deformability, too.

Discussion and Conclusion

Red blood cell deformability is an important microrheological parameter of the blood that can be altered in numerous cardiovascular, metabolic, hematological diseases and during inflammatory processes, ischemia-reperfusion, among others [35]. Red blood cell deformability can change due to the osmotic environmental alterations too, since any change in cell surface to volume ratio (while cells are swelling or shrinking) directly affect the deformability [1,2,11]. Osmotic gradient ektacytometry can provide information about the deformability changing in a wide range of osmolality [11]. However, the measuremental conditions, such as the applied shear stress, the range of osmolality changes, the used buffers, etc., all might affect the results and needs to be standardized. Furthermore, in biomedical research we have to face the inter-species (cross-species) differences of hemorheological parameters [29,36], even leading to modifications in the methodological standards depending on the species of the experimental/laboratory animal. For this issue, comparative studies are necessary in order to collect data at various measuremental conditions. Since red blood cell deformability shows colorful variety among species [36], the osmoscan curve part that reflects the zone of cell-swelling in hyposmolar environment (between EI max and EI min and the belonging osmolality) and also being related to the membrane stability properties, might be interesting to explore better the deformability characteristics.

In this study osmotic gradient ektacytometry data of human and some experimental/laboratory animals' blood were obtained at various shear stress values and analyzed in respect of the measurement conditions and further analyzing the osmoscan curves, with introducing newly calculated parameters. We could analyze various curve morphologies, focusing on the properties of membrane stability, the range between minimal and maximal EI and the related osmolality values.

The continuously measured elongation index values at a given shear stress in the function of osmolality give the characteristic osmoscan curve (Figure 1). The left, irregular part is considered to represent a mixture of cell fragments, 'ghosts', and still intact but swollen and nowise intact cells, since O min correlates well with the osmolality point at 50% lysis

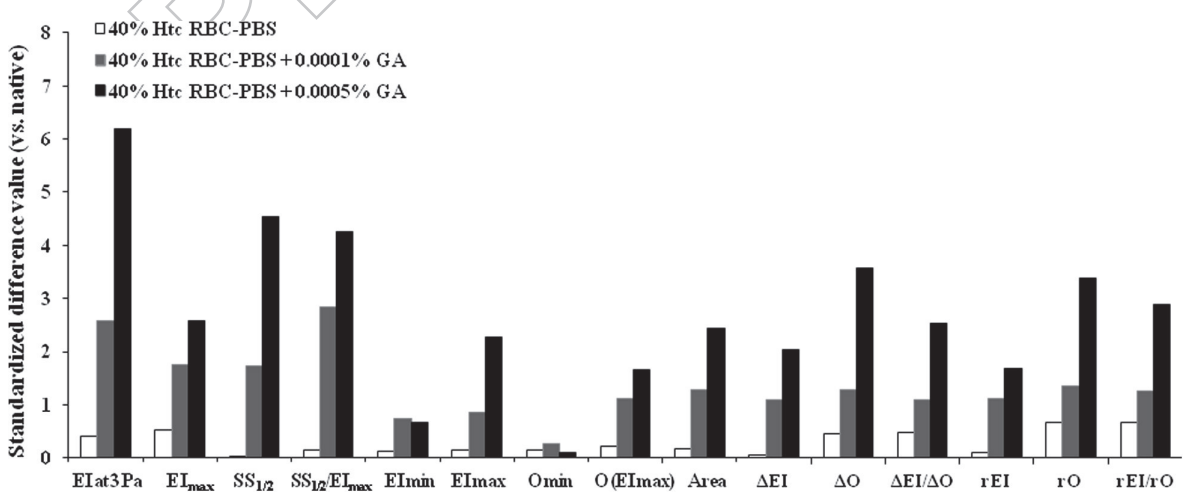

Figure 6. Standardized difference values of the suspensions vs. native sample (40% RBC-PBS; 40% RBC-PBS + 0.001% GA; 40% RBC-PBS + 0.005% GA) for various parameters.

during the osmotic fragility test [11]. Then, along the phase from the lower to the higher inflection point the process is represented, when the cells from their 'optimal' condition (EI max and belonging osmolality) are swelling by the decreasing osmolality, while ruptures. In the hyperosmotic direction the cells are shrinking with decreased deformability again, but this region is very wide, since physiologically the cells are liable to 'osmotic shock' in the kidney around the Henle loops.

Therefore, by our opinion, the zone between the minimal and maximal EI and the associated osmolality values may represent well the variability of red blood cell deformability changes that is determined also by the membrane stability. This zone is restricted (e.g. 120–300 mOsmol/kg), and thus, if the cells are rigid, this osmoscan curve zone can be distorted, as suggested previously [11]. The alterations in the hypososmotic range, where the cell-swelling occurs and the obtained EI values here can be associated with the cell's pliability, the deformability. It is supposed, that if these differences (e.g. ΔEI, ΔO, etc.) are small, the shifting from the optimal osmolality point is not tolerable well, and its magnitude is narrowed.

Another question is the sensitivity of the measurement. Which shear stress values are applicable and comparable to each other? How can the osmoscan curve morphology be interpreted more easily by calculated parameters? Are those parameters sensitive enough to describe differences of various species' data or of blood samples in various diseases, where the deformability is altered? We were driven by these questions when performing this study.

Although inter-species red blood cell deformability differences have been widely investigated [36], osmotic gradient ektacytometry data is hardly available [29]. In our previous comparative study on osmoscan data obtained at shear stress of 30 Pa, we also found that porcine EI-osmolality curves manifest in a higher osmolality range. The O (EI max) values were higher and EI max values were lower ($EI = 0.481 \pm 0.007$ at osmolality of 348.2 ± 15.5 mOsmol/kg), than those in rats ($EI = 0.509 \pm 0.014$ at osmolality of 311.2 ± 9.1 mOsmol/kg) or dogs ($EI = 0.513 \pm 0.007$ at osmolality of 288.6 ± 19.4 mOsmol/kg). Similar shifting to right occurred in murine blood samples, but associated with higher EI max values ($EI = 0.519 \pm 0.016$ at osmolality of 349.6 ± 44.8 mOsmol/kg). At the same time in the hypoosmotic range the difference was much smaller in EI min and O min parameters [29]. These data correlated well with our current results at 30 Pa.

Heo and co-workers [28] used another kind of precision ektacytometer to investigate shear stress dependency of the osmoscan measurements at 1, 2, 3, 5, 7, 10 and 20 Pa. They also found that the osmoscan curves are shifting to left and downwards with decreasing shear stress. In their method they used calculated values for the definitive osmolality points, and not a continuous manner. The LoRRca registers continuous EI and osmolality raw data, thus the data analysis is different [28]. In a shear stress range of 7–20 Pa they did not find significant differences, the O (EI max) decreasing appeared under 7 Pa (at 3–5 Pa it was about ~10%, at 2 Pa ~ 83%, and at 1 Pa only ~ 75% of the values measured at 7–20 Pa range) [28]. In our study we found continuously decreasing O (EI max) values with decreasing shear stress. Although there was no significant difference between 20 and 30 Pa, the decreasing tendency was nearly linear toward 1 Pa. The shear stress range under 5 Pa is very important and interesting, since physiologically the *in vivo* shear stress conditions rarely exceed 5–10 Pa [37]. However, the methodology originally uses higher shear stress values, such as 20 or 30 Pa [4,11].

Glutaradehyde treatment of red blood cells is a well-known and widely used easy method to cause the impairment of red blood cell deformability [30–32]. With the comparison of glutaraldehyde treated erythrocytes to normal, control erythrocyte we have planned to investigate that which parameter could demonstrate the highest and the most stable level of the difference between the two groups. Besides the traditional deformability parameters (e.g. Ei values at given shear stress, EI_{max}, $SS_{1/2}$) tested by normal ektacytometry we found that majority of the newly calculated osmoscan parameters showed relatively high values of standardized difference. It might reflect that these parameters seem to be sensitive indicators for the membrane rigidity changes, too.

Summarizing our results we can say that: (1) Osmoscan data tested at 20 or 30 do not differ significantly from each other; (2) Under shear stress of 20 Pa the EI max (and consequently the EI_{hyper}), the O (EI max), the EI min and the AUC nearly linearly decrease in the function of shear stress with different slope in rat, dog, pig and human blood. The O min values did not differ characteristically; (3) Measurements under shear stress of 3 Pa become unstable; (4) The differences between minimal and maximal EI and the related osmolality values, as well as their ratios, as new calculated parameters (ΔEI, ΔO, ΔEI/ΔO, EI max / EI min and O (EI max) / O min, etc.) can be suitable for further analysis of the osmoscan curve, and can extract more out of their information content together with other hemorheological parameters describing red blood cell deformability and membrane stability; and (5) Decreased erythrocyte deformability (by rigidifying with glutaraldehyde) can be reflected well with the following, calculated osmoscan parameters: ΔO, rO, rEI/rO, ΔEI/ΔO.

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References

[1] Meiselman HJ. Morphological determinants of red blood cell deformability. Scand J Clin Lab Invest 1981;41:27–34.

[2] Baskurt OK, Meiselman HJ. Blood rheology and hemodynamics. Semin Thromb Hemost 2003;29:435–50.

[3] Bessis M, Mohandas N, Feo C. Automated ektacytometry: a new method of measuring red cell deformability and red cell indices. Blood Cells 1980;6:315–27.

[4] Hardeman MR, Goedhart PT, Shin S. Methods in hemorheology. In: Baskurt OK, Hardeman MR, Rampling MW, Meiselman HJ, editors. Handbook of hemorheology and hemodynamics. Amsterdam: IOS Press; 2007. pp 242–66.

[5] Weed RI, LaCelle PL, Merrill EW. Metabolic dependence of red cell deformability. J Clin Invest 1969;48:795–809.

[6] Feo C, Phillips WM. The influence of suspension osmolality and erythrocyte volume on cell deformability. Nouv Rev Fr Hematol 1982;24:295–9.

[7] Araki K, Rifkind JM. The rate of osmotic hemolysis: a relationship with membrane bilayer fluidity. Biochim Biophys Acta 1981;645:81–90.

[8] Linderkamp O, Meiselman HJ. Geometric, osmotic, and membrane mechanical properties of density-separated human red cells. Blood 1982;59:1121–7.

[9] Yamamoto A, Niimi H. Effect of high osmotic media on blood viscosity and red blood cell deformability. Biorheology 1983;20:615–22.

[10] Scott MD, Kuypers FA, Butikofer P, Bookchin RM, Ortiz OE, Lubin BH. Effect of osmotic lysis and resealing on red cell structure and function. J Lab Clin Med 1990;115:470–80.

[11] Clark MR, Mohandas N, Shohet SB. Osmotic gradient ektacytometry: comprehensive characterization of red cell volume and surface maintenance. Blood 1983;61:899–910.

[12] Macey RI, Yousef LW. Osmotic stability of red cells in renal circulation requires rapid urea transport. Am J Physiol 1988;254:C669–74.

[13] Cynober T, Mohandas N, Tchernia G. Red cell abnormalities in hereditary spherocytosis: relevance to diagnosis and understanding of the variable expression of clinical severity. J Lab Clin Med 1996;128:259–69.

[14] Johnson RM, Ravindranath Y. Osmotic scan ektacytometry in clinical diagnosis. J Pediatr Hematol Oncol 1996;18:122–9.

[15] Bilto YY, Abdalla SS. Effects of selected flavonoids on deformability, osmotic fragility and aggregation of human erythrocytes. Clin Hemorheol Microcirc 1998;18:165–73.

[16] Bernassola F, Boumis G, Corazzari M, Bertini G, Citro G, Knight RA, Amiconi G, Melino G. Osmotic resistance of high-density erythrocytes in transglutaminase 2-deficient mice. Biochem Biophys Res Commun 2002;291:1123–7.

[17] Stookey JD, Klein A, Hamer J, Chi C, Higa A, Ng V, Arieff A, Kuypers FA, Larkin S, Perrier E, Lang F. RBC deformability and amino acid concentrations after hypo-osmotic challenge may reflect chronic cell hydration status in healthy young men. Physiol Rep 2013;1:e00117.

[18] Finkelstein A, Talbot H, Topsu S, Cynober T, Garcon L, Havkin G, Kuypers F. Comparison between a camera and a four quadrant detector, in the measurement of red blood cell deformability as a function of osmolality. J Med Bioeng 2013;2:62–5.

[19] Deuel JW, Lutz HU, Misselwitz B, Goede JS. Asymptomatic elevation of the hyperchromic red blood cell subpopulation is associated with decreased red cell deformability. Ann Hematol 2012;91:1427–34.

[20] Ballas SK, Smith ED. Red blood cell changes during the evolution of the sickle cell painful crisis. Blood 1992;79:2154–63.

[21] Delaunay J. The hereditary stomatocytoses: genetic disorders of the red cell membrane permeability to monovalent cations. Semin Hematol 2004;41:165–72.

[22] Syfuss PY, Ciupea A, Brahimi S, Cynober T, Stewart GW, Grandchamp B, Beaumont C, Tchernia G, Delaunay J, Wagner JC. Mild dehydrated hereditary stomatocytosis revealed by marked hepatosiderosis. Clin Lab Haematol 2006;28:270–4.

[23] Alanio-Bréchot C, Schischmanoff PO, Fénéant-Thibault M, Cynober T, Tchernia G, Delaunay J, Garçon L. Association between myeloid malignancies and acquired deficit in protein 4.1R: a retrospective analysis of six patients. Am J Hematol 2008;83:275–8.

[24] Pautard B, Feo C, Dhermy D, Wajcman H, Baudin-Chich V, Delobel J. Occurrence of hereditary spherocytosis and beta thalassaemia in the same family: globin chain synthesis and visco diffractometric studies. Br J Haematol 1988;70:239–45.

[25] De Franceschi L, Bachir D, Galacteros F, Tchernia G, Cynober T, Alper S, Platt O, Beuzard Y, Brugnara C. Oral magnesium supplements reduce erythrocyte dehydration in patients with sickle cell disease. J Clin Invest 1997;100:1847–52.

[26] Wandersee NJ, Birkenmeier CS, Gifford EJ, Mohandas N, Barker JE. Murine recessive hereditary spherocytosis, sph/sph, is caused by a mutation in the erythroid alpha-spectrin gene. Hematol J 2000;1:235–42.

[27] Bock I, Perrin J, Braun F, Garçon L, Lesesve JF. [A case of hereditary pyropoikilocytosis with mild expression and delayed onset]. Ann Biol Clin (Paris) 2012;70:483–8.

[28] Heo Y, Jung H, Shin S. Osmotic deformability of erythrocytes at various shear stresses. Clin Hemorheol Microcirc. 2013 Sep 4. [Epub ahead of print] doi. 10.3233/CH-131761 **[AQ4]**

[29] Nemeth N, Kiss F, Klarik Z, Miko I. Comparative osmotic gradient ektacytometry data on inter-species differences of experimental animals. Clin Hemorheol Microcirc 2014;57:1–8.

[30] Corry WD, Meiselman HJ. Modification of erythrocyte physicochemical properties by millimolar concentrations of glutaraldehyde. Blood Cells 1978;4:465–83.

[31] Mirossay L, Mojzis J, Jandoseková M, Lukacín S, Nicák A. Comparison of two methods in erythrocyte microrheology determination using glutaraldehyde-treated cells. Clin Hemorheol Microcirc 1997;17:187–92.

[32] Baskurt OK, Hardeman MR, Uyuklu M, Ulker P, Cengiz M, Nemeth N, Shin S, Alexy T, Meiselman HJ. Comparison of three commercially available ektacytometers with different shearing geometries. Biorheology 2009;46:251–64.

[33] Baskurt OK, Meiselman HJ. Data reduction methods for ektacytometry in clinical hemorheology. Clin Hemorheol Microcirc 2013;54:99–107.

[34] Stuart J, Stone PCW, Freyburger G, Boisseau MR, Altam DG. Instrument precision and biological variability determine the number of patients required for rheological studies. Clin. Hemorheol 1989;9:181–97.

[35] Toth K, Kesmarky G, Alexy T. Clinical significance of hemorheological alterations. In: Baskurt OK, Hardeman MR, Rampling MW, Meiselman HJ, editors. Handbook of Hemorheology and Hemodynamics. Amsterdam: IOS Press; 2007. pp 392–432.

[36] Windberger U, Baskurt OK. Comparative hemorheology. In: Baskurt OK, Hardeman MR, Rampling MW, Meiselman HJ, editors. Handbook of hemorheology and hemodynamics. Amsterdam: IOS Press; 2007. pp 267–85.

[37] Lipowsky HH. Microvascular rheology and hemodynamics. Microcirculation 2005;12:5–15.