

Effect of cell concentration on cell-specific oxygen fluxes in a coupling-control protocol using intact PBMCs

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Introduction

Cell concentration per volume is a variable which is usually utilized as a technical normalization variable in high resolution respirometry. However, level of standardization of cell concentration highly differs among research groups all over the world, with some standardizing cell concentrations while others do not standardize. Lack of standardization of cell concentration might be problematic, as has been previously reported in cultured endothelial cells [1]. However, no association was reported between different PBMC concentrations and routine respiration per million cells, whereas higher leak respiration was observed only at the lowest concentration, along with reduced electron transfer (ET) capacity at the lowest concentrations [2]. This study tested the impact of cell concentration on ROX-corrected cell-specific routine respiration, leak respiration, and ET capacity using intact PBMCs.

Methods

As part of the MitO₂Health study, 6 healthy women were recruited at Ulm University (mean age, 31.3 years; range, 25–36). The study was conducted in accordance with the Declaration of Helsinki and approved by the Ulm University Ethical Committee. After informed consent, venous blood was collected in K3EDTA tubes (Sarstedt S-Monovette). PBMCs were isolated within 30 minutes by Ficoll-Hypaque density-gradient centrifugation (GE Healthcare), washed three times with PBS, and cryopreserved in dimethyl sulphoxide/fetal calf serum (1:10; Sigma-Aldrich) at –80°C. Samples were analyzed within 2 weeks of cryopreservation.

For respirometry, PBMCs were thawed at 37°C for 1 minute and washed twice with prewarmed PBS + 10% FCS. Cells were counted manually using a Neubauer chamber and had >95% viability. Suspensions of 8, 6, 4, and 2 × 10⁶ cells/ml were prepared by serial dilution in prewarmed MirO5.

High-resolution respirometry was performed using the coupling control protocol (SUIT 003-D012), as previously described [3, 4]. Cell-specific oxygen fluxes (pmol/s per 10⁶ cells) were analyzed. Samples from each participant were measured in parallel across four 2-ml chambers of two Oxygraph O2ks (series F and G), with

concentrations randomly assigned to chambers. Experimenters were not blinded to cell concentration.

The effect of cell concentration on routine respiration, leak respiration, and ET capacity was assessed using linear mixed-effects models with subject-specific random intercepts, implemented with lme4 [5] in R (version 4.5.2). Cell concentration was modeled as a continuous variable, and all parameters were corrected for ROX. For subject 2, the 6×10^6 cells/ml measurement was missing and the 2×10^6 cells/ml concentration was measured in duplicate due to dilution error. ET capacity at 2×10^6 cells/ml was missing for subject 6 due to experimental error. All available measurements were included in the analyses.

Results and discussion

Cell-specific oxygen fluxes for routine respiration $M (SD) = 4.69 (0.73)$, leak respiration $M (SD) = 0.92 (0.16)$, and ET Capacity $M (SD) = 15.96 (1.92)$ were similar to previous reports with healthy participants [2, 6]. Linear mixed models with routine and leak respiration as outcomes revealed a decrease in cell-specific oxygen flux with increasing cell concentration. Surprisingly, the model with ET Capacity as outcome revealed no association between cell concentration and cell-specific oxygen flux (Figure 1).

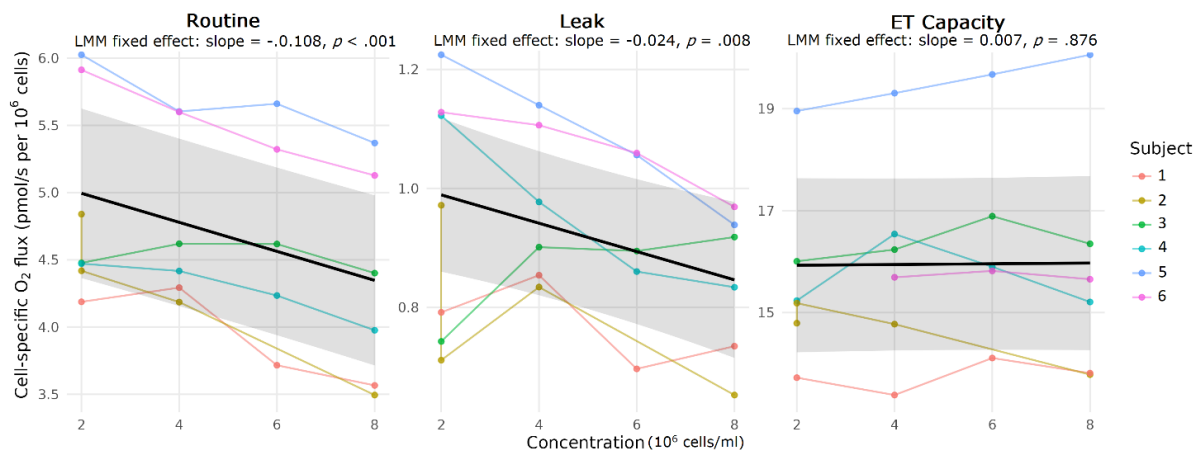


Figure 1. Spaghetti plots of cell-specific oxygen fluxes across cell concentrations. All values were ROX-corrected. The model statistics of each parameter is given above the figures. The black lines depict the average population-level fixed effects from the individual linear mixed models. Shaded area depicts the 95% confidence interval of the fixed effect. LMM = Linear mixed model, ET Capacity = Electron Transfer Capacity.

As time resolution was suggested to cause bias in determining accurate respiratory profiles of cells with low densities and low aerobic respiratory activities [1, 7], the cell-specific oxygen fluxes were calculated again with slope smoothing of 20 and 10s, in addition to 40s. This did not change the previous results (data not shown).

These results suggest that cell concentration is an important determinant of cell-specific routine and leak respiration in PBMCs. The underlying mechanisms remain unclear. Oxygen diffusion limitation, previously reported in cultured endothelial cells [1], is unlikely to explain our findings because PBMC size does not vary with cell concentration. Time resolution also did not account for the observed effect. Oxygen

backdiffusion may contribute, as higher cell concentrations consume oxygen in the chamber more rapidly; however, instrumental background correction was applied, and cell concentration did not affect ET capacity despite the lower oxygen concentration under this respiratory state. Cell crowding or clumping at higher concentrations [7] may partially explain the findings, but further experiments are needed to explore this possibility.

Although these findings require replication, they warrant standardizing cell concentration to improve the reproducibility of flux measurements. This is particularly important in case-control and pre-post studies, where differences in cell concentration may confound comparisons between groups or time points. Standardization across laboratories could further improve reproducibility and facilitate the establishment of reference values across different disease, lifestyle, and age groups. Consensus guidelines specifying standardized cell concentrations may therefore be warranted. Whether these findings extend to other cell types or isolated mitochondria remains unclear and should be investigated, with careful control of cell size.

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Author contributions: RNM designed the experiment, performed HRR measurements, supervised HRR measurements, performed statistical analyses and wrote the initial manuscript. LA isolated PBMCs, performed HRR measurements, analysed HRR data, and reviewed the manuscript. ITK conceptualized and coordinated the MitO₂Health project, acquired funding, and reviewed the manuscript.

Conflict of interest: None.

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