Hydrogen Peroxide Diffusion Across the Red Blood Cell Membrane Occurs Mainly by Simple Diffusion Through the Lipid Fraction

Florencia Orrico, Ana Clara Lopez, Nicolas Silva, Mélanie Franco, Isabelle Mouro-Chanteloup, Ana Denicola, Mariano Anibal Ostuni, Leonor Thomson, Matias Möller

► **To cite this version:**

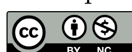
Florencia Orrico, Ana Clara Lopez, Nicolas Silva, Mélanie Franco, Isabelle Mouro-Chanteloup, et al.. Hydrogen Peroxide Diffusion Across the Red Blood Cell Membrane Occurs Mainly by Simple Diffusion Through the Lipid Fraction. Free Radical Biology and Medicine, 2024, 226, pp.389-396. 10.1016/j.freeradbiomed.2024.11.031 . hal-05527761

HAL Id: hal-05527761
https://hal.science/hal-05527761v1

Submitted on 25 Feb 2026

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC 4.0 - Attribution - Non-commercial use - International License

Hydrogen peroxide diffusion across the red blood cell membrane occurs mainly by simple diffusion through the lipid fraction

Florencia Orrico [a,b,c], Ana C. Lopez [a,b,c], Nicolás Silva [a,b,c], Mélanie Franco [d], Isabelle Mouro-Chanteloup [d], Ana Denicola [a,c], Mariano A. Ostuni [d], Leonor Thomson [b,c], Matias N. Möller [a,c,*]

[a] Laboratorio de Fisicoquímica Biológica, Instituto de Química Biológica, Facultad de Ciencias, Universidad de la República, Montevideo 11400, Uruguay

[b] Laboratorio de Enzimología, Instituto de Química Biológica, Facultad de Ciencias, Universidad de la República, Montevideo 11400, Uruguay

[c] Centro de Investigaciones Biomédicas (CEINBIO), Universidad de la República, Montevideo 11800, Uruguay

[d] Université Paris Cité and Université des Antilles, INSERM, BIGR, F-75015 Paris, France

* Correspondence to: Matias N. Möller, Laboratorio de Fisicoquímica Biológica, Instituto de Química Biológica, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, 11400, Uruguay.

E-mail address: mmoller@fcien.edu.uy

Florencia Orrico, forrico@fcien.edu.uy

Ana C. Lopez, anclalop@fcien.edu.uy

Nicolás Silva, nicolas.silva@pedeciba.edu.uy

Mélanie Franco, melanie.franco@inserm.fr

Isabelle Mouro-Chanteloup, isabelle.mouro-chanteloup@inserm.fr, https://orcid.org/0000-0002-6368-9904

Ana Denicola, denicola@fcien.edu.uy, https://orcid.org/0000-0002-5181-9708

Mariano A. Ostuni, mariano.ostuni@inserm.fr, https://orcid.org/0000-0001-6769-3786

Leonor Thomson, lthomson@fcien.edu.uy, https://orcid.org/0000-0002-2574-8520

Matias N. Möller, mmoller@fcien.edu.uy, https://orcid.org/0000-0002-3486-8217

Abstract

Hydrogen peroxide (H_2O_2) is an oxidant produced endogenously by several enzymatic pathways. While it can cause molecular damage, H_2O_2 also plays a role in regulating cell proliferation and survival through redox signaling pathways. In the vascular system, red blood cells (RBCs) are notably efficient at metabolizing H_2O_2. In addition to a robust antioxidant defense, we have recently determined that human RBCs also have a high membrane permeability to H_2O_2 that is independent of aquaporin 1 or aquaporin 3. In this work, we sought to further investigate the permeation mechanism of H_2O_2 through the membrane of human RBCs. First, we explored the role of other erythrocytic membrane proteins in H_2O_2 transport, including urea transporter B and ammonia transporter Rh proteins. However, no differences were found in H_2O_2 permeability in RBCs lacking these proteins compared to control RBCs. We then focused on the hypothesis that H_2O_2 diffuses through the lipid bilayer. To test this, we studied H_2O_2 permeability in RBCs from patients with Gaucher disease (GD), which accumulate sphingolipids in the membrane, affecting RBC morphology and deformability. We found that RBCs from GD patients exhibited lower H_2O_2 membrane permeability. In another approach, we treated normal RBCs with hexanol, which fluidizes the lipid fraction of the RBC membrane, and observed an increase in the permeability to H_2O_2. In contrast, hexanol had no effect in the rate of water efflux by aquaporin 1. Together, these results support the hypothesis that H_2O_2 diffusion through the RBC membrane occurs primarily through the lipid fraction.

Keywords: Hydrogen peroxide, red blood cell, erythrocyte, membrane, permeability, diffusion

Abbreviations: HbO_2, oxyhemoglobin; RBC, red blood cell; Prx2, peroxiredoxin 2; Pm, permeability coefficient; DMPC, dimiristoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; Chol, Cholesterol; DOPC, dioleoylphosphatidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; UT-B, urea transporter B; RhAG, Rh-associated glycoproteins; GD, Gaucher Disease; GCase, β-glucocerebrosidase; DPBS, Dulbecco´s phosphate buffer; HBSS, Hank's balanced salt solution; MCV, mean corspuscular volume; Hb, hemoglobin.

Introduction

Hydrogen peroxide (H_2O_2) is likely the most abundant oxidant in biology. Sources of H_2O_2 overlap with sources of superoxide because the latter rapidly dismutates by the action of the enzyme superoxide dismutase to oxygen and H_2O_2 (1). Therefore, H_2O_2 can be directly or indirectly produced enzymatically by NADPH oxidases, xanthine oxidase, and other enzymes, as a side reaction in mitochondrial respiration, and in spontaneous oxyhemoglobin (HbO_2) autoxidation (2). In blood vessels, H_2O_2 is produced mostly by endothelial NADPH oxidases and can have multiple physiological effects. For instance, H_2O_2 can induce vasorelaxation, and regulate cell proliferation, differentiation, and apoptosis (2–6). The concentration of H_2O_2 in plasma is still a matter of debate. A recent meta-analysis of published results suggests a steady state concentration of 1-5 μM (7) that could increase in certain conditions like exercise (8). However, it has long been recognized that red blood cells (RBCs) consume H_2O_2 very rapidly, so it is very unlikely that H_2O_2 could accumulate in blood. For instance, the half-life of H_2O_2 is estimated to be 35 ms in physiological conditions of 45% hematocrit (9). This high capacity to deal with H_2O_2 is given mainly by peroxiredoxin 2 (Prx2), which is present at a high concentration in the RBC (250-500 μM (10)), and reacts very rapidly with H_2O_2 ($k = 10^8$ $M^{-1}s^{-1}$ (11)). It is important to point out that for some experimental approaches, the physiological conditions cannot be used, and this has led to some confusion in the literature. Experimentally amenable conditions often involve low hematocrit and high H_2O_2 concentration. In these conditions, Prx2 is rapidly oxidized and cannot be recycled because NADPH is also depleted, and catalase becomes the dominant enzyme consuming H_2O_2 (10). Albeit reacting more slowly with H_2O_2 and being less abundant than Prx2, catalase (11 μM) is more abundant and slightly more reactive than glutathione peroxidase (1.4 μM), and it does not need a reductant as cosubstrate (10). This switch in H_2O_2-consuming enzyme dominance in RBCs depending on experimental conditions needs to be taken into account and can be taken advantage of to study the permeability of the membrane to H_2O_2 (9).

The consumption of extracellularly added H_2O_2 by RBCs clearly indicates that H_2O_2 can traverse the RBC membrane, but details on the mechanism of diffusion are still missing. We have previously studied the diffusion of H_2O_2 across pure lipid and human RBC membranes. Despite the low solubility of H_2O_2 in organic solvents (122000 times lower in hexadecane than in water (9)), H_2O_2 can diffuse across pure lipid membranes at similar rates than across cellular membranes.

The permeability coefficients (P_m) are 4.1×10^{-4} and 5.5×10^{-3} cm s^{-1} for liposome membranes composed of dimiristoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, cholesterol (DMPC:DPPG:Chol at a molar ratio 4:1:5) and dioleoylphosphatidylcholine, palmitoyloleoylphosphatidylglycerol, cholesterol (DOPC:POPG:Chol at a molar ratio 4:1:5) at 37 °C, and 2×10^{-4} and 1.6×10^{-3} cm s^{-1} for Jurkat T cells and human umbilical vein endothelial cells membranes at 37 °C (9,12,13). The P_m of human RBC membranes to H_2O_2 was 1.6×10^{-3} cm s^{-1} at 37 °C (9), in the high range of mammalian cell membrane permeability (14). Furthermore, we showed that H_2O_2 diffusion into human RBCs occurred without the facilitation by aquaporin 1 or aquaporin 3, by using RBCs devoid of these membrane proteins (9). We also demonstrated that no saturation in transport occurred up to 100 mM H_2O_2. Considering all this information, we proposed that H_2O_2 diffuses across the human RBC membrane through the lipid fraction, though recognizing that the involvement of other membrane proteins could not be ruled out (9). In particular, we suspected UT-B, the urea transporter that has also been observed to allow water passage (15), and Rh protein, which is associated with the transport of ammonia (16).

If H_2O_2 diffuses mainly across the lipid fraction, then RBCs containing abnormal lipid content, such as those from Gaucher Disease (GD) (17), could show differences in permeability to H_2O_2. GD is caused by β-glucocerebrosidase (GCase) deficiency. In RBCs, this manifests as a higher concentration of sphingolipids in the membrane (17) and altered rheologic properties, including enhanced blood viscosity, increased aggregation, and adhesion to endothelial cells (18).

In this work, we sought to further explore the mechanism of H_2O_2 permeation through the human RBC membrane. Therefore, we measured the permeability to H_2O_2 in human RBCs devoid of important membrane transport proteins, namely Kidd-null RBCs, deficient in UT-B, and Rh-null RBCs. Furthermore, we measured the permeability to H_2O_2 in RBCs from GD patients. Also, we used hexanol as a fluidizer of the lipid fraction and measured the effect on RBC membrane permeability to H_2O_2. The results support our previous hypothesis that H_2O_2 diffusion through the RBC membrane occurs preferentially through the lipid fraction.

Materials and methods

Materials

Chemical reagents were acquired from Sigma, Applichem, and Acros Organics. Work solutions of H_2O_2 were prepared daily and quantified by spectrophotometry ($\varepsilon_{240\ nm}$ = 39.4 M^{-1} cm^{-1} (19)). RBCs were washed by centrifugation at 900g for 10 minutes, discarding the supernatant and resuspending in either DPBS (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.67 mM KCl and 1.47 mM KH_2PO_4) or HBSS (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 1 g/L glucose, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$).

Blood samples

Studies were conducted in accordance with the Declaration of Helsinki. For assays with normal RBCs, blood samples were obtained from volunteer donors after informed consent at the Cátedra y Departamento de Medicina Transfusional, Hospital de Clínicas, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay. The research protocol was approved by the Ethics Committee of the Hospital. Packed RBCs were obtained as described before (10,20). Briefly, total blood was collected in primary bags containing CPD solution (105 mM citrate, 16 mM phosphate, and 129 mM dextrose, Terumo) and centrifuged at 2200 rpm at 20 °C to remove plasma and platelets. RBCs were preserved at 4 °C in SAGM solution (NaCl 8.77 g/L, glucose 9 g/L, mannitol 5.25 g/L, and adenine 0.3 g/L, Terumo). RBCs were used within one week of donation.

Rare RBCs

Kidd-null RBCs lacking urea transporter B (UT-B), Rh-null RBCs deficient in proteins from the Rhesus (Rh) complex, RBCs from patients with GD, and control normal RBCs for comparison were obtained by thawing cryopreserved samples from the Centre National de Référence pour les Groupes Sanguins (CNRGS), Paris, France. Regarding Kidd-null and Rh-null RBCs, only one donor of each mutation was analyzed, corresponding to samples previously characterized. The Kidd-null sample shows no UT-B protein by western-blot and no facilitated urea transport (21). The Rh-null patient had a deletion in the RhAG coding sequence resulting in a total absence of RhAG protein in the RBCs (22). For GD, samples from 14 patients were studied. Nine of these

patients had never received enzyme replacement therapy when blood samples were collected (NT, not treated), while the other five had been treated by enzymotherapy for at least 1 year (imiglucerase, Cerezyme, Genzyme Corporation, or velaglucerase alfa, VPRIV, Shire Human Genetic Therapies). Further data on GD donors is shown in Table 1. Before every experiment, RBCs were washed 3 times in the working solution by centrifugation at 900 g for 4 min at room temperature.

Table 1. Demographic characteristics, genetic characterization, and RBC characteristics of treated or not treated GD patients.

	NT GD	**T GD**
n	9	5
Male	2	3
Age (years)	43.2 ± 20.7	46.5 ± 7.3
	(18-86)	(32-51)
Mutations[a]		
N370S heterozygous	5	3
N370S homozygous	3	
Other	1	
Undetermined		2
RBC characteristics		
MCV (fL)	86.8 ± 6.8	87.8 ± 4.8
Hb (g/dL)	12.8 ± 1.7	16.1 ± 1.0

[a] N370S is the most common mutation in glucocerebrosidase (GBA1) associated to GD.

RBC treatment with hexanol

Hexanol solutions were prepared daily. For H_2O_2 permeability experiments, RBCs were washed in HBSS and diluted to 5% hematocrit. RBCs were then incubated with 0, 3, 6, and 12 mM hexanol for 30 minutes at room temperature. Stock 0.06% hematocrit suspensions of intact and lysed cells were then generated from every condition to continue with the H_2O_2 permeability protocol described below. For water permeability studies, 5% hematocrit RBCs in DPBS were treated with 0 or 18 mM hexanol in the same conditions.

Oxyhemoglobin concentration in supernatants was measured spectrophotometrically ($\varepsilon_{577\ nm}$ = 15 mM^{-1} cm^{-1} (23)) for hemolysis control, with no differences observed in the presence or absence of hexanol.

Determination of permeability coefficient to H_2O_2 in RBCs

The determination of the P_m to H_2O_2 in RBCs was conducted following the procedure described in Orrico et al. (9). In experiments performed to evaluate the effect of hexanol, RBCs were diluted in HBSS to a hematocrit of 0.06% after treatment. From this stock, suspensions of RBCs of increasing hematocrits (0.006%-0.024%) were generated and later mixed with 10 mM H_2O_2. The consumption of H_2O_2 by RBCs was measured in a Cary 50 spectrophotometer in a 1 mL quartz cuvette, following the decay in absorbance at 240 nm for 30 seconds at 37 °C. The same procedure was carried out with lysed cells, obtained by freezing part of the RBC stock. However, in this case, the range of hematocrits used was 10 times lower (0.0006-0.0024%) to obtain similar rates of reaction. Secondary graphs were generated by plotting these initial rates as a function of HbO_2 concentration in every sample. The slopes obtained (k_{RBC} and k_{lys} for intact and lysed RBCs, respectively) were used to calculate R, the k_{RBC}/k_{lys} ratio, and then P_m following equation 1.

$$Pm = \frac{k_{catalase}\,R}{\frac{S}{V}\,(1\text{-}R)} \qquad \text{(Eq. 1)}$$

S and V correspond to the surface area (1.4×10^{-6} cm^2) and volume (9×10^{-11} cm^3) of the RBC (24,25), and $k_{catalase}$ represents the pseudo-first order rate constant of H_2O_2 decomposition by catalase inside the RBC, calculated by extrapolating k_{lys} value to a condition of HbO_2 concentration of 20 mM. In this case, catalase can be assumed to be the main enzyme involved in H_2O_2 metabolization due to the low hematocrits and high H_2O_2 concentrations used in the experiments, as was previously demonstrated (10).

Determination of P_m in experiments with GD, Kidd-null and Rh-null RBCs was performed in the same manner, with minor variations in solutions and equipment. DPBS was used to wash RBCs and generate reaction mixes with H_2O_2. Simple spectrophotometrical reads were done in a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), while time courses were conducted using an SFM400 Stopped-Flow Spectrometer (Bio-Logic). Cryopreserved normal

RBCs were used as controls. When available, the determined mean corpuscular volume (MCV) was used in Eq. 1 instead of V.

Free HbO_2 in the supernatant was measured after treatment with H_2O_2, to evaluate possible RBC hemolysis. The concentrations of HbO_2 were equal to those determined in control RBCs treated with buffer, and less than 2% of the values quantified for lysed RBCs.

Assessment of osmotic water permeability in RBCs

RBCs were treated with hexanol in DPBS as described above. After being diluted to 0.5% hematocrit, RBCs were incubated with or without 0.5 mM mercuric chloride ($HgCl_2$) for 30 minutes at 10 °C. Water efflux was followed by mixing the cells with an equal volume of a hypertonic solution of 250 mM sucrose and measuring 90° light scattering at 650 nm. These measurements were performed in an SX Stopped-Flow Spectrometer (Applied Photophysics), for 2 seconds at 10 °C. The resulting time courses were fitted to an exponential model, where the constant (k_{water}) was used to compare between conditions.

Statistical analysis

Data were analyzed using GraphPad Prism 10.2.2 (GraphPad Software, Inc). Statistical analyses were performed by non-parametric Mann-Whitney or Kruskal-Wallis tests, when comparing two groups or more than two groups, respectively. Differences with $p < 0.05$ were considered statistically significant.

Results

Permeability of Kidd-null and Rh-null RBCs to H_2O_2

In our previous studies, aquaporins were discarded as H_2O_2 transporters in human RBCs (9). In continuity with this, we evaluated the possible role of other membrane proteins that could explain the relatively high H_2O_2 permeability and low activation energy of the process. H_2O_2 permeability was measured in Kidd-null RBCs, deficient in UT-B transporter, and Rh-null RBCs. Besides its canonical function as urea transporter, UT-B has also been reported to conduct water efficiently, accounting for 10% of the total osmotic water transport in human RBCs (15). Rh-associated

glycoproteins (RhAG) participates in the facilitated transport of ammonia in RBCs (16) and is absent in the Rh-null phenotype of the "regulator type", such as that used below (26).

.

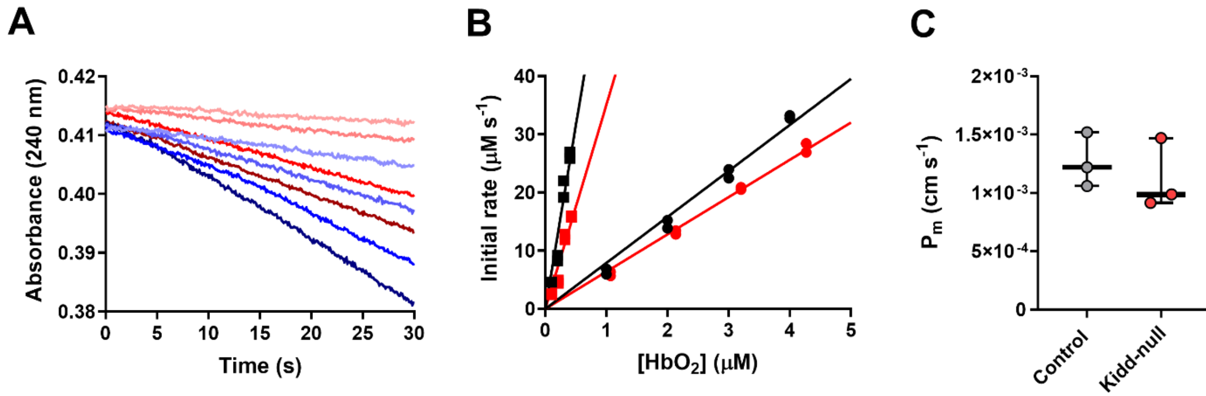


Fig 1. Permeability to H_2O_2 in Kidd-null RBCs. A. Consumption of 10 mM H_2O_2 by intact (blue tones) and lysed (red tones) Kidd-null RBCs at increasing cell densities (1.0-4.3 μM HbO_2 for intact RBCs and 0.1-0.4 μM HbO_2 for lysed RBCs) at 37 °C. The metabolization of H_2O_2 was measured by following the changes in its absorbance at 240 nm, for 30 seconds after being mixed with RBCs. **B.** Initial rates of H_2O_2 decomposition by intact (round symbols) and lysed (square symbols) normal RBCs (black) or Kidd-null RBCs (red), as a function of HbO_2 concentration in the sample. The slopes correspond to the observed constants, k_{RBC} for intact cells and k_{lys} for lysed cells, which were used to calculate R and P_m according to equation 1. **C.** Comparison of P_m determined for normal and Kidd-null RBCs (lines and bars represent mean and SD, respectively, from three experimental replicates). No statistically significant differences were observed.

The method to determine the P_m to H_2O_2 takes advantage of the dominant H_2O_2-metabolizing enzyme switch that occurs in RBCs at low hematocrit and high H_2O_2 concentration, where Prx2 is rapidly deactivated and catalase becomes dominant (9,10), and the enzyme latency concept (13,27,28). The latter states that while H_2O_2 diffuses into and out of the RBC, cytosolic catalase consumes incoming H_2O_2. Therefore, a steady state is achieved where the rate of diffusion into the cell equals the sum of the rates of outward diffusion and decomposition by catalase. Experimentally, the gradient of H_2O_2 established between the inside and outside of the RBC, and thus the P_m, can be calculated by measuring H_2O_2 consumption in intact versus lysed cells. This was performed by following the decay in absorbance of H_2O_2 at 240 nm (Fig 1A). The initial rates of these reactions were used to construct secondary plots (Fig 1B) to obtain k_{RBC} and k_{lys} and calculate P_m according to equation 1. As observed in Figure 1, lysed RBCs (square symbols)

metabolize H_2O_2 approximately 5 times faster than intact RBCs (round symbols) due to the absence of the permeability barrier of the membrane, which is maintained in cryopreserved samples, both normal and Kidd-null RBCs. However, when compared to control RBCs, no statistically significant differences were found between the P_m of the normal and Kidd-null phenotype ($1.3 \pm 0.2 \times 10^{-3}$ vs. $1.1 \pm 0.3 \times 10^{-3}$ cm s^{-1}, respectively). In the same manner, H_2O_2 permeability was evaluated in Rh-null RBCs, with no statistically significant differences found in comparison to control RBCs ($1.1 \pm 0.4 \times 10^{-3}$ vs. $1.1 \pm 0.3 \times 10^{-3}$ cm s^{-1}, respectively) (Figure 2). These results imply that neither UT-B nor RhAG are involved in facilitating H_2O_2 transport across the human RBC membrane.

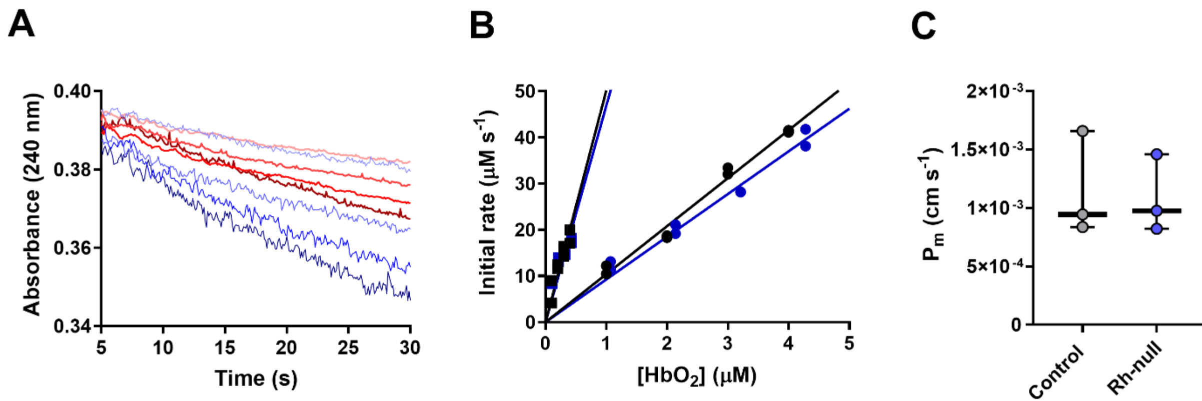


Fig 2. Permeability to H_2O_2 in Rh-null RBCs. A. Metabolization of 10 mM H_2O_2 by intact (blue tones) and lysed (red tones) Rh-null RBCs at increasing cell densities (1.0-4.3 μM HbO_2 for intact RBCs and 0.1-0.4 μM HbO_2 for lysed RBCs) at 37 °C. H_2O_2 consumption was followed by measuring changes in the absorbance at 240 nm for 30 seconds after being mixed with RBCs. **B.** Initial rates of H_2O_2 decomposition by intact (round symbols) and lysed (square symbols) normal RBCs (black) or Rh-null RBCs (blue), as a function of HbO_2 concentration in the sample. **C.** Comparison of P_m determined for normal and Rh-null RBCs (lines and bars represent mean and SD, respectively, from three experimental replicates). No statistically significant differences were observed.

Permeability to H_2O_2 in Gaucher disease RBCs

Focusing on the possibility of H_2O_2 passively diffusing through the lipid bilayer of the membrane, we studied the permeability of RBCs from GD patients presenting altered membrane lipid composition. GD is a lysosomal storage disease caused by a deficiency of the enzyme GCase, and therefore characterized by the accumulation of sphingolipids in various cells and tissues, especially monocytes and macrophages (29,30). RBCs from these donors have been reported to present

higher levels of GCase substrates, such as glucosylceramide and glucosylsphingosine, and other secondary metabolites of this enzyme (31–34). Consequently, they show an increase in the proportion of abnormally shaped cells and a reduction in deformability that suggests an increased rigidity in the lipid membrane of the RBCs (18).

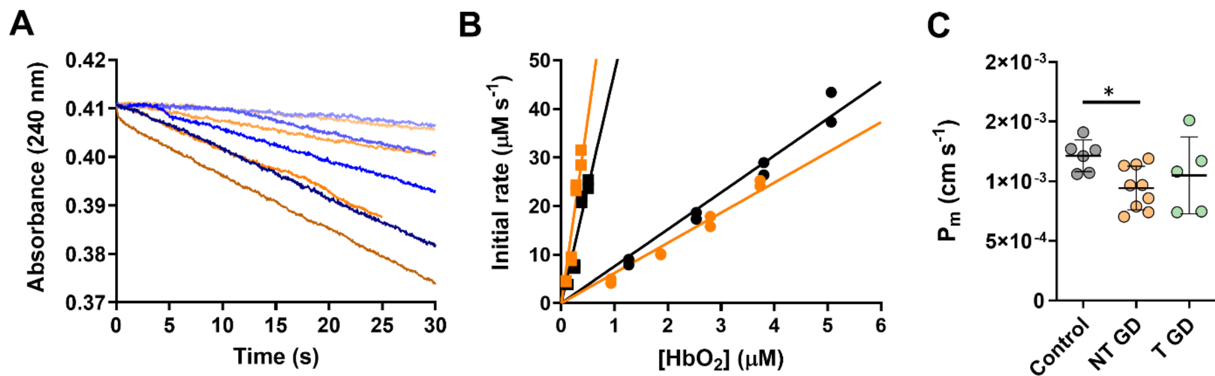


Fig 3. Permeability to H_2O_2 in Gaucher disease RBCs. A. Consumption of 10 mM H_2O_2 by intact (blue tones) and lysed (orange tones) GD RBCs at increasing cell densities (0.93-3.73 μM HbO_2 for intact RBCs and 0.09-0.37 μM HbO_2 for lysed RBCs) at 37°C. The metabolization of H_2O_2 was followed by absorbance measurements at 240 nm for 30 seconds, after mixing H_2O_2 with the RBCs. **B.** Initial rates of H_2O_2 decomposition by intact (round symbols) and lysed (square symbols) normal RBCs (black) or GD RBCs (orange), as a function of HbO_2 concentration in the sample. **C.** Comparison of P_m determined via enzyme latency method in control RBCs and RBCs from GD patients that had been treated (T GD) or not treated (NT GD) with enzyme replacement therapy. Each dot represents one patient: n = 6 for control, n = 9 for NT GD, and n = 5 for T GD. The lines and bars represent mean and SD, respectively. Statistically significant differences were found between control and NT GD (*$p < 0.05$).

The permeability to H_2O_2 was measured in these cells following the enzyme latency protocol, as described before. For these experiments, cryopreserved RBCs from GD patients were assessed and compared with normal RBCs stored in the same conditions. While control cells maintained their membrane permeability values in the range previously observed, the RBCs from the GD patients showed an overall decrease in the P_m (Fig 3). In addition, GD patients who had been treated with enzyme replacement therapy were also studied. In these cases, it is reported that RBCs regain normal values of lipid composition as well as normal deformability and morphological properties

(31,35). The decrease in P_m observed in RBCs from GD patients as compared with healthy donors ($0.9 \pm 0.2 \times 10^{-3}$ cm s^{-1} vs. $1.2 \pm 0.1 \times 10^{-3}$ cm s^{-1}, respectively) confirm that cells with an altered lipid composition suffer changes in H_2O_2 permeation across the membrane, supporting the hypothesis that the main mechanism is simple diffusion through the lipid fraction of the membrane. Moreover, despite the lack of statistical significance associated with data variability, the enzyme treatment induced a consistent increase in H_2O_2 permeability ($P_m = 1.0 \pm 0.3 \times 10^{-3}$ cm s^{-1}).

Effect of hexanol in H_2O_2 and water permeability of RBCs

Hexanol and other anesthetic organic compounds, like pentanol, heptanol, and tetracaine, have been reported to expand the area of the human RBC lipid membrane, thus increasing its fluidity (36,37). Consequently, the passage of molecules through the lipid bilayer can be affected by these agents in ways that depend on the type of transport. Particularly, they are expected to favor simple diffusion and probably decrease facilitated transport by proteins (36,38). Taking this into consideration, the permeability of human RBCs to H_2O_2 was studied in the presence of hexanol, to further investigate the mechanism of H_2O_2 permeation.

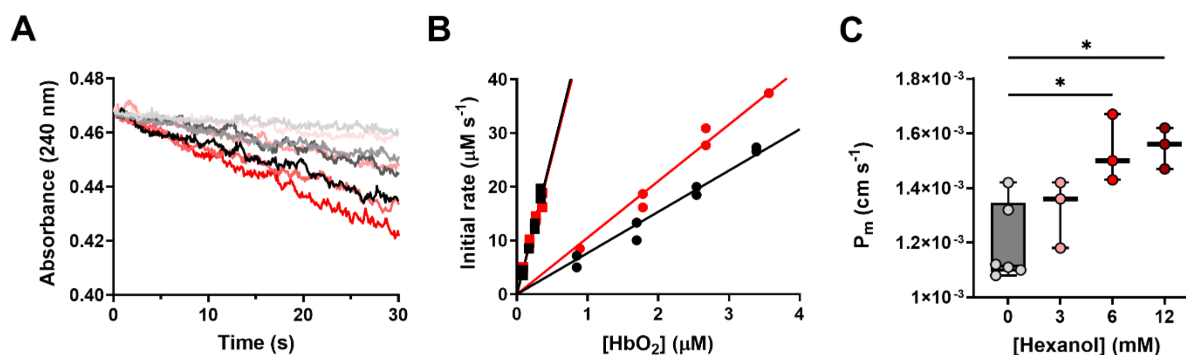


Fig 4. H_2O_2 permeability in hexanol-treated RBCs. A. Metabolization of 10 mM H_2O_2 by control RBCs (grey tones) and RBCs incubated with 12 mM hexanol (red tones) at increasing cell densities (0.8-3.6 µM HbO_2 for intact RBCs and 0.08-0.36 µM HbO_2 for lysed RBCs) at 37 °C. The reaction was measured by following the changes in H_2O_2 absorbance at 240 nm, for 30 seconds after being mixed with the RBCs. **B.** Initial rates of H_2O_2 consumption by intact (round symbols) and lysed (square symbols) control RBCs (black) or RBCs treated with 12 mM hexanol (red), as a function of HbO_2 concentration in the sample. The slopes are observed constants k_{RBC} for intact cells and k_{lys} for lysed cells, which were used to calculate R and P_m according to equation 1. **C.** Comparison of P_m determined

for control RBCs and RBCs treated with 3, 6 and 12 mM hexanol (one donor, each dot represents an experimental replicate, *$p < 0.05$). Lines and bars represent mean and SD, respectively.

The P_m of RBCs treated with 3, 6, and 12 mM hexanol was determined at 37 ºC using the same enzyme latency method as described before. The results show that incubation with hexanol increases the rate of H_2O_2 consumption in intact RBCs when compared to the control, evidenced by a faster decay in the absorbance at 240 nm (Fig 4A). In lysed RBCs, on the other hand, the initial rates of H_2O_2 decomposition are comparable between treated and non-treated cells (Fig 4B), indicating that the observed effect is given by the alteration of H_2O_2 passage through the membrane and not because of changes in catalase activity. The P_m calculated for each condition was $1.2 \pm 0.1 \times 10^{-3}$, $1.3 \pm 0.1 \times 10^{-3}$, $1.5 \pm 0.1 \times 10^{-3}$, $1.55 \pm 0.08 \times 10^{-3}$ cm s^{-1}, for 0, 3, 6 and 12 mM hexanol, respectively, showing an increase in RBC membrane permeability to H_2O_2 with higher hexanol concentrations (Fig 4C). Thus, the higher fluidity of the lipids in the membrane induced by hexanol appears to lead to a faster diffusion of H_2O_2 through the lipid fraction of the RBC membrane.
The effect of hexanol on human RBC membrane permeability was also studied for the case of water. This was especially helpful to compare with H_2O_2, as it is well known water is transported mainly by aquaporin 1 in human RBCs. To do this, water efflux was followed by mixing RBCs with a hypertonic sucrose solution and monitoring cell size changes by light scattering measurements in a stopped-flow apparatus.

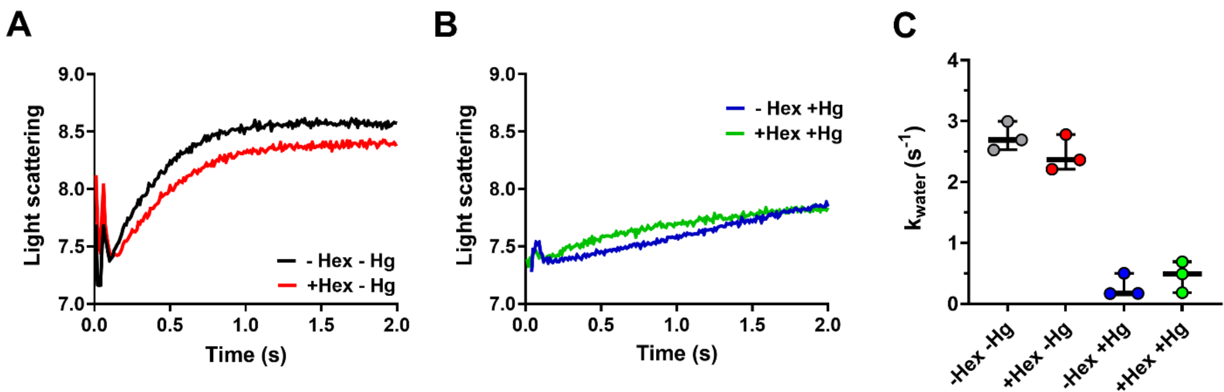


Fig 5. Osmotic water permeability in RBCs treated with hexanol. A. Time courses of changes in light scattering by RBC (0.5% hematocrit) after mixing with 250 mM sucrose (at 10°C). Water efflux mainly through aquaporin 1 causes a decrease in RBC size that is seen as an increase in light scattering. Control untreated RBCs are shown in black and 18 mM hexanol-treated RBCs in red. **B.** Same as A, but RBCs were treated with 0.5 mM $HgCl_2$ (in blue),

or 0.5 mM $HgCl_2$ and 18 mM hexanol (in green). **C.** Observed rate constants of water efflux (k_{water}) in experiments A and B (N =1, n =3). Lines and bars represent mean and SD, respectively.

In these assays, the decrease in cell size resulting from water efflux occurs both in control and hexanol-treated RBCs, marked by an increase in light scattering signal. Unlike H_2O_2 permeability, osmotic water permeability was not potentiated by hexanol. Both conditions yielded light scattering time courses with similar rate constants (k_{water}), with a slight but not statistically significant decrease in the rate of water efflux by hexanol (Fig 5A, Fig 5C). On the other hand, when aquaporin 1 was inhibited by $HgCl_2$, the incubation with hexanol had the opposite effect on water efflux. The addition of $HgCl_2$ resulted in a large decrease in permeation rates because water diffusion occurs mostly through the lipid bilayer, that is restricted at 10°C, the temperature of the assay. In this case, hexanol treatment results in a slight but not significant increase in water permeation rates (Fig 5B, Fig 5C).

The small but not significant decrease in water osmotic efflux rate by hexanol again supports the notion that H_2O_2 diffusion into RBCs is not facilitated by aquaporin 1. The small but not significant increase in water efflux rate by hexanol when aquaporin 1 was inhibited with $HgCl_2$ also substantiates that H_2O_2 crosses the RBC membrane by simple diffusion through the lipid fraction, with no need for facilitated transport by membrane proteins.

Discussion

RBCs are known to have a very powerful antioxidant system, and that is why they are considered a sink of H_2O_2 in the vasculature (39,40). In physiological conditions, the efficient metabolization of H_2O_2 in these cells is mainly the result of the reaction with Prx2, which reacts very rapidly (k = 10^8 $M^{-1}s^{-1}$ (11)) and is present at a high concentration (250-500 µM (10,41,42)), but is nonetheless limited by the entry of H_2O_2 to the RBC (9). The permeability of the human RBC membrane to H_2O_2 is quite high, with $P_m = 1.6 \times 10^{-3}$ cm s^{-1} at 37 ºC (9), in the high range of known cell permeabilities (14). Meanwhile, the mechanism of permeation is not fully characterized and is still an issue of debate. Aquaporins have often been reported as H_2O_2 transporters in other cell types, and some have been termed peroxiporins for this capacity (43–46). Of those present in human RBCs, aquaporin 3 is the most acknowledged as a peroxiporin (47,48), while data on aquaporin 1 has proven to be more contradictory (48–51). Our previous work, however, using human RBCs

deficient in AQP1 and AQP3 (Colton-null and GIL-null phenotypes, respectively), showed that neither aquaporin 1 nor aquaporin 3 are necessary for H_2O_2 permeation in human RBCs, but the involvement of other membrane proteins could not be discarded (9).

Urea transporter UT-B was an interesting candidate to transport H_2O_2 through the RBC membrane. With 14 to 24 × 10^3 copies per cell, UT-B is more than 10 times more abundant than aquaporin 3 in human RBCs (42). On top of that, it has a similar osmotic water unit permeability to aquaporin 1 and is responsible for 10% of the osmotic water transport in human RBCs (15). This is particularly relevant given the physicochemical similarities between water and H_2O_2 (52). Nevertheless, just as with aquaporin 1, our results on H_2O_2 permeability using Kidd-null RBCs devoid of UT-B showed no differences from normal human RBCs (Figure 1). Analogously to NPA and ar/R motifs in aquaporins, UT-B transporters also possess a selectivity filter region that allows them to discriminate between substrates via an energy barrier, thus preventing the passage of other small molecules like hydronium ions and ammonia (53,54). The distinctions in molecular size and composition of H_2O_2 with respect to urea and water, as well as different hydrogen bonding geometry, could block H_2O_2 transport through this kind of transporter. Similarly, our assays performed in Rh-null RBCs indicated the H_2O_2 permeability is maintained in the absence of RhAG ammonia transporters. Even though these results do not completely exclude the possibility that H_2O_2 could traverse the membrane through one of these transporters, they confirm that none of them is the exclusive or primary pathway for H_2O_2 entry into human RBCs.

In our preceding work, we also found that permeability to H_2O_2 in human RBCs is similar to that of lipid-only liposomes, as well as to water osmotic permeability in RBCs lacking aquaporin 1 (9,55). Furthermore, the consumption of H_2O_2 by RBCs showed no saturation when H_2O_2 concentrations were increased up to 100 mM (9). All these data supported the hypothesis that H_2O_2 can cross the membrane by a simple diffusion mechanism through the lipid bilayer. To keep investigating this hypothesis, the permeability of H_2O_2 was measured in RBCs from GD patients. These RBCs have an abnormal lipid content due to a deficiency in the enzyme GCase. Specifically, they present an increment in sphingolipid levels, most notably glucosylceramide, glucosylsphingosine, sphingosine, and sphingosine-1-phosphate (32–34). Therefore, if H_2O_2 diffusion across the RBC membrane occurs mostly through the lipid fraction, we expected to see a change in the membrane permeability to H_2O_2 of GD RBCs relative to normal RBCs. The results showed a decrease in the P_m values when compared to normal human RBCs. This is consistent

with the altered properties observed in these RBCs, which are 3 times less deformable than normal RBCs in shear stress conditions that simulate those of the physiological circulation. Also, they present anomalous shapes and are more prone to aggregation (18). These changes are suspected to be a consequence of a decrease in membrane elasticity due to the different lipid composition. In fact, studies in Gaucher-like macrophages indicate that the microviscosity of the cell membrane is increased in this phenotype, as well as the ratio of highly-ordered areas, while the hydration of the membrane is decreased (56). Naturally, it could be expected for the same to occur in RBCs. Since sphingolipids are known to be important constituents of lipid rafts, an increase in the membrane area occupied by these tightly packed lipid domains would mean an overall increase in rigidity that could be affecting H_2O_2 diffusion (56–58). The normal levels of sphingolipids can be restored if the patient is treated with enzyme replacement therapy, accompanied by a recovery in the rheologic properties of RBCs (31,35). The results of H_2O_2 permeability in RBCs from treated patients were too few and disperse to show statistical differences with not treated patients, but there was a slight increase in P_m, as expected.

Additional experiments were performed with hexanol-treated human RBCs, to keep gathering evidence on the connection between the lipid fraction of the membrane and H_2O_2 permeability. N-alkanols and similar anesthetic compounds such as hexanol are known to increase the fluidity of the lipid membrane (36,37), with different effects on permeability that vary depending on the solutes and their permeation mechanism. While facilitated transport of molecules shows a tendency to be inhibited by an increase in the membrane microviscosity, simple diffusion and active transport can be potentiated or present no effect, respectively (36,38). Permeability to H_2O_2 in human RBCs increased in the presence of hexanol, thus supporting the simple diffusion theory. Consistent with this, aquaporin-dependent water permeability was not increased by hexanol, with even a slight tendency to be reduced. On the other hand, the passage of water through the lipid bilayer, studied indirectly by inhibiting aquaporin 1, showed rates slightly higher with hexanol.

In summary, the present study helped further elucidate the permeation mechanism of H_2O_2 in the human RBC membrane with the following results: (a) rare RBCs lacking urea transporter UT-B present normal H_2O_2 permeability, so this protein is ruled out as a potential H_2O_2 transporter; (b) RBCs without ammonia transporter RhAG also have normal H_2O_2 permeability values, so they were discarded as well; (c) RBCs from GD patients, that present abnormal lipidic content and

rheological properties, showed a decrease in H_2O_2 membrane permeability; and (d) an increase in lipid bilayer fluidity by adding hexanol increases H_2O_2 permeability in human RBCs. These results add to our preceding findings (9), and reinforce the notion that H_2O_2 permeates RBC membranes primarily by simple diffusion and does not require membrane proteins for transport. Furthermore, these findings contribute to the ongoing debate about H_2O_2 transport mechanisms, challenging the prevailing view that aquaporins are the exclusive pathways for H_2O_2 passage across cellular membranes.

Acknowledgments

The authors acknowledge Dr Thierry Peyrard, head of the Centre National de Référence pour les Groupes Sanguins (CNRGS), for authorizing the thawing of rare blood samples Kidd-null and Rh-null. We thank Mrs Cecile Deleschaux and Mr Abdellah Nait for their technical assistance.

Funding and additional information

The authors acknowledge financial support from *Agencia Nacional de Investigación e Innovación* (ANII), Uruguay (grants FCE_2017_136043 to L.T. and M.N.M. and FMV_2019_155597 to L.T. and I.R., and a fellowship to A.C.L.) and to *Comisión Sectorial de Investigación Científica* (CSIC) (grants *Iniciación a la Investigación Científica* to F.O.; *Programa Grupos I+D*_2014_C632-348, and_2018_47, to A.D, CSIC I+D 2020-557 to M.N.M.). F.O. and A.C.L. received a scholarship from *Comisión Académica de Posgrados, Universidad de la República*. Authors thank the ECOS Sud Program, Project U20S02, for Ph.D. internships and PI missions.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions CRediT

Florencia Orrico: Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Visualization, Writing - Review & Editing. **Ana C. Lopez**, Investigation, Formal analysis. **Nicolás Silva**, Resources, Investigation. **Mélanie Franco**, Resources, validation, Writing -

Review & Editing. **Isabelle Mouro-Chanteloup**, Resources, validation, Writing - Review & Editing. **Ana Denicola**, Resources, Writing - Review & Editing, Funding acquisition. **Mariano A. Ostuni**, Conceptualization, Resources, Formal analysis, Writing - Review & Editing, Supervision, Funding acquisition. **Leonor Thomson**, Conceptualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition. **Matias N. Möller**, Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

References

1. Fridovich I. Superoxide Radical: An Endogenous Toxicant. Annual Review of Pharmacology and Toxicology. 1983 Apr 1;23(Volume 23, 1983):239–57.

2. Bretón-Romero R, Lamas S. Hydrogen peroxide signaling in vascular endothelial cells. Redox Biology. 2014 Jan 1;2:529–34.

3. Peshavariya H, Dusting GJ, Jiang F, Halmos LR, Sobey CG, Drummond GR, et al. NADPH oxidase isoform selective regulation of endothelial cell proliferation and survival. Naunyn-Schmied Arch Pharmacol. 2009 Aug 1;380(2):193–204.

4. Meijles DN, Cull JJ, Markou T, Cooper STE, Haines ZHR, Fuller SJ, et al. Redox Regulation of Cardiac ASK1 (Apoptosis Signal-Regulating Kinase 1) Controls p38-MAPK (Mitogen-Activated Protein Kinase) and Orchestrates Cardiac Remodeling to Hypertension. Hypertension. 2020 Oct;76(4):1208–18.

5. Li J, Stouffs M, Serrander L, Banfi B, Bettiol E, Charnay Y, et al. The NADPH Oxidase NOX4 Drives Cardiac Differentiation: Role in Regulating Cardiac Transcription Factors and MAP Kinase Activation. MBoC. 2006 Sep;17(9):3978–88.

6. Bretón-Romero R, González de Orduña C, Romero N, Sánchez-Gómez FJ, de Álvaro C, Porras A, et al. Critical role of hydrogen peroxide signaling in the sequential activation of p38 MAPK and eNOS in laminar shear stress. Free Radical Biology and Medicine. 2012 Mar 15;52(6):1093–100.

7. Forman HJ, Bernardo A, Davies KJA. What is the concentration of hydrogen peroxide in blood and plasma? Archives of Biochemistry and Biophysics. 2016 Aug 1;603:48–53.

8. Deskur E, Przywarska Izabela, Dylewicz P, Szczęśniak Ł, Rychlewski T, Wilk M, et al. Exercise-induced increase in hydrogen peroxide plasma levels is diminished by endurance training after myocardial infarction. International Journal of Cardiology. 1998 Dec 31;67(3):219–24.

9. Orrico F, Lopez AC, Saliwonczyk D, Acosta C, Rodriguez-Grecco I, Mouro-Chanteloup I, et al. The permeability of human red blood cell membranes to hydrogen peroxide is independent of aquaporins. Journal of Biological Chemistry. 2022 Jan 1;298(1).

10. Orrico F, Möller MN, Cassina A, Denicola A, Thomson L. Kinetic and stoichiometric constraints determine the pathway of H2O2 consumption by red blood cells. Free Radical Biology and Medicine. 2018 Jun 1;121:231–9.

11. Manta B, Hugo M, Ortiz C, Ferrer-Sueta G, Trujillo M, Denicola A. The peroxidase and peroxynitrite reductase activity of human erythrocyte peroxiredoxin 2. Archives of Biochemistry and Biophysics. 2009 Apr 15;484(2):146–54.

12. Makino N, Sasaki K, Hashida K, Sakakura Y. A metabolic model describing the H2O2 elimination by mammalian cells including H2O2 permeation through cytoplasmic and peroxisomal membranes: comparison with experimental data. Biochimica et Biophysica Acta (BBA) - General Subjects. 2004 Aug 4;1673(3):149–59.

13. Antunes F, Cadenas E. Estimation of H2O2 gradients across biomembranes. FEBS Letters. 2000 Jun 16;475(2):121–6.

14. Möller MN, Cuevasanta E, Orrico F, Lopez AC, Thomson L, Denicola A. Diffusion and transport of reactive species across cell membranes. In: Bioactive lipids in health and disease. 2019. p. 3–19.

15. Azouzi S, Gueroult M, Ripoche P, Genetet S, Aronovicz YC, Kim CLV, et al. Energetic and Molecular Water Permeation Mechanisms of the Human Red Blood Cell Urea Transporter B. PLOS ONE. 2013 dic;8(12):e82338.

16. Ripoche P, Bertrand O, Gane P, Birkenmeier C, Colin Y, Cartron JP. Human Rhesus-associated glycoprotein mediates facilitated transport of NH3 into red blood cells. Proceedings of the National Academy of Sciences. 2004 Dec 7;101(49):17222–7.

17. Bettioui T, Chipeaux C, Ben Arfa K, Héron S, Belmatoug N, Franco M, et al. Development of a new online SPE-HPLC-MS/MS method for the profiling and quantification of sphingolipids and phospholipids in red blood cells – Application to the study of Gaucher's disease. Analytica Chimica Acta. 2023 Oct 16;1278:341719.

18. Franco M, Collec E, Connes P, van den Akker E, Billette de Villemeur T, Belmatoug N, et al. Abnormal properties of red blood cells suggest a role in the pathophysiology of Gaucher disease. Blood. 2013 Jan 17;121(3):546–55.

19. Nelson DP, Kiesow LA. Enthalpy of decomposition of hydrogen peroxide by catalase at 25° C (with molar extinction coefficients of H2O2 solutions in the UV). Analytical Biochemistry. 1972 Oct 1;49(2):474–8.

20. Amen F, Machin A, Touriño C, Rodríguez I, Denicola A, Thomson L. *N*-acetylcysteine improves the quality of red blood cells stored for transfusion. Archives of Biochemistry and Biophysics. 2017 May 1;621:31–7.

21. Lucien N, Sidoux-Walter F, Olivés B, Moulds J, Pennec PYL, Cartron JP, et al. Characterization of the Gene Encoding the Human Kidd Blood Group/Urea Transporter Protein: EVIDENCE FOR SPLICE SITE MUTATIONS IN JknullINDIVIDUALS *. Journal of Biological Chemistry. 1998 May 22;273(21):12973–80.

22. Cherif-Zahar B, Raynal V, Gane P, Mattei MG, Bailly P, Gibbs B, et al. Candidate gene acting as a suppressor of the RH locus in most cases of Rh-deficiency. Nat Genet. 1996 Feb;12(2):168–73.

23. Winterbourn CC. [26] Oxidative reactions of hemoglobin. In: Methods in Enzymology. Academic Press; 1990. p. 265–72. (Oxygen Radicals in Biological Systems Part B: Oxygen Radicals and Antioxidants; vol. 186).

24. Park H, Lee S, Ji M, Kim K, Son Y, Jang S, et al. Measuring cell surface area and deformability of individual human red blood cells over blood storage using quantitative phase imaging. Sci Rep. 2016 Oct 4;6(1):34257.

25. Evans E, Fung YC. Improved measurements of the erythrocyte geometry. Microvascular Research. 1972 Oct 1;4(4):335–47.

26. Cartron JP. *RH* blood group system and molecular basis of Rh-deficiency. Best Practice & Research Clinical Haematology. 1999 Dec 1;12(4):655–89.

27. Marinho HS, Cyrne L, Cadenas E, Antunes F. The Cellular Steady-State of H2O2. In: Cadenas E, Packer L, editors. Methods in Enzymology. Academic Press; 2013. p. 3–19. (Hydrogen Peroxide and cell signaling, Part B; vol. 527).

28. Nicholls P. Activity of catalase in the red cell. Biochimica et Biophysica Acta (BBA) - Enzymology and Biological Oxidation. 1965 Jan 1;99(2):286–97.

29. Sidransky E. Gaucher Disease: Insights from a Rare Mendelian Disorder. Discov Med. 2012 Oct;14(77):273–81.

30. Grabowski GA. Gaucher disease and other storage disorders. Hematology. 2012 Dec 8;2012(1):13–8.

31. Dupuis L, Chipeaux C, Bourdelier E, Martino S, Reihani N, Belmatoug N, et al. Effects of sphingolipids overload on red blood cell properties in Gaucher disease. Journal of Cellular and Molecular Medicine. 2020;24(17):9726–36.

32. Chipeaux C, de Person M, Burguet N, Billette de Villemeur T, Rose C, Belmatoug N, et al. Optimization of ultra-high pressure liquid chromatography – tandem mass spectrometry determination in plasma and red blood cells of four sphingolipids and their evaluation as biomarker candidates of Gaucher's disease. Journal of Chromatography A. 2017 Nov 24;1525:116–25.

33. Nilsson O, Håkansson G, Dreborg S, Groth CG, Svennerhoim L. Increased cerebroside concentration in plasma and erythrocytes in Gaucher disease: Significant differences between Type I and Type III. Clinical Genetics. 1982;22(5):274–9.

34. Erikson A, Forsberg H, Nilsson M, Åström M, Månsson JE. Ten years' experience of enzyme infusion therapy of Norrbottnian (type 3) Gaucher disease. Acta Paediatrica. 2006;95(3):312–7.

35. Franco M, Reihani N, Marin M, De Person M, Billette de Villemeur T, Rose C, et al. Effect of velaglucerase alfa enzyme replacement therapy on red blood cell properties in Gaucher disease. Am J Hematol. 2017 Sep;92(9):E561–3.

36. Kutchai H, Chandler LH, Geddis LM. Effects of anesthetic alcohols on membrane transport processes in human erythrocytes. Biochimica et Biophysica Acta (BBA) - Biomembranes. 1980 Aug;600(3):870–81.

37. Feinstein MB, Fernandez SM, Sha'afi RI. Fluidity of natural membranes and phosphatidylserine and ganglioside dispersions: Effects of local anesthetics, cholesterol and protein. Biochimica et Biophysica Acta (BBA) - Biomembranes. 1975 Dec 16;413(3):354–70.

38. Seeman P. The membrane actions of tranquilizers. Pharmacol Rev. 1972 Jan 1;24.

39. Möller MN, Orrico F, Villar SF, López AC, Silva N, Donzé M, et al. Oxidants and Antioxidants in the Redox Biochemistry of Human Red Blood Cells. ACS Omega. 2023 Jan 10;8(1):147–68.

40. Low FM, Hampton MB, Winterbourn CC. Peroxiredoxin 2 and Peroxide Metabolism in the Erythrocyte. Antioxidants & Redox Signaling. 2008 Sep;10(9):1621–30.

41. Moore RB, Mankad MV, Shriver SK, Mankad VN, Plishker GA. Reconstitution of Ca(2+)-dependent K+ transport in erythrocyte membrane vesicles requires a cytoplasmic protein. Journal of Biological Chemistry. 1991 Oct 5;266(28):18964–8.

42. Bryk AH, Wiśniewski JR. Quantitative Analysis of Human Red Blood Cell Proteome. J Proteome Res. 2017 Aug 4;16(8):2752–61.

43. Watanabe S, Moniaga CS, Nielsen S, Hara-Chikuma M. Aquaporin-9 facilitates membrane transport of hydrogen peroxide in mammalian cells. Biochemical and Biophysical Research Communications. 2016 Feb 26;471(1):191–7.

44. Marchissio MJ, Francés DEA, Carnovale CE, Marinelli RA. Mitochondrial aquaporin-8 knockdown in human hepatoma HepG2 cells causes ROS-induced mitochondrial depolarization and loss of viability. Toxicology and Applied Pharmacology. 2012 Oct 15;264(2):246–54.

45. Bertolotti M, Bestetti S, García-Manteiga JM, Medraño-Fernandez I, Dal Mas A, Malosio ML, et al. Tyrosine Kinase Signal Modulation: A Matter of H2O2 Membrane Permeability? Antioxidants & Redox Signaling. 2013 Nov;19(13):1447–51.

46. Vieceli Dalla Sega F, Zambonin L, Fiorentini D, Rizzo B, Caliceti C, Landi L, et al. Specific aquaporins facilitate Nox-produced hydrogen peroxide transport through plasma membrane in leukaemia cells. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2014 Apr 1;1843(4):806–14.

47. Hara-Chikuma M, Satooka H, Watanabe S, Honda T, Miyachi Y, Watanabe T, et al. Aquaporin-3-mediated hydrogen peroxide transport is required for NF-κB signalling in keratinocytes and development of psoriasis. Nat Commun. 2015 Jun 23;6(1):7454.

48. Miller EW, Dickinson BC, Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. Proceedings of the National Academy of Sciences. 2010 Sep 7;107(36):15681–6.

49. Montiel V, Bella R, Michel LYM, Esfahani H, De Mulder D, Robinson EL, et al. Inhibition of aquaporin-1 prevents myocardial remodeling by blocking the transmembrane transport of hydrogen peroxide. Science Translational Medicine. 2020 Oct 7;12(564):eaay2176.

50. Bienert GP, Møller ALB, Kristiansen KA, Schulz A, Møller IM, Schjoerring JK, et al. Specific Aquaporins Facilitate the Diffusion of Hydrogen Peroxide across Membranes *. Journal of Biological Chemistry. 2007 Jan 12;282(2):1183–92.

51. Almasalmeh A, Krenc D, Wu B, Beitz E. Structural determinants of the hydrogen peroxide permeability of aquaporins. The FEBS Journal. 2014;281(3):647–56.

52. Bienert GP, Schjoerring JK, Jahn TP. Membrane transport of hydrogen peroxide. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2006 Aug 1;1758(8):994–1003.

53. Levin EJ, Cao Y, Enkavi G, Quick M, Pan Y, Tajkhorshid E, et al. Structure and permeation mechanism of a mammalian urea transporter. Proceedings of the National Academy of Sciences. 2012 Jul 10;109(28):11194–9.

54. de Groot BL, Frigato T, Helms V, Grubmüller H. The Mechanism of Proton Exclusion in the Aquaporin-1 Water Channel. Journal of Molecular Biology. 2003 Oct 17;333(2):279–93.

55. Mathai JC, Mori S, Smith BL, Preston GM, Mohandas N, Collins M, et al. Functional Analysis of Aquaporin-1 Deficient Red Cells: THE COLTON-NULL PHENOTYPE (∗). Journal of Biological Chemistry. 1996 Jan 19;271(3):1309–13.

56. Batta G, Soltész L, Kovács T, Bozó T, Mészár Z, Kellermayer M, et al. Alterations in the properties of the cell membrane due to glycosphingolipid accumulation in a model of Gaucher disease. Sci Rep. 2018 Jan 9;8(1):157.

57. Iessi E, Marconi M, Manganelli V, Sorice M, Malorni W, Garofalo T, et al. Chapter Five - On the role of sphingolipids in cell survival and death. In: Spetz JKE, Galluzzi L, editors. International Review of Cell and Molecular Biology. Academic Press; 2020. p. 149–95. (Cell Death Regulation in Health and Disease - Part A; vol. 351).

58. Sonnino S, Prinetti A, Mauri L, Chigorno V, Tettamanti G. Dynamic and Structural Properties of Sphingolipids as Driving Forces for the Formation of Membrane Domains. Chem Rev. 2006 Jun 1;106(6):2111–25.

 Click here to access/download;Graphical Abstract;graphic abstract 3.tif