COMPARATIVE STUDY OF TWO METHODS TO DETERMINE ERYTHROCYTE DIMENSIONS IN VERTEBRATES

(ESTUDIO COMPARATIVO DE DOS METODOS PARA DETERMINAR LAS DIMENSIONES DE ERITROCITOS EN LOS VERTEBRADOS)

Por L. Palacios
G. Viscor
J. Palomeque

Keywords: Erythrocyte dimensions, red blood cell size and shape red cell nucleus, smears, wet mounts.

SUMMARY

Two methods to determine erythrocyte dimensions in several vertebrate species were compared. The species analyzed were: pigeon, gull, hen, eel, rat and man. The first method was that of direct measure by means of a graduate eyepiece of cells in stained dry smears; the second method was a photomicrographic technique applied to erythrocyte wet mounts. A reduction of the dimensions of the blood cells during the fixation and staining procedures was observed and in some cases an alteration in erythrocyte shape was detected.

RESUMEN

Se compararon dos métodos para determinar las dimensiones eritrocitarias en varias especies de vertebrados: paloma, gaviota reidora, gallina doméstica, anguila común, rata de laboratorio y el hombre. El primer método fue el de medida directa de las células teñidas en una extensión de sangre por medio de un ocular micrométrico;

Unidad de Fisiología. Departamento de Bioquímica y Fisiología. División de Ciencias Experimentales y Matemáticas. Universidad de Barcelona.
Facultad de Biología. Avda. Diagonal, 645, E-08028 Barcelona. España.

An. Fac. Vet. León, 1987, 33, 53-60

el segundo método consistió en la medición sobre fotomicrografías obtenidas de suspensiones de hematíes en plasma. Se detectó una reducción de las dimensiones eritrocitarias durante el proceso de preparación del frotis sanguíneo y en algún caso se observó una alteración de la forma en los eritrocitos nucleados. Durante la determinación de las dimensiones de eritrocitos suspendidos en plasma, se mantienen las condiciones fisiológicas en el medio ambiente celular, obteniéndosar, obteniéndose de este modo resultados más fidedignos.

INTRODUCTION

Although it is not clear whether the size of red cells is of functional importance [14], it has been analyzed in many different species because it has proved interesting to compare red cell size in relation to animal activities and habits.

Frair [4] stated that sea turtles with larger lengths of upper shells have longer red cells by length-width ratio and volume. Abdel-Hamed et. al., [1], associated an increase in erythrocyte size with triploidy in the chicken. Kuramoto [9] found a correlation between erythrocyte dimensions and genome size in amphibians.

Generally the old hypothesis of Gulliver [6] that in mammals, larger animals have larger red blood cells, has been sustained or rejected by several authors in different zoological groups. This hypothesis is based on the fact that red cells give an indication of the surface available for exchange of gases in respiratory function; thus a small red blood cell may provide for a greater rate of exchange than a larger one, the former being from smaller animals with higher metabolic rates.

Traditionally the measurement of the dimensions of red blood cells were calculated using blood smears fixed and stained, and then measured by means of a calibrated eyepiece (ocular micrometer). There are many references in the bibliography using this technique: Hartman & Lessler [7] in fish, amphibians and reptiles; Abdel-Hamed et al., [1], Palomeque & Planas [11, 12] in birds; Kuramoto [9] in amphibians; Frair [4] and Otis [10] in reptiles, Altman & Dittmer [2] compiled data in 115 species of mammals and a great number of other vertebrates.

Apparently due to the fixation and staining processes the real dimensions of the red blood cells are affected, showing in this case a different size than in physiological conditions, as it is possible to deduct from preceding studies in man [13]. For this reason a photomicrographic method was applied to red cells suspended in autologous plasma, a modification of the technique described in human erythrocytes by Houchin et al. [8], to measure erythrocyte and nuclear dimensions. In order to compare the differences between the two methods, both were applied to each blood sample.

MATERIALS AND METHODS

The human blood was obtained from 8 healthy people of between 25-35 years of age of the staff of the Department by a puncture with a lancet on the tip of a finger. The rat blood was obtained by cardiac puncture. The bird blood was withdrawn from the radial wing vein. The avian species analyzed were the hen *Gallus g. domesticus*, the gull *Larus ridibundus* and the urban pigeon *Columba livia*. The hens were obtained from a commercial dealer and the others birds were captured in the city of Barcelona. The blood sampling was carried out after the first week of confinement of the birds in the laboratory. The hematological and body weight control showed no signifi-

Table 1
Dimensions of nucleated erythrocytes

Species	Length			Width			Ratio L/W	
	Smear	Wet	△ %	Smear	Wet	△ %	Smear	Wet
Hen	11.27●●●	12.28	−8.23	7.24●	7.47	−2.59	1.56●●●	1.65
(6)	±0.49	±0.55	±1.87	±0.44	±0.45	±4.51	±0.09	±0.12
Gull	12,59●●●	14.81	-14.89	6.49●●●	7.65	-14.86	1.94 ○	1.95
(5)	±0.66	±0.74	±2.69	±0.26	±0.53	±3.13	±0.13	±0.16
Pigeon	13.54●●●	14.16	−4.52	6.68●●	6.95	−4.22	2.03 ○	2.05
(6)	±0.72	±0.68	±1.92	±0.44	±0.47	±3.71	±0.14	±0.16
Eel (fw)	12.29●●●	14,49	−14.16	8.63●●●	9.56	-13.38	1.43●●●	1.52
(5)	±0.61	±0.38	±3.08	±0.62	±0.80	±0.84	±0.11	±0.17
Eel (bw)	12.75●●●	13.36	−4.77	9.19●●●	10.19	−7.88	1.40●●●	1.31
(9)	±0.45	±0.37	±3.67	±0.80	±0.41	±4.65	±0.14	±0.07

Table 1. Dimensions of the erythrocytes by the two methods.
Mean value ± standard deviation. Number of specimen in parenthesis.
△ = Percentage of increase of erythrocyte dimension in dry smears respect to wet preparations. (fw) = fresh water, (bw) = brackish water.
○ = NS, ● = $p < 0.01$, ●● = $p < 0.005$, ●●● = $p < 0.0005$.

Table 2
Nuclear erythrocyte dimensions in birds

Species	Length			Width			Ratio L/W	
	Smear	Wet	△ %	Smear	Wet	△%	Smear	Wet
Hen	4.75 ○	4.67	0.51	3.21 ○	3.14	1.64	± 1.49 ○	1.49
(6)	± 0.36	± 0.53	± 6.68	± 0.21	± 0.27	± 4.95	± 0.15	± 0.17
Gull	5.88 ●	6.21	– 5.16	2.37 ○	2.33	1.78	2.50 ●	2.68
(5)	± 0.45	± 0.47	± 4.00	± 0.19	± 0.18	± 7.20	± 0.32	± 0.32
Pigeon	6.06○	5.96	2.03	2.34 ○	2.34	0.04	2.60 ○	2.59
(6)	± 0.46	± 0.59	± 5.32	± 0.17	± 0.29	± 8.25	± 0.27	± 0.45

Table 2. Nuclear erythrocyte dimensions in birds.
Mean value ± standard deviation. Number of specimen in parenthesis.
△ = Percentage of increase of erythrocyte dimension in dry smears respect to wet preparations.
○ = NS, ● = $p < 0.01$.

Table 3
Erythrocyte dimensions of mammals

	Human (8)	Rat (7)
Smear	7.58 ± 0.40	7.01 ± 0.33
	●●●	●●●
Wet	8.24 ± 0.37	7.36 ± 0.32
△ %	− 8.08 ± 0.85	− 4.64 ± 0.99

Table 3. Erythrocyte dimensions of mammals.
Mean value ± standard deviation. Number of specimen in parenthesis.
△ = Percentage of increase of erythrocyte dimension in dry smears respect to wet preparations.
○ = NS. ●●● = $p < 0.0005$.

cant change while under laboratory conditions, which proved a complete adaptation. The eels *Anguilla anguilla* taken from the Ebro river delta were analyzed after a month of acclimatization under two different conditions; one group in fresh water and the other in brackish water (20°/$_{oo}$). The blood was removed by cardiac puncture.

METHOD OF MEASUREMENT OVER DRY SMEARS

The smears were prepared immediately after the blood was withdrawn from the animal. The staining method applied was the Diff-Quick (Harleco, USA) kit stain which is a modification of the well known Wright stain.

The cells were examined under immersion oil with a Nikon (Japan) microscope model SFR-Ke, and the dimensions measured by means of a calibrated eyepiece. Measurements of 150 erythrocytes of each species were taken from different smears.

PHOTOGRAPHIC METHOD ON WET MOUNTS

An heparinized glass capillary tube of blood was obtained from a freshly drawn sample. The capillary tube was centrifuged at 11.500 rpm during 5 minutes in a microcentrifuge (Hemofuge, Heraeus, West Germany). The capillary tube was cut with a glass cutter separating the plasma from the cells. The segment of the capillary tube containing the plasma was slightly joined with the blood sample; a small quantity of red cells was introduced by capillarity into the plasma tube. A mixing wire moved up and down within the tube with a magnet was used to homogenize the red cell in the suspension that was placed on a microscope slide. After the erythrocyte sedimentation, photomicrographs were taken (Nikon, Japan) using low sensibility film (Agfapan 25 ISO, Agfa, WG). A graduated scale from an objective micrometer (Olympus, Japan) was also photographed in each film at the same magnification and it was used as a reference to calculate the actual dimensions of the red blood cells.

MEASUREMENT OF THE NUCLEUS

With the preceding method it was impossible to observe the cell nucleus clearly enough in order measure it. Phase contrast and interference phase contrast observations were unsatisfactory.

For this reason the following procedure was added for the nucleated red cells: a diluted solution of the vital dye methylene blue, at a concentration 1: 20.000 in saline (NaCl 0.9%), was mixed in the same proportion with a sample of blood (1:1), the mixture was incubated in a double boiler at physiological temperature, after which the aforementioned method was applied. A suspension with a low erythrocyte concentration was obtained, photomicrographs of the stained red cells were then taken and the nuclear and cellular diameters were measured.

Statistical significance of the differences between the results of the two groups obtained by both methods was calculated using the Student'st test.

RESULTS

In table 1 the cellular dimensions obtained by both methods in the three species of birds and the two groups of eels are shown. Erythrocyte dimensions in the four species were similar. Both the cellular length and width obtained by the two methods were significantly different. In the dry smears the cellular dimensions were smaller than in the wet mounts, although the differences were not similar varying from 2% to 15%. From the lengh t/width ratio analysis (L/W) a small variation in cellular shape was observed, although it is only significant in the hen and eel. However, in most of the species a tendency toward roundness was detected in the dry smears.

The differences in nuclear dimensions between the two methods (table 2) ranged between 0% and 5% being statistically significant in only one case. Consequently the differences were smaller compared to cellular size variations. No significant differences were observed in the shape of the nucleus with the exception of gull red blood cells. A comparison of cellular dimensions obtained from photographs of stained (methylene blue) and non-stained erythrocytes showed no significant differences, proving that this vital dye at this concentration does not affect cellular size.

In the non-nucleated erythrocytes (table 3) the same trend was observed as in other species, with a statistically significant difference between the results obtained by the two methods, resulting in a reduction of the cellular diameter in the dry smears.

DISCUSSION

The results obtained on dry smears were in agreement with the literature, in birds [11], in mammals [2], and in the eel [15].

In humans it was possible to find references that agree well with the present data that were obtained by methods similar to the present study [5, 8, 17].

A decrease in erythrocyte size was observed in the smears compared to those in physiological conditions in all the species studied, although this was already observed in humans by Ponder [13]. The cause of this shrinking is probably due to drying and staining of the red cells during the preparation of the smear. Therefore the nuclear dimensions did not significantly change (table 2), perhaps because of the great viscosity

and density of their content [3], however a shortening in the gull erythrocyte nucleus was detected.

The percentage of erythrocyte size reduction varied according to species. This variability could be in relation to specific characteristics of the erythrocyte such as rheological and structural properties (deformability, elasticity of the membrane, osmotic resistence, development of cytoskeleton, etc.). Comparing the variations in the L/W ratio between the results obtained by dry smears and wet mounts, some alterations in the shape of the erythrocytes were detected. In the birds analyzed, gull and pigeon showed a negligible difference in erythrocyte shape, whereas there was a significant diffence ($p < 0.0005$) in the hen. Interestingly in a preceeding study [16] hen erythrocytes showed a greater deformability than the two other species of birds.

The L/W ratio in the eel denotes an opposite variation in the animals acclimatized to different environmental osmotic conditions. In the group of animals adapted to fresh water the variations in L/W showed the same trend observed in birds, but the ones acclimatized to brackish water presented an inverse variation, since the erythrocytes from the smears were more elongated than the ones from the wet mounts. The photomicrographic method on wet mounts, in contrast to the smears, allows the results to reflect differences in the erythrocyte shape probably due to the acclimatization process, a very important fact since the eel is an osmoconformer organism.

Although photomicrographic methods for determination of erythrocyte dimensions has been used in human clinical work for a long time, it has been little applied to other vertebrates. In this study definite differences between both methods (wet mount and dry smear) are seen. The measurement on erythrocytes suspended in autologous plasma maintain the physiological conditions in the environment of the erythrocyte during the determination of their dimensions, obtaining more reliable results.

REFERENCES

1) ABDEL-HAMED M.F., NEAT H.J. & BRILES W.E. (1971). Differences in erythrocyte measurements of intersex and normal adult chickens. *Poult. Sci.* 50(6), 1.847-1.854.
2) ALTMAN P.L. & DITTMER D.S. Eds. (1961). *Blood and other body fluids.* Federation of American Societies for Experimental Biology. Washington, D.C., 116-120.
3) CHIEN S., USAMI S., DELLENBACK R.J. & BRYANT C.A. (1971). Comparative hemorheology: Hematological implications of species differences in blood viscosity. *Biorheology* 8(1), 35-37.
4) FRAIR W. (1977). Sea turtle red blood cell parameters correlated with caparace lengths. *Comp. Biochem. Physiol.* 56A(4), 467-472.
5) GAEHTGENS P.; SCHMIDT F. & WILL G. (1981). Comparative rheology of nucleated and non-nucleated red blood cells: I. Microrheology of avian erythrocytes during capillary flow. *Pflügers Arch.* 390, 278-282.
6) GULLIVER G. (1875). Observations on the size and shapes of the red corpuscles of the blood of vertebrates, with drawings of them to uniform scale, and extended and revised tables of measurements. *Proc. Zool. Soc. London* 31(3), 474-495.
7) HARTMAN F.A. & LESSLER M.A. (1964). Erythrocyte measurements in fish, amphibia and reptiles. *Biol. Bull* 126(1), 83-88.
8) HOUCHIN D.N.; MUNN J.I. & PARNELL B.L. (1958). A method for the measurement of red cell dimensions and calculation of mean corpuscular volume and surface area. *Blood* 13(5), 1.185-1.191.
9) KURAMOTO M. (1981). Relationship between number, size and shape of red blood cells in amphibians. *Comp. Biochem. Physiol.* 69A(4), 771-775.

10) OTIS V.S. (1973). Hemocytological and serum chemistry parameters of the african puff adder *Bitis arietans*. *Herpetologica* 29(1), 110-116.
11) PALOMEQUE, J. & PLANAS J. (1977). Dimensions of erythrocytes of birds. *Ibis* 119(4), 533-535.
12) PALOMEQUE J. & PLANAS J. (1981). Erythrocyte size in some wild Spanish birds. *Rev. esp. Fisiol.* 37(1), 17-22.
13) PONDER E.T. & MILLER W.G. (1924). Measurement of diameters of erythrocytes: 1. Mean diameter of true red cells of man. *Q.J. Exp. Physiol.* 14(1), 67-83.
14) SCHMIDT-NIELSEN K. (1979). *Animal Physiology: Adaptation and environment.* Cambridge University Press, Cambridge, pp. 68.
15) SWARTS W. (1969). Blood studies in some marine teleosts. *Trans. Amer. Fish. Soc.* 98(2), 328-331.
16) VISCOR G.; FUENTES J. & PALOMEQUE J. (1984). Blood rheology in the pigeon (*Columba livia*), hen (*Gallus gallus domesticus*) and black-headed gull (*Larus ridibundus*). *Can. J. Zool.* 62(11), 2.150-2.156.
17) WESTERMAN M.P.; PIERCE L.E. & JENSEN W.N. (1961). A direct method for the quantitative measurement or red cell dimensions. *J. Lab. Clin. Med.* 57(5), 819-824.