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Early alterations in platelet mitochondrial function are associated with survival and organ failure in patients with septic shock☆,☆☆

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ABSTRACT

Introduction: The objective of the study is to determine if changes in platelet mitochondrial function in patients with sepsis are present early after presentation and the association of these changes with clinical outcomes and systemic metabolic function.
Materials and methods: This is a prospective observational cohort study of a convenience sample of patients with severe sepsis. Mitochondrial function of intact, nonpermeabilized platelets suspended in their own plasma was estimated using high-resolution respirometry. Unstimulated basal respiration, oligomycin-induced state 4, and maximal respiratory rate after serial titrations of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone were measured. Organ failure was estimated using Sequential Organ Failure Assessment score, and patients were followed up until 28 days to determine survival. Lactate levels were measured in all patients, and a subset of patients had lactate/pyruvate (L/P) ratios measured.
Results: Twenty-eight patients were enrolled, 21 of whom survived. Initial Sequential Organ Failure Assessment score and lactate levels were 8.5 (interquartile range [IQR], 6-10) and 2.3 (IQR, 1.2-3.5) respectively, whereas the median L/P ratio was 23.4 (IQR, 15.2-38). Basal and maximal respiratory rates were significantly higher among nonsurvivors compared to survivors ($P = .02$ and $P = .04$), whereas oligomycin-induced state 4 respiration was not statistically different between groups ($P = .15$). We found a significant association between maximal respiration and organ failure ($P = .03$) and both basal and maximal rates with initial lactate level ($P = .04$, $P = .02$), but not with L/P ratio.
Conclusions: Differences in platelet mitochondrial function between survivors and nonsurvivors are present very early in the hospital course and are associated with organ failure and lactate.

1. Introduction

In the setting of an aging patient population with an increasing comorbidity burden, the incidence of severe sepsis and septic shock is increasing [1]. Meanwhile, mortality remains high for the 2 conditions, at greater than 20% [1] and 40% [2], respectively. Decades of preclinical and clinical research have yielded significant improvement understanding of the pathophysiology of sepsis, particularly in regard to the critical role played by the inflammatory cascade. Unfortunately, clinical trials regarding specific pharmacologic therapies for the treatment of sepsis have had a checkered history and have not resulted in bringing novel, efficacious drugs into routine clinical practice. Given this history, investigations into relatively underinvestigated components of the pathophysiologic cascade are critical for the development of novel therapeutics.

Mitochondrial dysfunction is increasingly recognized as playing a critical role in the development and persistence of organ failure in sepsis [3,4]. This hypothesis may help explain many key features of death from sepsis including the notable lack of widespread apoptosis or necrosis within tissues, which suggests against hypoxia as the primary driver of organ failure. Although animal models of sepsis exhibit mitochondrial and subsequent organ dysfunction in widespread vital organs including the heart [5] and kidneys [6], access to the tissues necessary to study mitochondria has limited translation of these findings to humans. To date, human studies support the existence of mitochondrial dysfunction in humans with severe sepsis in a number of accessible cell lines, including leukocytes [7], platelets [8], and skeletal muscle [9].

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However, several questions remain underinvestigated and serve as the motivation for the present study. First, the timing of onset of altered mitochondrial respiration in sepsis remains unclear, which has implications for the design of interventional trials. Second, the association of platelet mitochondrial function and evidence of vital organ dysfunction or systemic perturbations in metabolism are lacking. To our knowledge, only a single small study by a single group has evaluated the prognostic potential of platelet mitochondrial alternations in sepsis [8]. In this study, we present a prospective cohort study of platelet mitochondrial function in patients with septic shock enrolled shortly after the initial diagnosis and resuscitation. The goal of this study was to test the hypothesis that alternations in early platelet mitochondrial function are associated with patient outcomes including survival and organ failure and changes in systemic evidence of metabolism.

2. Materials and methods

2.1. Study design

This was prospective observational study of a convenience sample of patients with severe sepsis enrolled at a single academic, tertiary care hospital from September 2013 to November 2014. Patients were eligible for inclusion if they had (1) a suspected or confirmed infection, (2) any 2 of 4 criteria of systemic inflammatory response as defined by the 2001 ACCP/SCCM Consensus Conference Committee [10], and (3) a mean arterial pressure of less than or equal to 65 mm Hg or systolic blood pressure less than 90 mm Hg after 2-L fluids or initiation of vasopressors. Exclusion criteria were (1) any primary diagnosis other than sepsis; (2) an established do-not-resuscitate status or advanced directives restricting aggressive care or treating physician deems aggressive care unsuitable; (3) cardiopulmonary resuscitation (chest compression or defibrillation) before enrollment; or (4) if the principal investigator was not available to conduct high resolution respirometry. All patients or their surrogates provided written informed consent, and the study was approved by the local institutional review board.

2.2. Platelet isolation

After consent, approximately 10 mL of whole blood was drawn into EDTA vaccuntainers and gently inverted 6 to 8 times. The blood was taken immediately to the laboratory where it was centrifuged within 15 minutes at 300*g* for 15 minutes at room temperature. The resulting platelet-rich plasma was transferred to a separate tube and centrifuge for an additional 5 minutes at 4500*g* at room temperature, which yielded a platelet pellet and nearly cell-free plasma.

2.3. Platelet mitochondrial measurements

We adapted previously published methods for the measurement of platelet mitochondrial function in intact platelets [8]. All measurements were performed by a single operator blinded to the clinical status of the patient. Because of a smaller volume of blood than used in previous reports and findings that suggest that platelet mitochondrial abnormalities in sepsis are more pronounced in and mediated by a yet unknown factor in the serum [7,8], all experiments were completed in the patient's plasma rather than buffered media. As a result, cells could not be permeabilized with digitonin. Therefore, the resultant oxygen consumption measurements do not represent classical mitochondrial states but are consistent with prior measurements reported in the literature [8] and consistent with recommendations for the assessment of mitochondrial function in whole cells by experts in the field [11].

Oxygen consumption was measured using a high-resolution respirometer (Oxygraph O2k; Oroboros Instruments, Innsbruck, Austria). The instrument was calibrated following manufacturer instructions using 2 mL of plasma. Simultaneously, the platelet pellet was gently resuspended in 100- to 200-μL plasma, and the platelet count of the resulting ultra–platelet-rich plasma was measured using an automated platelet counter (Cellometer AutoM10; Nexcelom Bioscience, Lawrence, MA). The necessary volume required to yield a final cell count of approximately 200×10^6 platelets/mL in the Oxygraph chamber was calculated and added to the chamber. This platelet count was chosen based on published data suggesting this concentration yields superior results [12]. The final platelet concentration in the chamber was measured and entered into the manufacturer provided software (DatLab 5.2; Oroboros Instruments, Innsbruck, Austria), which normalized the results to cell count following completion of the experiment, and the oxygen solubility constant in serum was estimated at 0.89. The chamber was closed, and after equilibration, the basal oxygen consumption rate of unstimulated platelets in plasma was estimated.

All chemicals for platelet mitochondrial experiments were purchased from Sigma-Aldrich (St Louis, MO). Oligomycin was added to the chamber (final concentration in chamber, 5 μmol/L), effectively blocking ATP synthase activity to measure oligomycin-induced state 4 (state 4o) respiration. Serial additions of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (1 μL; 20 mmol/L) were added until a maximal respiration rate was obtained. Of note, this concentration is significantly higher (~20-fold) than the concentration required when performing similar experiments in buffer but is consistent with work by other groups [13]. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone additions were continued until 3 consecutive additions failed to increase the respiration rate. Finally, rotenone and antimycin A (final concentrations, 0.6 μmol/L and 1.8 mmol/L, respectively) were added consecutively to arrive at a final, residual oxygen consumption rate that is independent of electron transfer chain activity. Given the fact that these measurements were performed in serum rather than buffer, this rate includes not only extra mitochondrial platelet oxygen consumption but also the oxygen consumption of the serum. This residual rate was subtracted from the basal, state 4o, and maximal respiration rates [11]. The cellular respiratory control ratio (RCR) was calculated by dividing the corrected maximal respiration rate by the state 4o rate. The cellular RCR similarly differs from isolated mitochondrial RCR as maximal FCCP-induced and state 4o respiratory rates are not identical to classical state 3 and state 4 rates [11].

2.4. Clinical measurements

Patient demographics, clinical characteristics, and outcomes were recorded by clinical research coordinators blinded to the results of the study measurements. Severity of illness at enrollment was estimated by the measurement of the Sequential Organ Failure Assessment (SOFA) score. All patients had lactate measurement performed as part of routine clinical care before inclusion in the study. Patients were followed up to 28 days to determine survival.

2.5. Lactate/pyruvate ratio and lactate clearance

In a subgroup of patients, additional samples were collected and processed at enrollment and 6 ($\pm$1) hours later according to protocols provided by LabCorp Clinical Trials (Cincinnati, OH), who performed the additional studies. Lactate/pyruvate (L/P) ratio was determined by dividing lactate by pyruvate. Relative lactate clearance was determined by calculating the difference between initial and delayed lactate and dividing by the initial lactate.

2.6. Outcomes and data analysis

The primary outcome of the study was the association between SOFA score and corrected platelet mitochondrial oxygen consumption at enrollment. Secondary outcomes included difference in platelet measurements between survivors and nonsurvivors, and the association between these same measurements and initial lactate, L/P ratio, and 6-hour relative lactate clearance. Simple descriptive statistics were used to summarize patient data. Associations were tested by using the

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