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RED BLOOD CELL WATER PERMEABILITY IN ELDERLY PEOPLE

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Abstract

Aim. To compare the red blood cell (RBC) diffusional water permeability (Pd) in elderly human subjects and mature subjects.

Patients and methods. 58 apparently healthy subjects, aged 65-80 years, were divided into two groups: 44 mature (35-64.9 years) and 14 elderly subjects (65-80 years). The morphological characteristics of RBCs were determined from light microscopic measurements and their Pd was measured by a NMR method. The inhibition of Pd induced by p-chloromercuribenzoate (PCMB) and the activation energy (Ea,d) of water diffusion across the RBC membrane were also determined.

Results. No significant differences between the RBCs of the two groups were found in regard with morphological parameters. Pd (10^{-3} cm/s) was in case of mature subjects ~ 3.1 at 15°C, 3.6 at 20°C, 4.2 at 25°C, 5.0° at 30°C, 6.1 at 37°C and 7.3 at 42°C, while for elderly subjects Pd was ~ 3.4 at 15°C, 3.9 at 20°C, 4.5 at 25°C, 5.3 at 30°C, 6.6 at 37°C and 7.9 at 42°C. Although rather small these differences were statistically significant: $p<0.004$ to $p<0.04$ at various temperatures. This means that RBCs from elderly people have a higher Pd. In agreement with this suggestion, the values of inhibition of water permeability induced by PCMB were higher for the RBCs from elderly individuals; however, the differences were not statistically significant. Ea,d was the same (~23 kJ/mol) for the RBCs from both groups. After incubation with PCMB Ea,d was ~ 37 kJ/mol for the mature individuals and ~ 31 kJ/mol for elderly individuals; however, the differences were not statistically significant.

Conclusion. A small, but statistically significant, increase in Pd of RBCs from elderly individuals was observed. This can be correlated with peculiarities of a less physically active organism.

Key words: aged people, water channel proteins, red blood cell, nuclear magnetic resonance.

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INTRODUCTION

In the past decades important progress in understanding the mechanisms of water permeability of biological membranes has been achieved (reviewed in refs. 1,2). Mercury-containing SH-modifying reagents, such as p-chloromercuribenzoate (PCMB) and p-chloromercuribenzene sulfonate (PCMBS), have played an important role in understanding the molecular basis of RBC water permeability. Macey and Farmer (3) made the initial discovery that such reagents are powerful inhibitors of the human RBC water permeability. A straightforward interpretation of these results was that mercurials react with SH groups in proteins associated with water channels, resulting in their closure (4).

The main discovery regarding the water permeability of membranes was the identification of water channels in membranes, represented by water channel proteins (WCPs). These are transmembrane proteins that have a specific three-dimensional structure with a pore that can be permeated by water molecules (2). The first WCP was discovered in the red blood cell (RBC) membrane by Benga and coworkers in 1985 in Cluj-Napoca, Romania, reported in publications in 1986 (5,6) and reviewed in subsequent years (7-16). This protein was later called aquaporin 1 (AQP1).

The recognition of the priority of Benga's group in the discovery of AQP1 and of the mistake of Benga being omitted from the 2003 Nobel Prize in chemistry (half of which was awarded "for the discovery of water channels") is growing. This is recognized by thousands of science-related professionals from hundreds of academic and research units who signed at www.ad-astra.ro, some of them, including George Palade (Nobel Laureate), adding thoughtful comments. Additional commentaries have been written by most distinguished Romanian (17-20) or foreign (21,22) scientists and by science journalists (23-27). As was recently stated in ref. 28: "In 1986 and 1988, the independent groups of Gheorghe Benga and Peter Agre, respectively, discovered the water channel proteins which later were called aquaporins… The detection of water-specific membrane channels in red blood cells belongs to the fundamental discoveries in the biology of the twentieth century (Benga et al., 1986a,b; Denker et al., 1988; Preston et al., 1992)."

Despite the important advances in understanding the structural determinants of water permeation through WCPs, particularly through AQP1, whose three-dimensional structure has been described (29), their physiological role in the RBC membrane is not fully understood (discussed in refs. 30-32). Investigations of RBCs from organisms of various ages could help shed light on the physiological significance of this transport process. As part of an extensive program of measurements of RBC water diffusional water permeability Benga's group has compared this parameter in fetal and adult guinea pig RBCs (33) and also in human fetal and maternal RBCs (34). It was concluded that RBC water permeability is a species characteristic (35), it is age-dependent in young organisms and has a genetic basis (36).

On the other hand, no comprehensive studies regarding the water

permeability of RBCs from aged people were performed. Such studies are important not only to better understand the physiological role of WCPs in the RBC membrane, but also to establish the reference values of parameters characterizing the RBC water permeability in healthy individuals for further studies of this characteristic of RBCs in subjects with various diseases.

We report here the first systematic studies of the RBC diffusional water permeability in elderly human subjects, compared to mature subjects; both groups consisted of healthy individuals, in order to exclude possible influences of various pathological conditions on the water permeability. In order to characterize this process properly the measurements were performed at various temperatures and the activation energy of water diffusion was also evaluated.

PATIENTS AND METHODS

Subjects

Fifty-eight apparently healthy individuals (31 females and 27 males) aged 35-80 years were included in the study. They were recruited in the Departments of Cardiology, Balneophysiotherapy and Rheumatology of "The New St. John" Suceava County Emergency Hospital, Romania. All patients had previously signed an informed consent. The study was approved by the Local Ethics Committee.

Blood samples

Samples of venous blood were obtained by venipuncture. The volume of blood taken for analyses was kept (for ethical reasons) at a minimum but sufficient to provide meaningful parameter estimates.

Blood samples were collected into heparin (15 IU.mL^{-1}), refrigerated immediately and used within 72h. The RBCs were isolated by centrifugation, washed three times in medium S: 150 mmol.L^{-1} NaCl, 5.5 mmol.L^{-1} glucose, 5 mmol.L^{-1} Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4, and suspended at a haematocrit of 30—40% in the same solution, supplemented with 0.5% (w/v) bovine serum albumin.

For incubation with PCMB the washed RBCs were suspended at a haematocrit of 10% in medium S and maintained at 37°C for 60 min. After incubation, the RBCs were washed three times in medium S to remove the reagent. Finally, the RBCs were suspended in medium S, supplemented with 0.5% bovine serum albumin, at a haematocrit of 30 - 40%.

Morphological measurements

The mean cell volume (V) was calculated from measurements of haematocrit and mean cell count using either a cell counter or a Thoma counting chamber to avoid errors in an electronic counter due to variations in cell sizes (37).

The cell surface area (A) was calculated from the mean cell volume and diameter according to the formula (38):(1) where D is the mean corpuscular diameter and V is the mean cell volume.

$$A = \frac{\pi D^2}{2} + \frac{4V}{D}$$

D was measured in isotonic suspensions of cells using a Nikon Eclipse 80i light microscope (Nikon Corporation, Tokyo, Japan) with an

Olympus Color View 1 CCD camera, using the CellD computer software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

The observed water proton relaxation time of the erythrocyte (T'_{2a}) and the water diffusion exchange time (T_e) were measured by the Mn^{2+}-doping ^1H NMR method (39-41) on a Bruker minispec 20 NMR spectrometer as previously described (42-44).

The transverse relaxation time of the water inside the cell (T_{2i}) was measured on packed cells (haematocrit 95%), from which the supernatant, with no Mn^{2+} added, had been removed by centrifugation. Samples for NMR measurements of the water proton relaxation time T'_{2a} (the prime denotes the fact that this is a composite relaxation time) were prepared by gentle mixing of 0.4 ml RBC suspended in medium S (haematocrit 30-40%) and 0.2 ml doping solution (40 mM $MnCl_2$, 100 mM NaCl). As shown by Conlon and Outhred (39) T'_{2a} and T_{2i} are the parameters used to estimate the water diffusion exchange time (T_e), according to the equation:

$$1/T_e = 1/T'_{2a} - 1/T_{2i} \quad (2)$$

The membrane permeability for water diffusion, P_d, is related to $1/T_e$ by the expression involving the cell water volume V_w (Vx0.7) and the cell surface area (A):

$$P_d = (V_w/A) \text{ x } (1/T_e) \quad (3)$$

The inhibition of water diffusion across the red cell membrane (in samples incubated with PCMB) was calculated using the formula:

$$\% \text{ Inhibition} = \text{ x } 100 \quad (4)$$

Other details of measurements of water permeability and of the activation energy of the water diffusion ($E_{a,d}$), were previously described (45,46).

Statistical methods

Data were expressed as mean ± standard deviation (SD). Differences between groups were analysed by Student's t test, using the Microsoft Office Excel software (Microsoft Corporation, Redmond, USA); p values ≤ 0.05 were considered statistically significant.

RESULTS

The subjects were divided in two groups. The first group included 44

Table 1. Morphological parameters of red blood cells*

Group of subjects	Number of red blood cells (10^6/mm^3)	Haematocrit (%)	Diameter (μm)	Volume (μm^3)	Surface Area (μm^2)	Vw/A Ratio
All subjects	4.73 ± 1.11 (n = 55)	40.6 ± 6.7 n = 55	7.3 ± 0.3 n = 57	85.0 ± 8.4 n = 55	130.3 ± 8.2 n = 55	0.46 ± 0.03 n = 57
Subjects 35-64.9 years	4.71 ± 1.24 (n = 41)	40.6 ± 7.5 n = 41	7.3 ± 0.3 n = 43	84.9 ± 7.7 n = 41	130.8 ± 7.2 n = 41	0.45 ± 0.02 n = 43
Subjects 65-80 years	4.78 ± 0.57 n = 14	40.7 ± 3.3 n = 14	7.2 ± 0.3 n = 14	85.4 ± 10.5 n = 14	128.6 ± 10.7 n = 14	0.47 ± 0.04 n = 14

Table 2. Values of water diffusional permeability Pd of red blood cells*

Group of subjects	Pd (cm x s-1 x10^3) Temperature (°C)					
	15	20	25	30	37	42
All subjects	3.17 ± 0.40 n = 57	3.68 ± 0.45 n = 57	4.27 ± 0.47 n = 116	5.00 ± 0.55 n = 116	6.23 ± 0.66 n = 174	7.37 ± 0.77 n = 174
Subjects 35-64.9 years	3.11 ± 0.41 n = 44	3.62 ± 0.46 n = 44	4.22 ± 0.49 n = 88	4.96 ± 0.57 n = 88	6.15 ± 0.67 n = 132	7.26 ± 0.78 n = 132
Subjects 65-80 years	3.39 ± 0.33 n = 13	3.87 ± 0.38 n = 13	4.45 ± 0.40 n = 28	5.30 ± 0.70 n = 28	6.62 ± 0.70 n = 42	7.85 ± 0.75 n = 42

*For details of preparation, measurements and calculations see Materials and Methods. The values are means ± SD; n= number of NMR measurements

mature individuals aged 35-64.9 years (24 females and 20 males) and the second group included 14 elderly subjects, aged 65-80 years: 7 females and 7 males.

The morphological parameters of RBCs are listed in Table 1. No significant differences in any of such parameters were observed in elderly subjects compared with mature subjects.

The NMR parameters and the estimates of P_d for the RBCs are listed in Table 2. Although the differences in P_d values between the two groups are rather small, these differences are statistically significant: $p<0.01$ at 15 °C; $p<0.04$ at 20 °C and 25 °C; $p<0.004$ at 37 °C and $p<0.02$ at 42 °C, by the Student's t test. This means that RBCs from elderly people have a higher water diffusional permeability.

From Table 3 it is obvious that PCMB is a powerful inhibitor of water diffusion in the RBCs of both groups. The degree of inhibition was higher for the RBCs of elderly subjects at all temperatures, however the differences were not statistically significant.

Systematic studies were performed to determine the values of the activation energy of water diffusion ($E_{a,d}$) in the RBCs from individuals of the two groups (Table 4). In the absence of PCMB it is clear that no significant differences between the two groups regarding this parameter exists. In the presence of PCMB a lower value of $E_{a,d}$ for the RBCs from elderly subjects was noticed; however the differences were not statistically significant.

DISCUSSION

The largest series of determinations of water diffusional permeability of human RBCs available in the literature was reported previously (47) and was proposed that the P_d values can be taken as references for studies of water permeability. The P_d values reported here in RBCs from mature subjects (35 — 64.9 years) are in good agreement with those reference values.

This is the first extensive comparative study of water permeability of RBCs from elderly individuals compared to mature subjects. It is obvious

Table 3. Inhibition of red blood cell water permeability induced by p-chloromercuribenzoate*

Group of subjects	PCMB	Measurement Temperature (°C)	Pd (cm x s-1x10^3)	Inhibition (%)
All subjects	-	25	4.3 ± 0.5 (n = 116)	48.2 ± 9.4
	+	25	2.2 ± 0.4 (n = 27)	
	-	30	5.0 ± 0.6 (n = 116)	43.4 ± 12.7
	+	30	2.9 ± 0.6 (n = 27)	
	-	37	6.3 ± 0.7 (n = 174)	38.7 ± 11.9
	+	37	3.9 ± 0.7 (n = 27)	
Subjects 35-64.9 years	-	25	4.2 ± 0.5 (n = 88)	47.1 ± 9.6
	+	25	2.2 ± 0.5 (n = 20)	
	-	30	5.0 ± 0.6 (n = 88)	42.1 ± 13.6
	+	30	2.9 ± 0.5 (n = 20)	
	-	37	6.2 ± 0.7 (n = 132)	36.6 ± 11.8
	+	37	4.0 ± 0.7 (n = 20)	
Subjects 65-80 years	-	25	4.6 ± 0.7 (n = 28)	51.2 ± 8.9
	+	25	2.3 ± 0.3 (n = 7)	
	-	30	5.3 ± 0.7 (n = 28)	47.3 ± 9.4
	+	30	2.9 ± 0.3 (n = 7)	
	-	37	6.6 ± 0.7 (n = 42)	45.6 ± 10.2
	+	37	3.7 ± 0.6 (n = 7)	

For details of preparation, measurements and calculations see Materials and Methods. The values are means ± SD; n= number of NMR measurements.

Table 4. The activation energy of water diffusion in red blood cells*

Group of subjects	PCMB	Ea (kJ/mol)
All subjects	-	23.8 ± 1.7 (n = 58)
	+	35.5 ± 9.6 (n = 27)
Subjects 35-64.9 years	-	23.9 ± 1.7 (n = 44)
	+	37.1 ± 0.4 (n = 20)
Subjects 65-80 years	-	23.0 ± 2.3 (n = 14)
	+	30.9 ± 9.3 (n = 7)

*For details of preparation, measurements and calculations see Materials and Methods. The values are means ± SD; n= number of NMR measurements.

from the results reported here that, compared with RBCs of mature individuals, RBCs from elderly people have a higher water diffusional permeability. This is documented by the higher values of P_d.

The higher water permeability of RBCs from elderly individuals is also suggested by the higher values of the degree of inhibition of water diffusion induced by PCMB. Although not statistically significant (probably due to a too small number of measurements), the differences between the RBCs of the two groups of individuals concerning the degree of inhibition by PCMB are in agreement with a higher water permeability of the RBCs from elderly people.

There is a general agreement that the inhibition of RBC water permeability induced by mercurials is due to the closure of water channels and the residual water permeability is due to water diffusion across the lipid bilayer (3-6). As pointed out by Stein (48), the values of permeability measured for specific molecules under conditions of maximal inhibition define the basal permeability, in the absence of any specific transport pathway; i.e. in case of RBC water permeability the basal permeability corresponds to diffusion of water across the lipid bilayer. Consequently, the percent inhibition of RBC water permeability is due to the water channel proteins. From Table 3 it is obvious that RBCs from both groups have similar values of the basal permeability to water: 2.2 — 2.3 cm x s^{-1} x10^{-3} at 25 °C, 2.9 cm x s^{-1}x10^{-3} at 30 °C and 3.7 — 4.0 cm x s^{-1}x10^{-3} at 37 °C. This means that the higher water permeability of RBCs from elderly individuals is due to a higher contribution of WCPs.

Other parameter of interest is the value of the activation energy for water diffusion ($E_{a,d}$) across the membrane. In the absence of PCMB it is clear that no significant differences between the two groups regarding this parameter exists. In the presence of PCMB a lower value of $E_{a,d}$ for the RBCs from elderly subjects was noticed, however the differences were not statistically significant. Schafer and Andreoli (50) described two components of the activation energy for water diffusion across a membrane: one is the energy required for a water molecule to break the hydrogen bonds formed with neighboring molecules, while the other is the activation energy for water diffusion across the membrane. A low value of $E_{a,d}$ for a molecule suggests that the diffusion of the molecule is specialized with respect to the hydrophobic lipid bilayer, i.e. water channel proteins are involved. A higher water permeability of the RBC membrane could be due to a greater number of channels; hence in parallel with a higher P_d value one would expect a lower value of $E_{a,d}$. This is what we observed in the case of RBCs from elderly individuals after incubation with PCMB.

Although the difference between the two groups regarding Ea,d is not statistically significant, possiblydue to a too small number of samples, the difference is in the "right" direction.

The increased water permeability of RBCs from aged people reported here, although small, but statistically significant, deserves appropriate attention.

Benga and Kuchel have previously (30-32) provided possible explanations for the fast exchange of water across the RBC membrane, and, consequently, for

the physiological role of WCPs in the red blood cell. The first explanation is denoted as the "oscillating sieve" hypothesis and it states that known membrane undulations of the RBC membrane (at frequencies up to 30 Hz with displacements up to 0.3 μm) are energetically favoured by the high water permeability of the membrane. The second explanation, denoted as the "displacement" hypothesis, is based on the known rapid exchange across the RBC membrane of ions (such as Cl^- and HCO_3^-) and solutes (such as glucose), all of whose molecular volumes are significantly greater than that of water. The RBC membrane has been "naturally selected" to be very permeable to water so that the rapid entry and exit of solutes of molecular size greater than water allows concomitant displacement of water thus obviating a change in cell volume which would affect the concentration of reactants and hence the rates of enzymic and binding reactions, and cell flexibility" (30).

The two above mentioned explanations are related to molecular- and cellular- scale events; however, there is a third explanation that applies to the whole-body system: the organisms that are more physically active have higher metabolic rates or higher mean rates of circulation of their blood and also have higher RBC water permeability (30-32). This was obvious from comparative studies of RBC water permeability in over 30 animal species taking man as reference (see refs. 2, 35, 36 for reviews and 42-44, 50 for original reports).

Based on the results reported here we suggest that in case of elderly people a relatively higher water permeability of the RBC membrane compared to mature subjects is required in order to favour the membrane undulations and the rapid entry or exit of solutes of molecular size greater than water, in conditions of an organism which is less physically active, probably has lower metabolic rates and lower mean rates of blood circulation. Further detailed studies will be very useful to elucidate these aspects.

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References

1.Benga Gh. Birth of water channel proteins — the aquaporins. Cell Biol Int 2003; 27(9):701-709.

2.Benga Gh. Water channel proteins (later called aquaporins) and relatives: past, present and future. IUBMB *Life* 2009; 61(2): 112-133.

3.Macey RI, Farmer REL. Inhibition of water and solute permeability in human red

cells. Biochim Biophys Acta 1970; 211:104—106.
4.Macey RI. Transport of water and urea in red blood cells. Am J Physiol 1984; 246(3 Pt 1):C195-203
5.Benga Gh, Popescu O, Pop VI, Holmes RP. p-Chloromercuribenzene sulfonate binding by membranes proteins and the inhibition of water transport in human erythrocytes. Biochemistry 1986; 25(7): 1535-1538.
6.Benga Gh, Popescu O, Pop VI, Muresan A, Mocsy I, Brain A, Wrigglesworth J. Water permeability of human erythrocytes. Identification of membrane proteins involved in water transport. Eur J Cell Biol 1986; 41: 252-262.
7.Benga Gh. Water transport in human red blood cells. Prog Biophys Mol Biol 1988; 51(3): 193-245.
8.Benga Gh. Water exchange through the erythrocyte membrane. Int Rev Cytol 1989; 114: 273-316.
9.Benga Gh. Membrane proteins involved in the water permeability of human erythrocytes: binding of p-chloromercuribenzene sulfonate to membrane proteins correlated with nuclear magnetic resonance measurements. In Benga Gh, ed. Water Transport in Biological Membranes. CRC Press, Boca Raton, 1989: 41-61.
10.Benga Gh. The first water channel protein (later called aquaporin 1) was first discovered in Cluj-Napoca, Romania. Romanian J Physiol 2004; 41(1-2): 3-20.
11.Benga Gh. The discovery of the first water channel protein (later called aquaporin 1) in the human red blood cell membrane in 1985 in Cluj-Napoca, Romania, a few years before Peter Agre (2003 Nobel Prize in chemistry). In: Gârban Z, Dragan P, Gârban Gabriela, eds. Metal Elements in Environment, Medicine and Biology. Proc. 6th Int. Symp., Timisoara, Nov. 8-10, 2004. Eurobit Publishing House, Timisoara, 2004, VI: 61-74.
12.Benga Gh. Canalele proteice pentru apa (aquaporine) din membranele celulare: de la descoperirea lor la Cluj-Napoca în 1985 la premiul Nobel pentru chimie 2003. Craiova Medicala, 2004; 6(2): 201-203.
13.Benga Gh. Water channel proteins: from their discovery in 1985 in Cluj-Napoca, Romania, to the 2003 Nobel Prize in Chemistry and their medical implications. In: Zinn D, Savoie M, Lin KC, El-Badawy El-S, Benga Gh, eds. Proceedings of the 9th World Multi-Conference on Systemics, Cybernetics and Informatics, 2005; 10: 99-104.
14.Benga Gh. Water channel proteins: from their discovery in 1985 in Cluj-Napoca, Romania, to the 2003 Nobel Prize in Chemistry and their implications in molecular medicine. Keyo J Med 2006; 55(2): 64-69.
15.Benga Gh. Water channel proteins: from their discovery in 1985 in Cluj-Napoca, Romania, to the 2003 Nobel Prize in Chemistry. Cell Mol Biol 2006; 52(7): 25-31.
16.Benga Gh. Water channel proteins (aquaporins and relatives): twenty years after their discovery in Cluj-Napoca, Romania. Acta Endocrinologica 2006; 2(3): 323-336.
17.Schneider Fr. Twenty years since the discovery of the first waterchannel protein by Gheorghe Benga's group in Romania. Fiziologia (Physiology) 2005; 15(4): 29-30.
18.Caproiu MT. The 2003 Nobel Prize in chemistry eluded the Romanian chemist Gheorghe Benga who first discovered the aquaporins. Rev chim 2006; 57(4): 439-443.
19.Gozariu L. Comments on the discovery of water channels, selected for the 2003 Nobel Prize in chemistry, and the regrettable omission of Benga Gh. Acta

endocrinologica 2006; 2: 377-380.
20.Frangopol P, Gavrila L. Memorandum regarding the discovery of the first water channel protein by Gheorghe Benga in Romania, a few years before Peter Agre (2003 Nobel Prize in chemistry). Rom J Genetics 2005; 1(2): 101-102
21.Vandenberg JI, Kuchel PW. Nobel Prizes for magnetic resonance imaging and channel proteins. Med. J. Australia 2003; 179: 611-613.
22.Sha'afi R.I. Opening Address to the Symposium Water Channel Proteins: from their Discovery in 1985 in Romania to the 2003 Nobel Prize in Chemistry and their implications in Molecular Medicine Systems. In: Zinn D, Savoie M, Lin KC, El-Badawy ElS, Benga Gh, eds. Proceedings of the 9th World Multi-Conference on Systemics, Cybernetics and Informatics 2005; 10: 128-129.
23.Rognerud K.K. Kemipriset vållar ny strid. Dagens Nyheter 10 December 2003. (Accessed December 11, 2003, at site www.dn.se/DNet/jsp/polopoly.jspd=597&a=212849&previous RenderType=6.)
24.Bradley D. Nobel controversy, again and again. The Alchemist, 13 October 2003 (Accessed November 4, 2003, at site www.chemweb.com/alchem/articles/1063812326697.html).
25.Blackman A. Leading lights in science prize-fight. Chemistry Matters November 2003 and Otago Daily Times 3 November 2003 (Accessed December 12, 2003, at site www.odt.co.nz/cgi-bin/search-display-story-onlinenewdate=03Nov2003&object=NTL02J7657LS&type=html&WORDS=benga&DB=Editorial).
26.Nobel mystery. Comment, The King's College Newsletter, Issue No. 151, January 2004: 4. (Accessed February 20, 2004, at site http://www.kcl.ac.uk/depsta/ppro/comment/151.pdf).
27.Kapoun J. Ohlédnutí za týdnem Nobelových cen (3): Chemie. Science WORLD 2003.
(Accessed January 24, 2004, at site www.scienceworld.cz/sw.nsf/ID/0BC262AFF133FFD0 C1256 DC1003018F3).
28.Wolburg H, Wolburg-Buchholz K, Fallier-Becker P, Noell S, Mack AF. Structure and functions of aquaporin-4-based orthogonal arrays of particles. In: Kwang W. Jeon, ed. Int Rev Cell Mol Biol, Burlington: Academic Press, 2011; 287:1-41.
29.Murata K, Mitsuoka K, Hirai T, Walz T, Agre P, Heymann JB, Engel A, Fujiyoshi Y. Structural determinants of water permeation through aquaporin 1. Nature 2000; 407(6804): 599-605.
30.Kuchel PW, Benga Gh. Why is the transmembrane exchange of water in the red blood cell so fast? B Mol Med 2003; 15-16: 29-34.
31.Benga Gh, Kuchel PW. Physiological significance of water channel proteins in the red blood cell membranes: analysis at the level of the cell and of the whole-body systems. The 9th World Multi-Conference on Systemics, Cybernetics and Informatics, Orlando, Fl. 2005; X: 105-110.
32.Kuchel PW, Benga Gh. Why does the mammalian red blood cell have aquaporins? BiosSystems 2005; 82(2): 189-196.
33.Benga Gh, Borza T, Matei H, Hodor P, Frentescu L, Ghiran I, Lupse C. Comparative nuclear magnetic resonance studies of diffusional water permeability of red blood cells from different species. VIII. Adult and fetal guinea pig (*Cavia porcellus*). Comp Haematol Int 1995; 5: 106-111.
34.Benga Gh, Frentescu L, Matei H, Tigan S. Comparative nuclear magnetic resonance studies of water permeability of red blood cells from maternal venous and newborn umbilical cord blood. Clin Chem Lab Med

2001; 39(7): 606-611.
35.Benga Gh. Water channels in membranes. Cell Biol Int 1994; 18(8): 829-833.
36.Benga Gh. Diffusional water permeability of red bloods cells from various vertebrate species. B Mol Med 2001; 7-8: 27-42.
37.Jain NC. Schalm's veterinary hematology. Philadelphia: Lea and Febiger, 4th edn, 1986.
38.Kondi V. Laboratorul clinic — hematologie. Editura Medicala, Bucuresti, 1981.
39.Conlon T, Outhred R. Water diffusion permeability of erythrocytes using an NMR technique. Biochim Biophys Acta 1972; 288(2):354—361.
40.Morariu VV, Benga Gh. Evaluation of a nuclear magnetic resonance technique for the study of water exchange through erythrocyte membranes in normal and pathological subjects. Biochim Biophys Acta 1977; 469(3):301—310.
41.Morariu VV, Benga Gh. Water diffusion through erythrocyte membranes in normal and pathological subjects: nuclear magnetic resonance investigations. In: Benga Gh, Baum H, Kummerow FA, eds. Membrane Processes: Molecular Biology and Medical Applications. Springer Verlag, New York, 1984: 121-139.
42.Benga Gh, Matei H, Borza T, Porutiu D, Lupse C. Comparative nuclear magnetic resonance studies of diffusional water permeability of red blood cells from different species. V - Rabbit (*Oryctolagus cuniculus*). Comp Biochem Physiol 1993; 106B (2): 281-285.
43.Benga Gh, Borza T, Popescu O, Porutiu D, Matei H. Comparative nuclear magnetic resonance studies of diffusional water permeability of red blood cells from sheep and cow. Comp Biochem Physiol 1993; 104B(3): 589-594.
44.Benga Gh, Chapman BE, Gallagher CH, Cooper D, Kuchel PW. NMR studies of diffusional water permeability of red blood cells from macropodid marsupials (kangaroos and wallabies). Comp Biochem Physiol 1993; 104A(4): 799-803.
45.Benga Gh, Chapman BE, Hinds L, Kuchel PW. Comparative NMR studies of diffusional water permeability of erythrocytes from some animals introduced to Australia: rat rabbit and sheep. Comp Haematol Int 1994; 4:232-235.
46.Benga Gh, Pop VI, Popescu O, Hodârnau A, Borza V, Presecan E. Effects of temperature on water diffusion in human erythrocytes and ghosts-nuclear magnetic resonance studies. Biochim Biophys Acta 1987; 905(2): 339-348.
47.Benga Gh, Pop VI, Popescu O, Borza V. On measuring the diffusional water permeability of human red blood cells and ghosts by nuclear magnetic resonance. J Biochem Biophys Methods 1990; 21(2):87-102.
48.Stein WD. Transport and diffusion across cell membranes. Academic Press ,Orlando, Fl., 1986.
49.Schafer JA, Andreoli TE. Principles of

water and nonelectrolyte transport across membranes. In: Andreoli TE, Hoffman JF, Farnestil DD, eds. Membrane physiology. New York: Plenum, 1980: 177—190.
50.Benga G, Chapman BE, Kuchel PW. Comparative NMR studies of diffusional water permeability of red blood cells from different species. XV. Agile wallaby (*Macropus agilis)*, red-necked wallaby (*Macropus rufogriseus*) and Goodfellow's tree kangaroo (*Dendrolagus goodfellowi*). Comp Biochem Physiol 2009; 154A:105-109.