

## Does hemoglobin affect measures of mitochondrial respiration in red blood cells? Comment to “Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress”

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**Title:** Does hemoglobin affect measures of mitochondrial respiration in red blood cells? Comment to “Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress”

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No conflicts of interest to disclose.

**Contributions**

LBG conceived the idea. WTW, ACB, and LBG reviewed the literature, and discussed the basic principles. WTW and LBG performed the calculations. LBG wrote the first draft. WTW, ACB, and LBG reviewed and revised all subsequent drafts. WTW, ACB, and LBG read and approved the final version of the manuscript.

We are intrigued by the Connes group's interesting study of the retention of mitochondria in mature red blood cells (RBC) in 61 patients with sickle cell anemia (SCA).<sup>1</sup> Using the sophisticated techniques of Image Stream (Amnis, MK II) and Mitotracker Red CMXRos Dye (Invitrogen) in combination with flow cytometry, they reported that a significant percentage of mature sickle cells in these patients retained mitochondria.<sup>1</sup> This presence of mitochondria in human erythrocytes is a unique phenomenon, heretofore considered to be a characteristic reserved for red cells in nonmammalian vertebrates (e.g., avians, fish, amphibians, reptiles).<sup>2</sup>

However, we were particularly fascinated by one of their primary aims; specifically, "to investigate the functionality of these mitochondria".<sup>1</sup> Mitochondrial function was assessed with high-resolution respirometry, which according to the authors, resulted in "detectable mitochondrial oxygen consumption in sickle mature RBC, but not in healthy RBC." The authors concluded that their data showed the presence of functional mitochondria in mature sickle RBC, "which could favor RBC sickling and accelerate RBC senescence, leading to increased cellular fragility and hemolysis".<sup>1</sup> The presence of functional mitochondria in human erythrocytes is an interesting observation with important implications. For example, the Connes group noted that the propensity of RBC to sickle when deoxygenated was greater in the SCA subgroup with a high percentage (13%) of mitochondria retained in mature RBC.<sup>1</sup> These are provocative findings with great potential for both increased understanding of basic mechanisms and application to practice.

Oxygen consumption rate ( $J_{O_2}$ ) is, of course, a valid indicator of mitochondrial function, particularly when flux is modified by agents known to inhibit or accelerate various steps in the oxidative pathway, as was done by the Connes group here.<sup>1</sup>

However, in such assessments, care must be taken to ensure the validity and accuracy of the  $J_{O_2}$  measurement itself. The Connes group used intact red cells in their polarographic measurement of RBC mitochondrial  $J_{O_2}$ <sup>1</sup>, basically following the methods of Sjövall et al.<sup>3</sup>, and Stier et al.<sup>2</sup>, with Stier being a coauthor of this current study. Briefly, 100 $\mu$ L of packed RBC was added to 1mL of a potassium-based respiratory buffer (MiR05, see below), and transferred into an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) set at 37°C and containing another 1mL of MiR05. Subsequently, a variety of standard mitochondrial respiratory measures were made, all based on  $J_{O_2}$ . Of significant concern here is the  $O_2$  associated with hemoglobin; i.e., oxyhemoglobin ( $HbO_2$ ), in the red cells incubating in these respiration chambers. This store of  $O_2$  was apparently ignored in the authors' calculation of  $J_{O_2}$ . The errors introduced by this omission are potentially quite large and variable depending on hemoglobin content,  $O_2$  binding parameters (e.g.,  $P_{50}$ ), the oxygen tension ( $PO_2$ ) at which the analysis is being done, and the duration of a given assay. The discussion below will be restricted to a simple presentation of the overall problem. Basic concepts and details related to how the Oroboros respirometer calculates  $J_{O_2}$  are clearly and completely described in the user's manual.

Clark type  $O_2$  electrodes such as that used in the Oroboros instrument generate an electrical signal proportional to the oxygen tension,  $PO_2$  (mmHg), which, in turn, is proportional to the concentration of dissolved  $O_2$  ( $nmolO_2 \cdot mL^{-1}$  or  $\mu M$ ). As stated in Sander<sup>4</sup>: "... the amount of dissolved gas is proportional to its partial pressure in the gas phase. The proportionality factor is called Henry's law constant." The value of the Henry constant ( $\mu mol \cdot mmHg^{-1}$ ); i.e., solubility, depends on temperature and other factors such

as ionic strength. Pure water at 37°C exposed to (and in equilibrium with) room air at 1.0 atmosphere pressure contains about 213  $\mu\text{M}$  of dissolved  $\text{O}_2$  ( $213 \text{ nmolO}_2\cdot\text{mL}^{-1}$ ).<sup>4</sup> If instead, MiR05 medium is the solution of interest, the higher ionic strength reduces  $\text{O}_2$  solubility (“salting out”). In the Oroboros literature this is taken into account by the “ $F_M$ ” factor, which is 0.92 for MiR05. Accordingly, in our example:  $[\text{O}_2] = 0.92 * 213 \mu\text{M} = 196 \mu\text{M}$  at 1.0 atmosphere and 37°C. This value is roughly similar to the initial  $[\text{O}_2]$  shown in Figure 2 in Stier et al.<sup>2</sup>. If we now multiply  $196 \text{ nmolO}_2\cdot\text{mL}^{-1}$  by a total medium volume of 2.0 mL, we get 392  $\text{nmolO}_2$  in the respiratory chamber when the assay begins. Below we will compare this soluble  $\text{O}_2$  mass to the  $\text{O}_2$  mass bound to Hb.

When gas exchange with the environment is prohibited in the Oroboros,  $\text{O}_2$  consumption decreases the  $\text{PO}_2$  hence proportionally decreasing the electrode signal. The progressive fall in  $\text{PO}_2$  across time ( $\text{mmHg}\cdot\text{min}^{-1}$ ), linked by the Henry constant ( $\mu\text{mol}\cdot\text{mmHg}^{-1}$ ) to the corresponding fall in  $[\text{O}_2]$  ( $\mu\text{M}$  or  $\text{nmolO}_2\cdot\text{mL}^{-1}$ ), along with the medium volume (mL), allows the calculation of  $J_{\text{O}_2}$  ( $\text{nmolO}_2\cdot\text{min}^{-1}$ ). Such calculations have been routinely made and reported in a vast literature, which began rapidly expanding shortly after Leland Clark developed his  $\text{O}_2$  electrode with a cellophane covering in the 1950s.<sup>5</sup>

However, the presence of an  $\text{O}_2$  buffering molecule such as Hb in the respiratory chamber complicates the above straightforward and linear relationships in two ways: 1) the Hb mass available to bind  $\text{O}_2$  in each assay must be determined, and 2) the  $\text{HbO}_2$  binding parameters must be evaluated. Obviously, accounting for the utilization of this  $\text{O}_2$  pool will involve a nonlinear relationship between the electrode signal and  $\text{O}_2$  concentration, and thereby  $J_{\text{O}_2}$ . We can approximate the Hb-bound  $\text{O}_2$  by assuming a

normal mean corpuscular hemoglobin concentration of  $34 \text{ g}\cdot\text{dL}^{-1}$  {similar to the SCA patients in Figure 6C<sup>1</sup> if the 10-fold unit error is overlooked}, and a Hb molecular weight of  $67000 \text{ g}\cdot\text{mol}^{-1}$ . With these values, the  $100 \text{ }\mu\text{L}$  of RBC in the assay contains a Hb mass of  $0.507 \text{ }\mu\text{mol}$ . At the onset of the assay, assuming  $1.0$  atmosphere barometric pressure,  $0.2095$  mole fraction of  $\text{O}_2$  in dry air, and a  $37^\circ\text{C}$  assay temperature (thus  $47.1 \text{ mmHg}$  water vapor pressure at the air:water interface), gives a medium (MiR05)  $\text{PO}_2 = (760 - 47.1) \times 0.2095 = 149 \text{ mmHg}$  at the start of the assay. Thus, the Hb will initially be almost fully saturated, holding  $4 \text{ O}_2\cdot\text{Hb}^{-1} \times 0.507 \text{ }\mu\text{mol Hb} = 2030 \text{ nmol O}_2$ , which is  $5.2$  times greater than the soluble  $\text{O}_2$  in the medium (see above). As  $\text{J}_{\text{O}_2}$  commences early in the assay the medium  $\text{PO}_2$  will fall, drawing predominantly from the soluble pool. With time and ongoing  $\text{J}_{\text{O}_2}$ , however, the continuous fall in  $\text{PO}_2$  will elicit significant  $\text{O}_2$  dissociation from Hb, which will dampen changes in  $\text{PO}_2$  and result in an underestimation of the true  $\text{J}_{\text{O}_2}$  (if  $\text{O}_2$  bound to Hb is ignored). Because in a given assay the  $\text{PO}_2$  is progressively falling, the errors will confound not only the absolute rates but also the relative differences between what Stier et al.<sup>2</sup> call “routine, oxphos, leak, ETS, and non-mito”  $\text{O}_2$  consumption. In a comparative study, like that of Esperti et al.<sup>1</sup>, such errors may be amplified when the two RBC groups have different Hb contents and/or  $\text{O}_2$  binding kinetics. In any case, standard polarographic procedures used to measure  $\text{J}_{\text{O}_2}$  by red blood cells must be viewed with extreme caution.

In practice, the mitochondrial respiration measurements may be largely made at high partial pressures of  $\text{O}_2$  where the oxyhemoglobin dissociation curve is quite flat.<sup>6</sup> However, a decrease of even  $0.5\%$  in %Saturation would release  $\approx 5.2 \text{ nmol}$  of  $\text{O}_2$ , an amount that would be about one-third of the decline in  $\text{O}_2$  content occurring during

“endogenous” mitochondrial function in king penguin RBC; see Figure 2 in Stier et al.<sup>2</sup> Of course, the further the  $PO_2$  declines during the respiratory measurements, the closer the approach to a steeper portion of the  $HbO_2$  dissociation curve. Accordingly, we propose that hemoglobin must be accounted for in quantitative measures of mitochondrial respiration in RBC.

We also noted that RBC were suspended in respiratory buffer MiR05 {0.5 mM EGTA, 3mM  $MgCl_2$ , 60 mM K-lactobionate, 20 mM taurine, 10 mM  $KH_2PO_4$ , 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin ( $1\text{ g}\cdot\text{L}^{-1}$ ), pH 7.1}, a solution commonly used for studies of mitochondria, which are intracellular organelles.<sup>1</sup> Accordingly, we wondered 1) whether a respiratory solution more closely resembling plasma might be more appropriate for RBC, and 2) whether glucose should be provided as a fuel.

Further, for quantitative mitochondrial respiratory analyses, it is important to be certain about the volume of RBC used in the assays. In this context, how an exact volume of RBC is assayed is unclear. If packed RBC are transferred, air displacement pipetting is error-laden, and if packed cells are resuspended, the amount of supernatant retained with prior centrifugation is uncertain.

Finally, we saw no mention of any pharmacotherapy for the SCA patients. Would any such treatments have potential ramifications for either the presence or function of mitochondria in the RBC of this group?

Once again, we acknowledge and appreciate the research of Esperti et al.<sup>1</sup>, but we also believe that their methods for mitochondrial respirometry deserve a candid discussion.

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