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Water Efflux in Red Blood Cells of Sickle Cell Patients under Spontaneous Deoxygenation

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Abstract

The water transport through Red Blood Cells (RBC) membrane has been previously studied in Sickle Cell Disease (SCD) using oxygenated RBC or under complete deoxygenation. In this work, the water efflux in RBC of sickle cell patients was studied under spontaneous deoxygenation conditions. With that purpose, a magnetic resonance method was used to evaluate the water exchange time (τ_e) and the permeability through the erythrocyte membrane (P) measuring the spin-spin relaxation time (T_2) in doped and non-doped RBC. Carr-Purcell-Meiboon-Gill (CPMG) pulse sequence was used to measure T_2 in a magnetic resonance console coupled to one homogeneous magnet system (0.095 T). An increase of the water transport in RBC from sickle cell patients was observed and characterized with a τ_e value of 15.2 ± 0.8 ms. The abnormal activation of the P_{sickle}, Gardos, and potassium chloride cotransporter channels starting from deoxygenation, as well as, the possible appearance of new pores due to the increase of the hemoglobin-membrane interaction, are suggested to explain this abnormal transport phenotype. The change of the water volume to surface ratio (V/S) in the sickle cells is also suggested to be considered in P calculation under deoxygenation. The results obtained in this work increase the fundamental knowledge about molecular mechanism involved in SCD and could be useful in the development of new methods for diagnostic and treatment evaluation.

Keywords

Red Blood Cells, Water Permeability, Nuclear Magnetic Relaxation, Sickle Cell Disease

1. Introduction

After one century, SCD is still an important worldwide health problem without a final

solution [1]. Its complexity, genetic origin [2] and the different molecular processes involved in its pathophysiology [3] have contributed to this situation. The presence of an abnormal hemoglobin (HbS) inside the RBC, causing a polymerization process under deoxygenation, is recognized as the molecular process contributing most to the pathogenesis of SCD [4], and for this reason many efforts have been made in relation to its study [5] [6] [7] [8] [9]. Nevertheless, the deoxygenation also causes cell damage, dehydration and shrinking [10].

Different membrane abnormalities have been documented in RBC from sickle cell patients [11]: loss of lipid bilayer asymmetry, altered rate of phosphatidylcholine flip-flop, abnormal spectrin-actin skeleton, as well as, defective association between ankyrin and spectrin *in situ* on sickle cell inside out vesicles. These cause functional abnormalities as an abnormal transport phenotype [11] [12]: increased influx of calcium, reduced efflux permeability to non-electrolytes as glycerol and ethylene glycol and net loss of monovalent cations. Specially, the net loss of monovalent cations has been directly related with direct cellular dehydration because of the cellular response to maintain the osmotic equilibrium [12].

The water transport in RBC has been widely studied before [13]-[18]. Especially, the water efflux and influx through the membrane of RBC from sickle cell patients has been studied in oxygenated erythrocytes [19] and in cells under total deoxygenation [20], however, no studies under spontaneous deoxygenation have been performed. On the other hand, after deoxygenation, no values of τ_e and no proper values of P have been reported.

The deoxygenation pattern (deoxygenation % and speed) defines the HbS polymerization, as well as, the RBC shape change [2] [4]. In this work, we study τ_e after HbS polymerization under spontaneous deoxygenation conditions. Different molecular mechanisms are considered to explain the observed behaviour, and the necessity of an adequate evaluation of the V/S to calculate P in sickle cells, after deoxygenation, is discussed.

2. Materials and Methods

After the approval of the hospital ethical committee, and the obtaining of the informed consent, samples of RBCA (containing adult normal hemoglobin, HbA) and RBCS (containing HbS) were obtained [5]. Whole blood samples were obtained by venipuncture and immediately heparinized. After removing the plasma and leukocytes, RBC were washed three times with phosphate buffered saline (PBS, pH 7.4, Sigma Chemicals Co.) [5] [6] [7] [8] [9]. Supernatants were removed by centrifugation (500 g, 10 min) and decanting. The obtained washed RBC were resuspended (Hematocrit 45%, PBS (pH 7.4), 0.5% of BSA) and one portion was centrifuged (1000 g, 30 min, 25°C) eliminating the supernatant to obtain packed RBC. 500 μl of packed RBC were taken and T_2 for this sample was measured (T_{2a}).

300 μl of resuspended RBC were mixed with 600 μl of the manganese chloride stock solution (MnCl$_2$(aq)) to obtain a final concentration of 2.5 mM. 500 μl of doped RBC

were taken and T_2 for this sample was measured (T_{2d}).

Starting from the measured values of T_{2a} and T_{2d}, τ_e and P were calculated as follows [13]:

$$\frac{1}{\tau_e} = \frac{1}{T_{2d}} - \frac{1}{T_{2a}} \tag{1}$$

$$P = \frac{V}{S} \cdot \frac{1}{\tau_e} \tag{2}$$

where V (0.63×10^{-10} cm^3) and S (1.42×10^{-6} cm^2) are the water volume inside the RBC and the surface of the RBC, respectively. $V/S = 4.4 \times 10^{-5}$ cm.

The magnetic resonance measurements were performed in a magnetic resonance console (MARAN DRX, Oxford Instruments, UK). T_2 was measured using CPMG pulse sequence with a resonance frequency of 4.0353 MHz (homogeneous permanent magnet, $B_0 = 0.095$ T), 10 and 20 μs for 90° and 180° pulses respectively, 128 scans, 50% for the RF power amplifier and the receiver gains, 3 s of relaxation delay and echo time of 0.2 ms (doped RBC) and 2 ms (packed RBC).

A student's test (t-test) was performed to compare main values with $\alpha = 0.05$.

3. Results and Discussion.

The values of T_{2d}, τ_e and P in oxygenated RBCA were measured for two temperature values (25°C and 29°C) (see Table 1).

The τ_e and P values for RBCA water efflux, shown in Table 1, are coherent with previous reports in the literature (τ_e = (9.8 - 14.0) ms; $P = (3.3 - 4.7) \times 10^{-3}$ cms^{-1}) [16]. The independence with the temperature is due to the activation energy characterizing

Table 1. T_{2d}, τ_e and P values corresponding to oxygenated RBCA for two temperature values (25°C ± 1°C and 29°C ± 1°C).

Individuals	T_{2d} (ms)	τ_e (ms)	P (10^{-3} cms^{-1})	T (°C)
1	9.93 ± 0.53	12.00 ± 0.23	3.70 ± 0.18	29 ± 1
2	11.67 ± 0.76	13.00 ± 0.46	3.30 ± 0.16	29 ± 1
3	11.89 ± 0.94	13.7 ± 1.2	3.25 ± 0.29	29 ± 1
4	10.60 ± 0.50	11.30 ± 0.67	3.92 ± 0.20	29 ± 1
5	11.83 ± 0.63	12.80 ± 0.84	3.40 ± 0.22	29 ± 1
6	11.02 ± 0.80	12.19 ± 0.75	3.60 ± 0.18	29 ± 1
7	14.75 ± 0.69	16.30 ± 0.84	2.68 ± 0.13	29 ± 1
8	12.87 ± 0.50	13.93 ± 0.58	3.16 ± 0.13	29 ± 1
9	13.71 ± 0.96	15.0 ± 1.1	2.94 ± 0.21	29 ± 1
10	11.28 ± 0.40	11.99 ± 0.42	3.67 ± 0.13	25 ± 1
11	12.16 ± 0.55	13.32 ± 0.66	3.31 ± 0.16	25 ± 1
12	14.98 ± 0.43	16.47 ± 0.53	2.67 ± 0.01	25 ± 1
13	14.14 ± 0.60	15.21 ± 0.53	2.89 ± 0.10	25 ± 1
Main values	12.37 ± 1.60	13.6 ± 1.6	3.27 ± 0.39	-

the process (E_a = 26 kJ·mol^{-1}) [18] and its influence in Equation (3).

$$\tau_e = \tau_{e0} \exp\left(-\frac{E_a}{KT}\right)$$

(3)

τ_{e0}, T and K are the initial water exchange time, the absolute temperature and the Boltzman constant, respectively.

The P values in oxygenated and spontaneously deoxygenated RBCA (24 h, 36°C, 50% of deoxygenation) are shown in **Figure 1**. Because of the deoxygenation inhibits the KCC (K$^+$ and Cl$^-$ cotransporter) channel activation in RBCA [12], causing τ_e increases, these cells have a reduced P values after spontaneous deoxygenation. It was not taken into account and/or discussed in previous results [20].

The values of T_{2d}, τ_e and P in oxygenated RBCS are summarized in **Table 2**. P
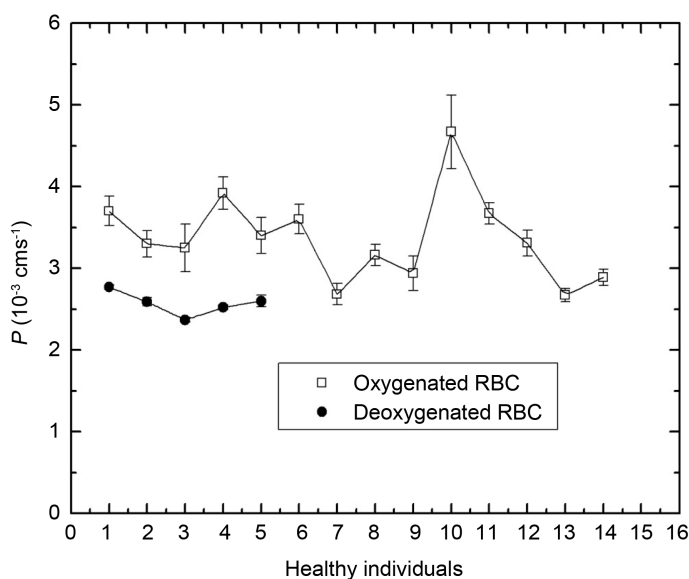

Figure 1. Efflux water permeability in oxygenated (100% of oxygenation) and spontaneously deoxygenated (50% of deoxygenation) RBC from healthy individuals. Red blood cells have intracellular concentrations and P was determined starting from τ_e and V/S ratio (Equation (2)). τ_e was measured using the T_2 values of doped (2.5 mM MnCl$_{2(aq)}$) and not doped RBC samples (Equation (1)). T_2 was measured using CPMG.

Table 2. T_{2d}, τ_e and P values in oxygenated RBCS.

Sickle Cell patient	T_{2d} (ms)	τ_e (ms)	P (10^{-3} cms^{-1})	T (°C)
1	16.01 ± 0.80	18.7 ± 1.2	2.35 ± 0.16	29 ± 1
2	17.34 ± 0.30	20.3 ± 1.1	2.16 ± 0.15	29 ± 1
3	21.71 ± 0.70	26 ± 1	1.69 ± 0.12	29 ± 1
4	17.16 ± 0.96	20.3 ± 1.3	2.17 ± 0.14	29 ± 1
5	16.27 ± 0.96	19.5 ± 1.3	2.26 ± 0.16	29 ± 1
6	18.84 ± 0.88	23.2 ± 1.3	1.89 ± 0.11	29 ± 1
7	15.4 ± 1.1	18.2 ± 1.5	2.43 ± 0.21	29 ± 1
Main values	17.5 ± 2.1	20.8 ± 2.7	2.13 ± 0.26	29 ± 1

decreases ($(2.13 \pm 0.26) \times 10^{-3}$ cms^{-1}) and τ_e increases ($\tau_e = 20.89 \pm 2.77$ ms) if compared with the values obtained for healthy individuals ($P = (3.27 \pm 0.39) \times 10^{-3}$ cms^{-1} and $\tau_e = 13.63 \pm 1.67$ ms) (see **Figure 2**).

The increase in τ_e and P reduction in oxygenated RBCS, compared with healthy individuals, match with previous reports ($P = (1.61 \pm 0.39) \times 10^{-3}$ cms^{-1} and $\tau_e = 29.2 \pm 7.3$ ms) [19], and are closely related to:

- The lipid peroxidation in the lipid bilayer.
- The hemoglobin interaction with membrane Band 3 protein.
- The oxidation of Band 3 sulfhydryl groups in the 15 kDa transmembrane fragment.

Because of simple diffusion only supports the 10% of the water transport through RBC membrane, the lipid peroxidation in the lipid bilayer cannot explain the observed P reduction in 34%. The sulfhydryl group oxidation is caused by the spontaneous generation of oxygen radicals and has been considered as the main contribution to P reduction [19]. The hemoglobin-membrane interaction is established with the Band 3 protein, at the same position as the main pore (4.5 Å) for water transport in RBC membrane; and, therefore, could cause an occlusion of this channel [20]. It has been demonstrated that this interaction is stronger in oxygenated sickle cell patient RBC than in cells from healthy controls [21]. As a consequence, the occlusion could contribute to the observed P reduction in oxygenated RBCS.

The values of T_{2d} and τ_e in RBCS, under spontaneous deoxygenation, are summarized in **Table 3**.

The spontaneous deoxygenation decreases τ_e values ($\tau_e = 15.2 \pm 1.0$ ms) of the RBCS compared with the results obtained before deoxygenation ($\tau_e = 20.8 \pm 2.7$ ms)
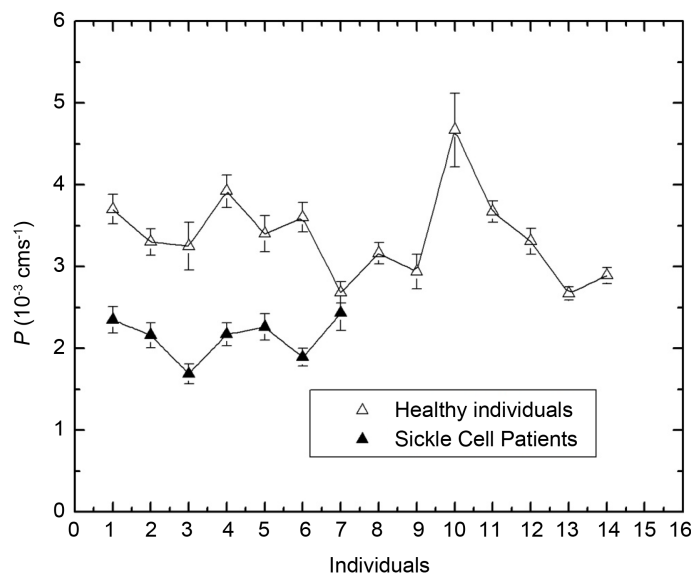

Figure 2. Efflux water permeability in oxygenated (100% of oxygenation) RBC from healthy individuals and sickle cell patients. Red blood cells have intracellular concentrations and P was determined starting from the τ_e and V/S ratio (Equation (2)). τ_e was measured using the T_2 values of doped (2.5 mM MnCl$_{2(aq)}$) and not doped RBC samples (Equation (1)). T_2 was measured using CPMG.

(see Figure 3).

Even though it is possible to find reports of the water transport for deoxygenated RBCS ($P = (1.58 \pm 0.06) \times 10^{-2}$ cms^{-1}) [20], it does not affect the novelty of our results because in these experiments:

- The reported experimental values of efflux water permeability were 10 times higher than the established values for human RBC [16].
- Induced complete deoxygenation was employed to provoke the HbS polymerization (0.5 mM, sodium dithionite).
- The τ_e values are not reported.

The explanation of decreased τ_e, as a consequence of the spontaneous deoxygenation, is related with the hemoglobin-membrane interaction and the activation of the P$_{sickle}$,

Table 3. Values of T_{2d} and τ_e in RBCS under spontaneous deoxygenation.

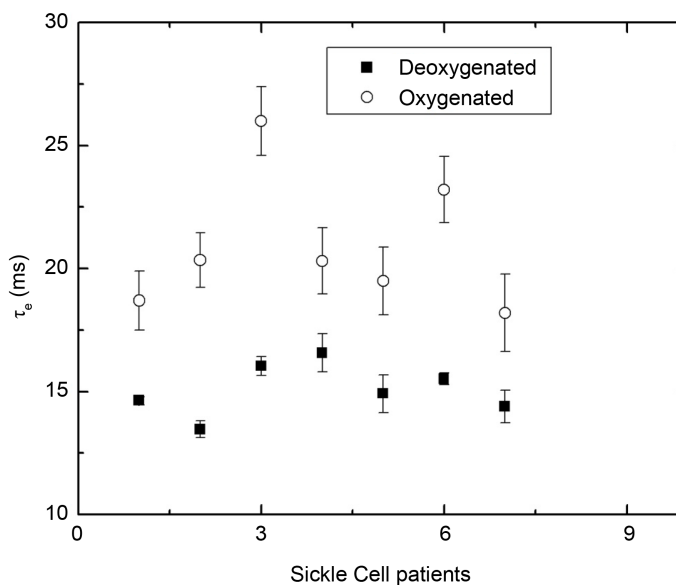| Sickle Cell patient	T_{2d} (ms)	τ_e (ms)	T (°C)
1	11.69 ± 0.11	14.63 ± 0.17	25 ± 1
2	11.32 ± 0.24	13.47 ± 0.34	25 ± 1
3	13.04 ± 0.25	16.04 ± 0.39	25 ± 1
4	13.26 ± 0.49	16.58 ± 0.77	25 ± 1
5	12.17 ± 0.50	14.92 ± 0.76	25 ± 1
6	12.57 ± 0.16	15.51 ± 0.24	25 ± 1
7	11.83 ± 0.45	14.39 ± 0.67	25 ± 1
8	13.3 ± 2.1	16 ± 3	25 ± 1
Main values	12.41 ± 0.77	15.2 ± 1.0	25 ± 1

Figure 3. Water exchange time in oxygenated (100% of oxygenation) and spontaneously deoxygenated (50% of deoxygenation) RBC from sickle cell patients. Red blood cells have intracellular concentrations and τ_e was measured using the T_2 values of doped (2.5 mM MnCl$_{2(aq)}$) and not doped RBC samples (Equation (1)). T_2 was measured using CPMG.

Gardos, and KCC channels. The hemoglobin-membrane interaction could cause the appearance of pores starting from high order structure of the Band 3 protein [20]. We have previously demonstrated the increase of hemoglobin-membrane interaction with HbS polymerization under spontaneous deoxygenation conditions [22]. Now, we are strongly suggesting this increase could provoke the opening of new pores for the water efflux, which could explain the τ_e decrease after the agglutination process.

In RBCS, decreased oxygen partial pressure (PO_2) provokes the P_{sickle} activation, causing a K^+ efflux, as well as, Na^+ and Ca^{2+} influx. When the Ca^{2+} concentration reaches a threshold, the Gardos Channels (about 150 per cells) are activated causing a fast K^+ and Cl^- efflux [12]. As a difference with RBCA, in the RBCS the deoxygenation inhibits the KCC channel until PO_2 = 40 mmHg, provoking it increases again when PO_2 < 40 mmHg [12]. After 24 h of spontaneous deoxygenation (PO_2 < 40 mmHg [23], 50% of deoxygenation) KCC is reactivated in our experiments contributing to the net ions loss. The efflux of ions is coupled with a water efflux to maintain the osmotic equilibrium. The cellular dehydration increases the HbS concentration, which facilitates the HbS polymerization, the increase in the hemoglobin-membrane interaction, and the RBC shrinking.

On the other hand, it is important to consider that we are determining P (Equation (2)) for RBCA and RBCS using the same V/S ratio, as usual in the literature [19]. We consider this is an adequate procedure in oxygenated RBCS taking into account that: the Mean Corpuscular Volume (MCV) and Surface Area (S) do not have a statistically significant variation in these cells [24]-[29]. For deoxygenated RBCS is reported a MCV decreasing [30] [31] [32] [33] [34] and S remains constant [31], which could change the V/S ratio and affect the P values. For this reason, in this work we are only reporting the τ_e values after deoxygenation for RBCS. To evaluate P, it is necessary to determine the V/S behavior in RBCS after deoxygenation. It could be done by performing a magnetic resonance experiment which includes a magnetic field gradient, studying the water self-diffusion coefficient behavior for short interpulse delay time in CPMG or stimulated echo pulse sequences [35]. Another way to measure P is starting from the effective water self-diffusion coefficient measured in the RBC, as well as, in the extracellular and intracellular medium [35]. Starting from this last determination and measuring, in the same sample, the τ_e values using the method described in this work, it is also possible to determine V/S using Equation (2). It will be the subject of forthcoming works.

Not separated variations in water transport have been found in density-separated cell fractions of RBCS [19] [20], for that reason our study has not considered it.

4. Conclusion

The HbS polymerization process, under spontaneous deoxygenation conditions, leads to an increase in the water transport through the RBC membrane from sickle cell patients, characterized by τ_e values of 15.2 ± 0.8 ms. The abnormal activation of the P_{sickle}, Gardos, and KCC channels starting from deoxygenation, as well as, the possible appearance of new pores due to the increased hemoglobin-membrane interaction could

explain this abnormal transport phenotype. The results obtained in this work increase the fundamental knowledge about molecular mechanism involved in SCD and could be useful in the development of new methods for diagnostic and treatment evaluation.

Acknowledgements

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