Mitochondrial Dysfunction in Sepsis

*David Brealey, BSc, MRCP, and Mervyn Singer, MD, FRCP**

Address
*Bloomsbury Institute of Intensive Care Medicine, Wolfson Institute of Biomedical Research, University College London, Gower Street, London, WC1E 6BT, UK.
E-mail: m.singer@ucl.ac.uk

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Sepsis is an increasingly common problem, particularly among critically ill patients. Mechanisms by which sepsis induces organ dysfunction have not been elucidated. The coexisting findings (unique to sepsis) of metabolic acidosis yet increased tissue oxygen tensions suggest cellular availability but decreased use of oxygen (tissue dysoxia). Because mitochondria use more than 90% of total body oxygen consumption for adenosine triphosphate (ATP) generation, a bioenergetic abnormality is implied. Cell and animal data have shown that nitric oxide (and its metabolites), produced in considerable excess in patients with sepsis, can affect oxidative phosphorylation by inhibiting several of its component respiratory enzymes. Human data are scarce. However, in skeletal muscle biopsies taken from patients with sepsis, we have recently demonstrated a relationship between increased nitric oxide production, antioxidant depletion, reduced respiratory chain complex I activity, and low ATP levels. These findings correlated with severity of disease and outcome and support the notion that mitochondrial dysfunction resulting in bioenergetic failure may be an important factor in the pathophysiology of sepsis-associated multiorgan failure. However, a reasonable argument can be made that the reduction in energy supply could represent a last-ditch adaptive response to ongoing inflammation, resulting in a cellular shutdown analogous to hibernation that allows eventual restoration of organ function and long-term survival in patients fit enough to survive the acute phase.

Introduction

Sepsis is defined as the systemic inflammatory response to infection. It is caused by the resultant organ dysfunction and is the predominant cause of mortality in the intensive care unit (ICU). Unlike other common causes of death, mortality rates are increasing [1], and the incidence of sepsis is predicted to increase.

Patients who die of sepsis usually succumb to the ensuing multiorgan failure instead of the initial acute inflammation. The multiorgan dysfunction syndrome (MODS) is defined as altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention. Many insults may cause MODS; sepsis is the most common. Other recognized insults are diverse and include trauma, burns, pancreatitis, cardiac arrest, hemorrhage, and surgery.

The multiorgan dysfunction syndrome is considered the end result of severe, persistent inflammation. However, precise mechanisms by which inflammation results in organ dysfunction are elusive. Features of sepsis, including a decrease in oxygen extraction and lactic acidosis, caused investigators to conclude that MODS was a result of tissue hypoxia caused by microvascular disruption. Vasoactive mediators released during the septic process, such as nitric oxide (NO), leukotrienes, and prostaglandins, produce changes in blood vessel tone, resulting in blood flow redistribution away from nutrient capillaries. Coexisting capillary leak would further contribute to hypovolemia and increase diffusion distances for oxygen to travel from blood to cell. Activation of neutrophils, platelets, and the various coagulation cascades would result in widespread intravascular obstruction. All of these mechanisms would thus render the tissues ischemic. Unable to generate aerobic adenosine triphosphate (ATP), the organs would fail.

Although vascular hyporeactivity (decreased responsiveness to catecholamine vasopressors) and microvascular flow abnormalities clearly exist in patients with sepsis, the development of significant tissue hypoxia as the cause of multiorgan failure is undermined by the findings of:

1. Minimal cell death in postmortem samples taken from the failed organs of patients with sepsis [2••].
2. Recovery from sepsis is associated with (near-) complete recovery of organ function, even in organs whose cells have poor regenerative capacity [3].
3. Increased tissue oxygen tensions in various organs (muscle, gut, bladder) in animals [4,5] and patients with sepsis [6•].

These paradoxic findings suggest that vascular dysfunction may be an important trigger rather than a direct cause of organ failure. Thus, other explanations must be sought.

The findings of decreased oxygen extraction and increased tissue oxygen tensions prompted investigation of an alternate hypothesis that organ dysfunction is a result of impaired cellular usage of oxygen rather than inadequate delivery. Because more than 90% of body oxygen consumption is used by the mitochondrial respiratory chain in

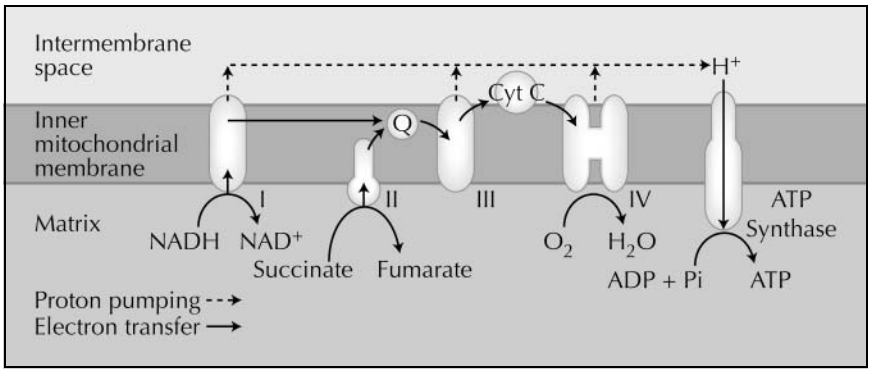

Figure 1. Schematic representation of mitochondrial respiratory chain showing electron transfer and proton pumping across the inner mitochondrial membrane. ADP—adenosine diphosphate; ATP—adenosine triphosphate; Cyt C—cytochrome C; Pi—inorganic phosphate; Q—ubiquinone.

the process of oxidative phosphorylation, a disruption of this process with a resultant decrease in ATP production is a plausible unifying explanation for the mechanism underlying organ dysfunction. Support for this cellular dysoxia theory comes from several cell culture models and clinical studies. For example, Rosser *et al.* [7] found that maximal oxygen consumption increased markedly in hepatocytes exposed to endotoxin at 6 hours, but decreased significantly by 24 hours. In a patient study, Kreymann *et al.* [8•] noted that increasing sepsis severity was associated with progressive decreases in oxygen consumption.

Mitochondria

Mitochondria are intracellular organelles. Their primary roles are production of ATP and control of cell death. They are also involved in Ca^{2+} and H+ homeostasis, intracellular signaling, and heat production. Mitochondria are composed of a central matrix enclosed by an inner and outer mitochondrial membrane.

The mitochondrial respiratory chain (Fig. 1) is located in the inner mitochondrial membrane and is composed of four individual enzyme complexes (complexes I to IV). These complexes transfer electrons from NADH and succinate (produced by the citric acid [Krebs] cycle) down a redox gradient, finally reducing molecular oxygen to water. The transfer of electrons allows complexes I, III, and IV to translocate protons from the mitochondrial matrix to the intermembrane space, generating a proton gradient. This gradient is used by ATP synthase (complex V) to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate.

For several decades, biochemists have studied the respiratory chain through the use of specific inhibitors. The recognition that one of these inhibitors (NO) was a biologically active molecule raised the possibility of physiologic/pathologic inhibition in vivo. This is particularly pertinent in patients with sepsis in whom NO is produced in considerable excess.

Nitric Oxide

Nitric oxide is a free radical gas produced in numerous cell types by the action of NO synthase (NOS) on L-arginine, or derived from endogenous NO donors, such as nitrosothiols. NO is an important mediator in many physiologic processes such as control of vascular tone, inhibition of platelet aggregation, signaling, memory formation, and enhancing neutrophil and macrophage cytotoxicity.

There are three NOS isoforms; two constitutive (eNOS and nNOS) and one inducible (iNOS). The constitutive isoforms maintain a background level of NO to perform normal physiologic functions. The inducible isoform has very little activity in health. However, its production can be markedly and rapidly upregulated in response to stress.

Nitric oxide is metabolized in several ways. It can be oxidized to form nitrite and nitrate; measurement of tissue or plasma nitrite/nitrate (NOx) levels is often used as a surrogate for increased NO production because direct NO measurement is very difficult. NO may be scavenged by hemoglobin to form nitrosyl-hemoglobin, which is subsequently metabolized to methemoglobin. Reaction with protein thiol groups to create nitrosothiols and with free radicals (*eg*, with superoxide [O_2^-] to generate peroxynitrite [$ONOO^-$]) are biologically important pathways of NO metabolism.

Nitric Oxide and Sepsis

Macrophage activation is an early event in the septic process, resulting in upregulation of iNOS production and the release of proinflammatory cytokines such as tumor necrosis factor and interleukin-1. These cytokines can upregulate iNOS at distant sites. This occurs within a few hours of the cell being stressed.

Plasma and tissue NOx levels increase in patients with sepsis in conjunction with the severity of inflammation. In adult patients with sepsis, plasma NOx levels correlated inversely with systemic vascular resistance, systolic blood pressure, and global oxygen extraction ratios, and correlated directly with plasma lactate, plasma endotoxin concentration, cardiac output, vasopressor requirements, and severity of organ dysfunction. By inhibiting synthesis of NO or its action on guanylate cyclase, the hyporesponsiveness to vasopressors could be reversed and blood pressure restored. Unfortunately, an as yet unpublished clinical trial of NOS inhibition showed an overall adverse

event, caused in large part by wrong selection of dose and target patient population.

Mitochondria in Cell Models of Sepsis and Inflammation

The addition of NO donors to cell cultures or intact mitochondria causes a rapid decrease in oxygen consumption that can be reversed by addition of NO scavengers such as oxyhemoglobin. This early inhibition in respiration occurs at mitochondrial complex IV where NO competes with oxygen for the same binding site. NO has been found to decrease oxygen consumption in heart, liver, and brain mitochondria, renal tubules, macrophages, myocytes, neurones, and brown adipocytes. Although the initial inhibition of respiration is likely caused by this binding of NO to complex IV, a more prolonged exposure may result in more persistent inhibition of the other complexes, in particular complex I [9].

Inhibition of the respiratory chain causes the complexes to become maximally reduced. This allows electrons to leak from the chain, reacting with molecular oxygen to generate O_2^-. This leak probably occurs at complexes I and III. Mitochondria exposed to NO or isolated from septic animals show increased rates of O_2^- generation. Superoxide rapidly reacts with NO to generate the powerful oxidant, peroxynitrite, which is capable of denaturing proteins, cleaving DNA, and causing prolonged/irreversible inhibition of the respiratory chain and ATP synthase. Evidence of $ONOO^-$ production during the inflammatory process has been found in animal models and patients [10,11•].

Reduced glutathione is a potent antioxidant that protects the respiratory chain against inhibition by reactive species. Glutathione is one of the predominant intramitochondrial antioxidants responsible for maintaining protein thiol groups in a reduced form and for detoxifying $ONOO^-$ and hydrogen peroxide (H_2O_2). The addition of proinflammatory cytokines and NO donors to cell culture models resulted in a loss of glutathione and mitochondrial complex activities. Complex I appears particularly susceptible to glutathione depletion [9]. A loss of muscle and plasma glutathione is a well-recognized phenomenon in patients who are critically ill [12••,13]. Several studies have found that the NO-mediated inhibition of complex I may be prevented or reversed by the addition of exogenous GSH [14]. However, no studies have attempted to replete intramitochondrial GSH.

The $NO/ONOO^-$-mediated inhibition of the respiratory chain results in a decrease in the proton gradient, and thus a decrease in ATP synthesis. This may be compounded further by inhibition of ATP synthase by $ONOO^-$. Several studies have confirmed this finding. Brookes *et al*. [15•] demonstrated that the addition of NO to isolated rat brain mitochondria resulted in a rapid but partially reversible loss of ATP. Similar data have been obtained in chondrocytes, lymphoma cells, and pneumocytes.

Mitochondrial Function in Animal Models of Sepsis

Data obtained from animal models are often contradictory, with some models showing an increase in mitochondrial activity, some finding no change, and others showing a decrease. As demonstrated by Kantrow *et al*. [16], this may be explained partially by the different techniques used to examine mitochondrial activity. The timing of mitochondrial function measurement in relation to the duration of sepsis may be an important factor [17••].

Enhanced mitochondrial function

Some short-term studies have demonstrated increased oxygen consumption and enhanced mitochondrial function in septic models [18–21]. The study by Dawson *et al*. [21] involved rats injected with a large dose of intravenous endotoxin (70 mg/kg) and observed until death (mean duration of 4 hours). Mitochondria were isolated from liver, skeletal, and cardiac muscle of the dead rats, and mitochondrial function was determined for another 4 hours. Although hepatic mitochondria stopped functioning within 30 minutes of death, there was either no change or increases in mitochondrial respiration from all tissues studied.

Mixed findings

In a rat cecal ligation and puncture model, Kantrow *et al*. [16] found that hepatocyte oxygen consumption was reduced at 16 hours, compared to sham controls. This could not be reversed by a 5-minute incubation with the NOS inhibitor, L-NAME, and may thus reflect a more persistent inhibition of respiration. The investigators also isolated mitochondria from the septic hepatocytes yet here demonstrated a significant increase in complex I and II mediated respiration. Incubation with an NO donor resulted in a reduction in respiration rates. These results typify the difficulty in interpreting data obtained from animal models. The authors suggested that techniques used to purify mitochondria may only isolate robust mitochondria from the septic animals, with dysfunctional mitochondria destroyed or excluded.

Several studies have found temporal variation in mitochondrial activity with initial increases followed by later decreases. Kurose *et al*. [22] isolated rat livers and perfused them with rhodamine-123, a dye that fluoresces with an intensity proportional to mitochondrial energization. The livers were then perfused with 1 μg/mL of endotoxin for 60 minutes. After 10 minutes there was an initial increase in fluorescence; it then progressively decreased until the end of the experiment. This decrease in membrane potential could be prevented by infusion of the NOS inhibitor, L-NMMA. Likewise, Tanaka *et al*. [23] investigated rats

injected with a lethal (at 24 hours) or sublethal intravenous dose of *Escherichia coli*. After 6 hours, both septic groups had higher NAD:NADH ratios compared to controls; this change in mitochondrial redox potential suggests increased activity of the respiratory chain. However, by 12 hours, this ratio had decreased in the lethal group yet remained elevated in the nonlethal group.

Diminished mitochondrial function

Most laboratory studies, particularly those using long-term sepsis models (>16 hours), have reported a decrease in mitochondrial function, often with ultrastructural changes seen on electron microscopy. These are summarized in a previous review of the topic [17••]. Recent studies have repeated this finding [24–28]. Callahan *et al.* [24] reported a significant decrease (44%) in diaphragmatic mitochondrial respiration from an endotoxic rat model at 48 hours but not at 24 hours. Administration of an NOS inhibitor (L-NAME) or a superoxide dismutase mimetic (PEG-SOD) to the rats prevented these changes.

Crouser *et al.* [26] designed an anesthetized septic feline model that specifically attempted to exclude any confounding effect of coexisting tissue hypoxia. They exposed a segment of ileum, cannulated the superior mesenteric artery to maintain a steady oxygen delivery, and then administered a bolus of intravenous endotoxin to 50% of the cats. After 2 hours, oxygen delivery to the ileal segment was similar to the control animals, yet there was a decrease in oxygen extraction and an elevation in critical oxygen delivery (DO_{2crit}), the point at which oxygen consumption becomes delivery-dependent. The endotoxin-treated animals displayed significant mitochondrial damage that was unseen in controls, ranging from mild (mitochondrial swelling) to severe (disruption of the membrane). There was a significant negative correlation between the oxygen extraction ratio and the degree of damage, implicating damaged mitochondria to defective oxygen usage.

Direct assessment of in vivo mitochondrial function is possible using near-infrared spectroscopy to measure the redox state of cytochrome aa_3 (a component of complex IV) or nuclear magnetic resonance spectroscopy to assess changes in, for example, ATP:ADP or ATP:phosphocreatinine (PCr) ratios. Schaefer *et al.* [29,30] demonstrated a dose-response relationship between endotoxin and a reduction of intestinal cytochrome aa_3, despite a maintenance of blood flow, which suggests a direct (or rapidly mediated indirect) impairment of oxidative phosphorylation. Skeletal muscle cytochrome aa_3 became progressively reduced in a primate model of gram-negative sepsis, despite the maintenance of DO_2 [31]. This correlated with myocyte mitochondrial structural changes, progressive swelling and distortion, and membrane fragmentation before death.

Mitochondria from some tissues are more resistant than others to inhibition by endotoxin. In a rabbit model, Gellerich *et al.* [32] found that reduced respiration and complex I activity in mitochondria isolated from skeletal muscle required a 50% higher dose than needed to achieve an equivalent effect on myocardium. Myocardial complex I and II activities also decreased with increasing doses of *E. coli* administered to baboons [32].

Adenosine Triphosphate Levels in Sepsis

Adenosine triphosphate is used by the cell to drive energetically unfavorable reactions (*eg*, protein and DNA synthesis, pumping ions up a concentration gradient). Cells hydrolyze ATP to ADP and Pi (inorganic phosphate) with a resultant release of energy. Under normal circumstances, ADP is rapidly converted back to ATP by the mitochondria, thus maintaining a high ATP:ADP ratio. However, in times of stress, ADP may be hydrolyzed to adenosine monophosphate (AMP) with a loss of a further phosphate and energy release (although less than released by the hydrolysis of ATP). For short periods of time, muscle ATP levels can be maintained using muscle PCr; this can act as a phosphate reserve, rapidly phosphorylating ADP back to ATP ($PCr + H^+ + ADP \leftrightarrow creatine + ATP$).

Animal models have demonstrated that sepsis is often associated with low tissue ATP levels; however, few models have examined the cause. Rat gastrocnemius muscle PCr:Pi and ATP:Pi ratios declined 48 hours after cecal ligation and puncture compared to sham-operated animals [33]. To investigate whether this was caused by impaired mitochondrial function, the investigators electrically stimulated the muscle and measured the time of recovery for PCr and ATP. Compared to controls, recovery time was shorter at 24 hours but prolonged by 48 hours, suggesting that mitochondrial function was initially enhanced during sepsis but later decreased. The Tanaka study [23] mentioned previously investigated rats administered lethal or sublethal doses of *E. coli*. After 12 hours, the lethal group had a metabolic acidosis and significantly lower hepatic ATP levels compared to control and sublethal groups. This was also associated with significantly increased ADP and AMP levels and a decrease in the NAD:NADH ratio (redox state), which indicates impaired mitochondrial respiration.

The notion that sepsis is associated with an initial acceleration in mitochondrial activity followed by a decrease in the sicker, longer-duration animals is plausible; this relates to the different hormone profiles found in the acute and subacute stress response to sepsis and suggests a possible interaction between hormonal control, mitochondrial activity, and metabolic rate.

A decline in tissue ATP levels appears to be related to excess production of NO. The decrease in hepatic ATP in an endotoxic rabbit model could be attenuated by infusion of the NO scavenger, carboxy-PTIO [34]. Similarly, iNOS inhibition with L-canavanine prevented the endotoxin-induced decrease in hepatic, renal, and jejunal ATP levels in a rat model [35]. However, conflicting results were

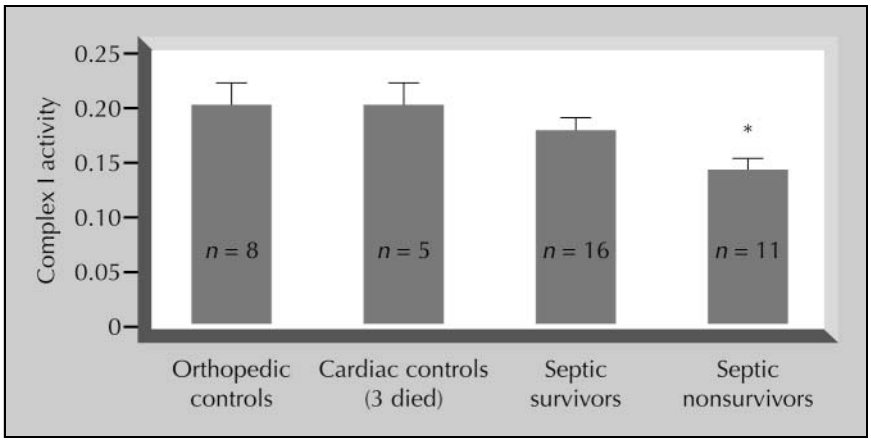

Figure 2. Complex I activity in orthopedic controls, patients with cardiogenic shock, and septic survivors and nonsurvivors. *Asterisk* denotes P value < 0.05 compared to orthopedic controls. Complex I activity is expressed as a ratio to citrate synthase activity to control for mitochondrial content of the tissue.

found when vasopressor agents were used to maintain blood pressure. Revelly *et al.* [36] maintained jejunal mucosal ATP levels in an endotoxic pig model with norepinephrine, whereas Levy *et al.* [37] found that epinephrine and norepinephrine exaggerated the decrease in hepatic and renal ATP levels in an endotoxic rat model.

Mitochondrial Dysfunction in Human Sepsis

Scanty data have been available on mitochondrial function in human sepsis, although the weight of evidence has been increasing in recent years. A small study performed by Poderoso *et al.* [38] on mitochondria isolated from the skeletal muscle of patients with septic shock indicated an impairment in complex I mediated respiration, whereas other small studies or case series have reported lower ATP levels [39–41].

Mitochondrial dysfunction has also been inferred from studies investigating changes in arterial ketone body ratio. A decrease in the ratio of acetoacetate to β-hydroxybutyrate reflects a decrease in the ratio of hepatic NAD to NADH, inferring that the respiratory chain is inhibited. This ratio progressively decreases in patients who are dying of sepsis [42]. A decrease in this ratio also correlated with an increase in plasma nitrite, an indicator of NO production [43].

Human endothelial cells exposed to serum obtained from patients with sepsis demonstrated lower rates of mitochondrial respiration [44••] compared to cells incubated with serum obtained from healthy volunteers. Respiration could be increased in the cells exposed to septic serum (but not in the controls) with the addition of the NO inhibitor, L-NMMA, or the PARS inhibitor, 3-aminobenzamide (mentioned later).

We recently reported evidence of mitochondrial dysfunction in skeletal muscle biopsies obtained from 28 patients with severe sepsis admitted to the ICU [12••]. Compared to biopsies obtained from patients undergoing elective orthopedic surgery (controls), the patients with sepsis had an increased NOx level and decreased levels of GSH, complex I, and ATP (Figs. 2 and 3). These also correlated with norepinephrine requirements to maintain an adequate blood pressure and the degree of organ dysfunction. Low complex I activity and a low ATP were predictive of a poor outcome. These changes were not seen in patients with cardiogenic shock of similar disease severity (data unpublished). The ATP level was higher (although insignificant) in eventual septic survivors compared to orthopedic controls but significantly lower in eventual nonsurvivors; however, these septic groups could not be distinguished clinically.

The question remains as to the relevance of these findings in skeletal muscle to other more vital organs such as liver and kidney. There are logistic and ethical difficulties in performing a validation study in humans, particularly when serial sampling of vital organs is required. Therefore, we have created an awake, fluid-resuscitated long-term (3-day) fecal peritonitis rat model that carries a 40% mortality and displays many of the human physiologic, biochemical, and histologic characteristics of sepsis. The findings from this model will be shortly submitted for publication. Liver, kidney, and skeletal muscle bioenergetic data mirrored each other and the data obtained from human skeletal muscle. This gives some confidence to the significance of skeletal muscle data to the body as a whole.

Poly-(ADP)-ribosyl Synthetase

Poly-(ADP)-ribosyl synthetase (PARS) is a nucleotide polymerizing enzyme involved in the repair of DNA. It cleaves NAD to ADP ribose and nicotinamide, attaching the ADP ribose group to a protein before extending this group into a nucleic acid-like polymer, poly-ADP-ribose. Activation of PARS is thought to rapidly deplete the cell of NAD, thus limiting glycolysis and the Krebs cycle of substrate. ATP is also required for the cell to resynthesize NAD. Several sepsis models have demonstrated the benefits of PARS inhibition [45–50], although other wider-ranging anti-inflammatory effects may be responsible for the demonstrated benefit [48–50] rather than its effects on cellular NAD and ATP.

Conclusions

The incidence of sepsis is rising, and it is the most common cause of mortality in the ICU. Most patients who die

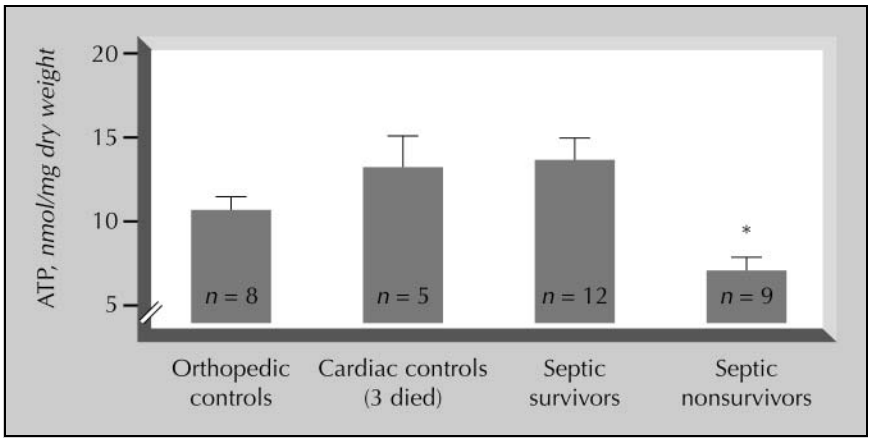

Figure 3. Adenosine triphosphate (ATP) concentration in orthopedic controls, patients with cardiogenic shock, and septic survivors and nonsurvivors. *Asterisk* denotes *P* value < 0.05 compared to orthopedic controls.

of sepsis die from the associated organ dysfunction rather than the initial acute inflammatory episode. Mechanisms by which organ dysfunction occur have yet to be fully established, although the decrease in oxygen extraction, the increase in tissue oxygen tension (unique to sepsis), and the lack of cell death suggests that the problem is more of cellular inability to use oxygen than one of compromised tissue oxygen delivery. Because more than 90% of body oxygen consumption is used by the mitochondrial respiratory chain in the process of oxidative phosphorylation, this suggests that failure of the bioenergetic process may be central to the pathophysiology of MODS. Increased NO and its metabolites (such as peroxynitrite), known inhibitors of the respiratory chain, are produced during the septic process and are associated with increasing levels of mitochondrial and organ dysfunction. Inhibiting NOS or scavenging free NO can prevent or reverse these changes. In cell models, the antioxidant GSH has a protective role against mitochondrial inhibition, particularly for complex I. Human data are scarce but supportive of these findings. The importance of complex I inhibition is increasingly apparent; this complex appears to be primarily involved in numerous other disease processes such as Parkinson's disease and Friedrich's ataxia.

The field is muddied by the considerable variability of in vivo, ex vivo, and in vitro septic/endotoxic models, the duration and severity of the septic insult, the degree of resuscitation, and interspecies differences. However, the literature suggests a temporal variation in mitochondrial function during sepsis, with initial increases in activity, followed in hours or days by decreased activity.

The intriguing findings of organ failure despite a lack of cell death, with eventual (near-) complete recovery of organ function if the patient survives the insult, suggest that bioenergetic failure may represent an adaptive, last-ditch protective response. A programmed cell shutdown, analogous to hibernation or estivation, would allow continuing integrity of the cell with the potential to recover when the insult has passed and inflammation has abated. The converse situation of cells attempting to function normally in the face of altered oxygen and substrate supplies, oxidant damage, and so on, would likely hasten the onset of necrotic or apoptotic processes, a significant level of which would augur against recovery in organs whose cells have poor regenerative capacity. Thus, the paradigm of multiple organ success is not as preposterous as it sounds.

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