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REVIEWS

Factors affecting the pathophysiology of sepsis, an inflammatory disorder: Key roles of oxidative and nitrosative stress

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Abstract

Sepsis, one of the primary causes of mortality in the intensive care units, occurs due to the host's dysregulated immune responses to an infection. Consequently, persistent systemic inflammation along with suppressed adaptive immunity ensues, resulting in deranged metabolism, recurrent infections, tissue damage and multi-organ failure. The uncontrolled oxidative stress mediated by the imbalance between the generation of reactive oxygen species and their neutralization by the host's antioxidant system is involved in inflammation-induced damage. The profound deleterious effects in the host range from mitochondrial dysfunction and endothelial damage to reduced cardiac output. Therefore, antioxidant therapy was actively considered to have therapeutic benefits in sepsis patients. Although some success has been obtained with the use of antioxidants in sepsis patients, considerable ambiguity persists that prevents their routine use. Another key molecule that may dictate the outcome and prognosis during sepsis is nitric oxide (NO). This pleiotropic molecule plays a central role in inflammation and in leukocyte recruitment at the site of inflammation. NO is synthesized by three different isoforms of nitric oxide synthases (NOS) and significantly high and sustained levels of NOS2 have been reported in sepsis. Abundant literature supports the protective roles of NO during sepsis; however, there is uncertainty in various reports. The administration of NO donors in clinical trials for sepsis treatment has encountered limited success. NO, during sepsis, acts like a double-edged sword: increased NO levels can result in hypotension, whereas reduced levels contribute to poor organ perfusion and an elevated susceptibility to infection. Therefore, several parameters need to be evaluated, while considering the potential of antioxidant and NO-based therapy during sepsis.

Keywords: Sepsis, reactive oxygen species, reactive nitrogen species, nitric oxide synthase, antioxidants, mitochondrial dysfunction

Introduction

Inflammation is a cascade of host defense responses initiated upon recognition of irritants, pathogens and damaged host cells. Inflammation not only acts as a self-protective host immune response to harmful stimuli but is also essential for healing of damaged tissues and recovery of normal cellular homeostasis such as during wounds and infections. Based on the symptoms and the duration of the inflammatory responses, inflammation can be broadly classified into acute and chronic inflammation.[1] Acute inflammation lasts for a few days to a week and it manifests the four classic cardinal signs of inflammation as indicated in table 1: rubor (redness), tumor (swelling), calor (fever) and dolor (pain). It was defined for the first time by Roman anthropologist Cornelius Celsus as "*rubor et tumor cum calore et dolore*" in 1st century AD. A fifth cardinal sign of inflammation "functiolaesa" i.e. "disturbance of function" was defined by Rudolph Virchow in 1858.[2] Due to the increased knowledge and recent advancements, the physiological basis of these manifestations can be explained at the macroscopic level. When infection is detected, inflammatory mediators such as chemokines, cytokines, vasoactive amines and proteolytic enzymes are released. These inflammatory mediators act on local blood vessels and bring about changes in micro-circulation such as vasodilation, which involves dilation of venules and arterioles followed by increased vascular permeability for extravasation of leukocytes and plasma proteins leakage,[3] leading to increased blood flow and leukocyte infiltration into the inflamed tissues.[4]

A typical example of acute inflammation is microbial

infection, which is usually recognized by pattern recognition receptors present on innate immune cells. Infections are often sensed by tissue resident macrophages, which are activated upon the recognition of pathogen-associated molecular patterns and damage associated molecular patterns related to infection and host cell damage respectively. The activated tissue resident macrophages further secrete inflammatory mediators and initiate a complex cascade of inflammatory responses. A variety of inflammatory mediators and oxidizing molecules such as reactive nitrogen species (RNS) and reactive oxygen species (ROS) are secreted during the inflammatory process. One of the immediate effects of this process is the activation of the endothelium of blood vessels and the selective extravasation of the neutrophils. Neutrophils, upon reaching the site of infection, are activated and further attempt to eradicate the infection by secreting noxious molecules such as RNS, ROS, proteinases, cathepsins etc.[3] As these highly toxic molecules cannot discriminate between foreign and the host targets, collateral tissue damage is inevitable during inflammation.[5]

The symptoms of chronic inflammation, unlike acute inflammation, can persist from months to years and the escalation of symptoms may vary depending on the type of tissue. During chronic inflammation, the inflammatory cascade is not completely resolved leading to persistent tissue damage, malfunction and organ dysfunction.[1] The molecular and cellular basis of chronic inflammation differs based on the type of inflamed cells and organs.[6] It can either occur due to recurrent infections, exposure to low levels of toxic chemicals or due to the failure of the acute inflammatory response to completely eliminate the inflammatory stimuli, for example asthma, gout etc. Additionally, tissue damage due to persistent autoimmune responses can also lead to chronic inflammatory disorders such as type 1 diabetes and rheumatoid arthritis. While regulated inflammation is beneficial to the host, persistent inflammation can become detrimental. A classic example of extensive host tissue damage due to uncontrolled inflammation is sepsis.

Sepsis

Sepsis, a dysregulated inflammatory disorder, is a leading cause of critical illness and mortality worldwide with approximately 53 million deaths annually.[7] According to an Indian epidemiological study on sepsis carried out from 2006 to 2011, one out of every four patients in ICU suffers from this syndrome. Sepsis-induced mortality in India ranges from 40-70%.[8] Sepsis can be defined as a highly heterogeneous, complex syndrome caused by physiological, pathological and biochemical abnormalities in host response to infection. Sepsis occurs when an infection exceeds the barrier of local tissue containment and induces a series of dysregulated host immune responses. During sepsis, both the pro- and anti-inflammatory responses occur early and co-exist simultaneously. Although, the net effect of these two competing processes is manifested predominantly by the early hyper-inflammatory responses. The expression of the genes involved in the regulation of the adaptive immune response are highly down-regulated during sepsis.[9] Impaired recovery and the subsequent mortality of sepsis patients occur due to persistent inflammation-induced organ damage as well as recurrent secondary nosocomial infections[10]

The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) developed in 2016, defined sepsis as a *"life threatening organ dysfunction caused by a dysregulated host immune response to an infection"*. Sepsis-3 consensus further defined organ dysfunction by an increase in the Sequential Organ Failure Assessment (SOFA) score of 2 points or more, which is associated with in-hospital mortality >10%. This score is based on six different independent scores, including respiratory, cardiovascular, hepatic, renal, coagulation and neurological systems. Sepsis-3 redefined septic shock as a subset of sepsis in which profound circulatory and metabolic disorders occur and is associated with a higher risk of mortality than sepsis alone. Clinically, patients with septic shock are on vasopressors to maintain a mean arterial pressure ≥65 mmHg. Additionally, the serum lactate levels are greater than 4 mmol/L (>18mg/dL) in the absence of hypovolemia with in-hospital mortality rates more than 40%.[11] Along with primary defects in immune cell functions; metabolic and neurological abnormalities, altered complement activation and coagulation pathway, calcium homeostasis and redox imbalance are considered as a central hub in the pathophysiology of sepsis with pleiotropic effects and connection with various pathways.[10] The list of genetic deficiencies that predispose individuals to sepsis is listed in Table 2.

Biomarkers of sepsis are likely to be useful for identification of sepsis as well for the selection of specific therapy to benefit the sepsis patients and the assessment of patient response to the specific therapy.[15] A few biomarkers exist that have proven to be useful, but are not reliable in the

Table 1: Hallmark signatures of acute inflammatory responses

Common Name	Latin Name	Reason
Heat	*calor*	Vasodilation
Redness	*rubor*	Vasodilation
Swelling	*tumor*	Enhanced vascular permeability and granulation tissue
Pain	*dolor*	Physical and chemical stimulation of pain receptors (nociceptors)
Loss of function	*functiolaesa*	Pain, fibroplasia and metaplasia and damage to the tissue structure

Table 2: Genetic predisposition towards sepsis

Gene name	Description
IRAK4	• IRAK4 is activated by IL-1R/Toll-like receptor superfamily (TIRs) and leads to NFκB and MAPK3 signaling pathways. • Patients with IRAK4 deficiency of autosomal recessive type are prone to develop septicemia, followed by mild or delayed signs of sepsis.[12]
IKBKG	• IKBKG gene product regulates the activity of NFκB. • Mutations in this gene results in X-linked recessive EDA-ID (anhidrotic ectodermal dysplasia with immunodeficiency). • Such patients are prone to develop septicemia, followed by mild or delayed signs of sepsis.[12]
NFKBIA	• The *NFKBIA* gene provides instructions for making the alpha subunit of the IKK protein complex, which is a group of related proteins that regulates the activity of nuclear factor-kappa-B. • Mutations in this gene are typified as autosomal dominant EDA-ID, making the patients prone to develop septicemia, followed by mild or delayed signs of sepsis.[12]
Caspase 12	• Caspase 12 is a member of cysteine-aspertate protease, involved in apoptosis and processing of inflammatory cytokines. • Gene-targeted knock-out in C57BL/6 mice rendered them resistant to peritonitis and septic shock by reducing the production of a number of pro-inflammatory cytokines. • IFNγ plays important role in these mice as ablating their function with neutralizing antibodies against IFNγR deprive them of sepsis resistance.[13]
NOS2	• NOS2 codes for inducible nitric oxide synthase, which produces nitric oxide from L-arginine. The absence of this enzyme in gene-targeted knockout mice reduces their survivality in an infection-induced peritonitis mouse model of sepsis.[14]

diagnosis of sepsis. Several efforts have been made to come up with different biomarkers for diagnosis of sepsis as well as for monitoring the treatment. Approximately, 180 biomarkers have been evaluated for use in sepsis over the last five decades.[15] A recent review summarizes various sepsis biomarkers that have been used for the prognosis of morbidity and mortality. It suggests the use of three-biomarkers, i.e. procalcitonin (PCT), interleukin-6 (IL-6) and soluble triggering receptor expressed on myeloid cells-1(sTREM-1) to diagnose sepsis before it becomes life-threatening.[16] Table 3 summarizes the various biomarkers used in the diagnosis of sepsis and its different stages.

Sepsis is a complex heterogeneous syndrome and several factors are involved in the pathogenesis of sepsis. Due to improved antibiotic treatments, better and safer organ support and effective intensive care medicines, there is increase in survival of sepsis patients. However, there

Table 3: Commonly used biomarkers for sepsis and its different stages

Name	Mechanism of Action
Biomarkers of hyper-inflammatory phase of sepsis	
Pro-inflammatory cytokine IL6	Increased plasma IL6 is associated with increased mortality. It stimulates the production of C-reactive protein and granulopoiesis.[17]
Chemokine IL8	Useful to monitor infection, sepsis and mortality in burn patients. It is required for neutrophil recruitment.[18]
C-reactive protein (CRP)	Highly sensitive for screening early-onset of sepsis in adults and to detect the recovery of sepsis patients post-surgery. CRP enhances inflammation by complement activation.[19]
Procalcitonin (PCT)	Used for the diagnosis of invasive bacterial infection and to determine the efficacy of antibiotic treatment.[20]
Soluble triggering receptor expressed on myeloid cells 1 (sTREM-1)	Specific indicators of myeloid leucocyte recruitment due to bacterial infection. It serves as a good indicator of risk of sepsis severity in patients not yet expressing symptoms. It differentiates sepsis from shock.[16]
Biomarker of activated neutrophils	
CD64	Enhanced expression during infection and is used to detect activated state of blood neutrophils at early stage with 77% specificity and 75% sensitivity in sepsis patients.[21]
Biomarkers of immune suppression phase	
HLA-DR	Reduced expression on monocyte is the most reliable marker for assessment of immune status in critically ill patients. It serves as an indicator of immune paralysis and susceptibility to secondary infections.[22]
PD-L1	Reduced expression on monocyte is associated with increased mortality. It is a prognostic marker for shock patients.[23]
Biomarkers of sepsis induced organ dysfunction	
Lactate	Widely used biomarker for detection of septic shock. High serum lactate levels ≥4 mmol/L or 18 mg/dL, in the absence of hypovolemia, indicates mitochondrial dysfunction and impaired liver function.[17, 24]
Paraoxonase-1	Reduced amount of serum paraoxonase-1, an anti-inflammatory, antioxidant and detoxifier, is associated with increased multi organ dysfunction syndrome and cardiovascular insufficiency.[25]

is no specific therapy to directly treat sepsis induced dysregulated host responses.[26] Human recombinant activated protein C, the only FDA-approved drug i.e., with anti-thrombotic, anti-inflammatory and profibrinolytic activity, was withdrawn due to increased risk of bleeding and no reduction in mortality rate of sepsis patients in subsequent confirmatory trials.[27, 28] These observations demonstrate the complexity of this syndrome and major problems in the field. This review summarizes the roles of oxidative and nitrosative stress during sepsis.

Oxidative stress and mitochondrial dysfunction in the pathogenesis of sepsis

Results from clinical and pre-clinical experimental studies have demonstrated the existence of overwhelming oxidative stress in sepsis patients.[29, 30] Oxidative stress results from the imbalance between the generation and participation of reactive species and the effectiveness of

host antioxidant defense system to neutralize it, leading to overload of oxidants in cells. Furthermore, according to 'redox hypothesis', oxidative stress is defined as reversible oxidation of intra- and extra-cellular thiols.[31] RNS, e.g. nitric oxide (NO) and peroxynitrite (ONOO-), and ROS, e.g. such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), are the principal molecules involved during oxidative stress-induced cellular dysfunction (Fig.1).[32] Under normal conditions, mitochondria are the central source of reactive species. In complex IV of the mitochondrion electron transport chain, defective reduction of O_2 to H_2O results in the generation of superoxide (O_2^-). Normally O_2^- is converted by manganese superoxide dismutase (SOD) to H_2O_2, which is further catalyzed by catalase (CAT) into H_2O. However, imbalance in the ratio of SOD and CAT has been associated with the oxidative stress and morbidity in sepsis patients.[33] In addition, phagocytic cells also express membrane-bound nicotinamide adenine dinucleotide phosphate oxidase that generates O_2^- and myeloperoxidase, which converts H_2O_2 and Cl to hypochlorous acid (HOCl) and OH.[32]

Mitochondria are not only the major source of cellular ROS production, but are also highly vulnerable to the

Fig. 1: The main enzyme and substrates involved in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells

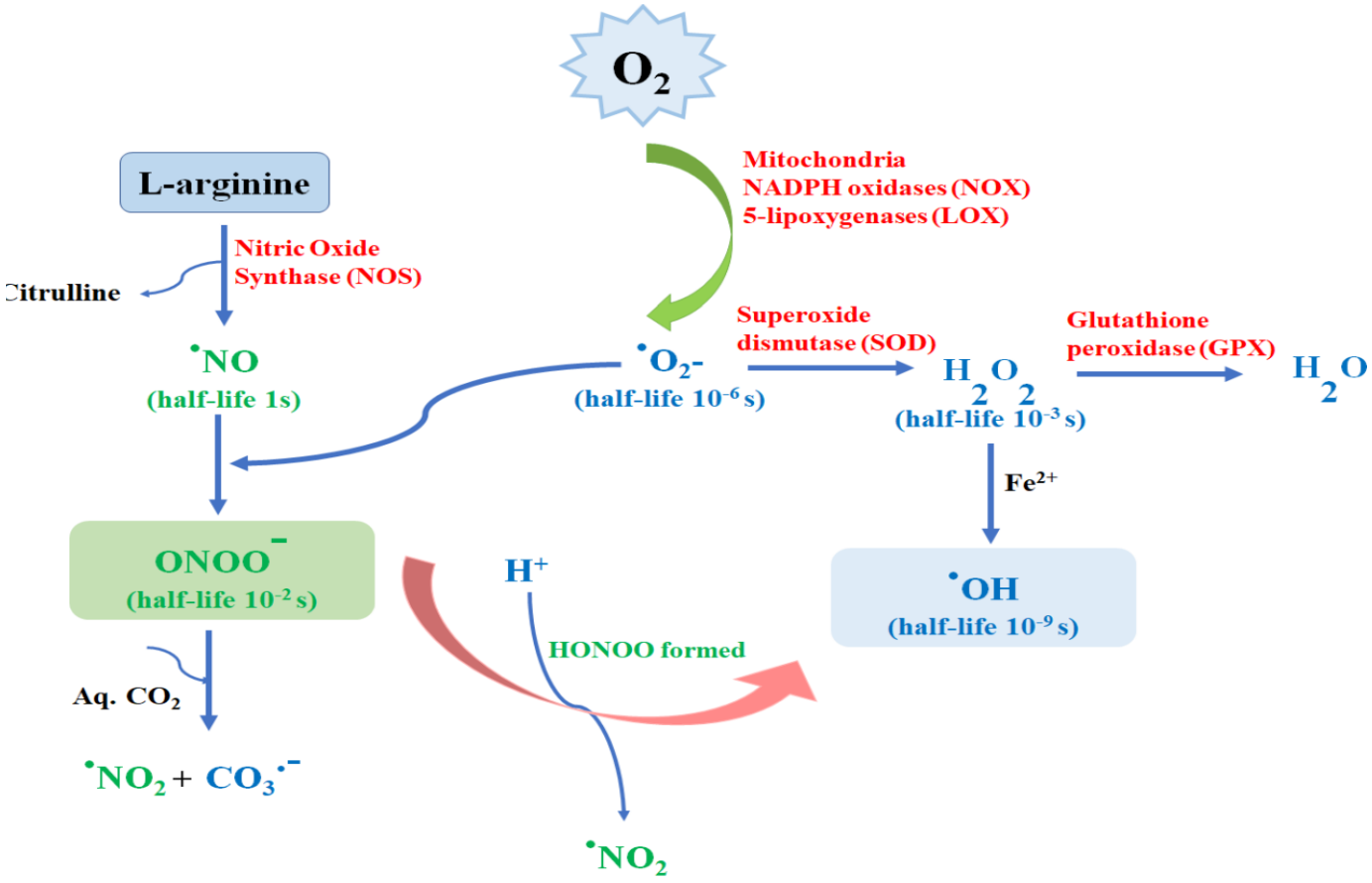

Figure 1: The main enzymes and substrates involved in generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in cells. The conversion of L-arginine to citrulline by Nitric Oxide Synthases (NOS) results in the production of nitric oxide (NO). NO can readily react with the superoxide radical ($^{\cdot}O_2^-$) to generate the highly reactive peroxynitrite ($ONOO^-$), which can also react with CO_2 to form nitrite ($^{\cdot}NO_2$) and carbonate ($CO_3^{\cdot-}$) radicals. $ONOO^-$ radicals can combine with H^+ ions to form Peroxynitrous acid (HONOO) which can generate more hydroxyl and nitrite radicals. Mitochondria are the main cellular organelles where superoxide radicals are generated. They can also be produced by the enzymes NADPH oxidases (NOX) or Lipooxygenases (LOX). Superoxide dismutase (SOD) converts the highly reactive superoxide radical into peroxide which is finally metabolized into water by the action of Glutathione peroxidase (GPX). Hydrogen peroxide can also result in the production of reactive hydroxyl ($^{\cdot}OH$) species via Fenton reactions using Fe^{2+}. All major RNS are indicated in green while major ROS are indicated in blue. The half- lives of the species are given in brackets.

rapid destruction caused by cellular oxidants leading to mitochondrial dysfunction.[32, 34] Mitochondrial dysfunctions are related to loss of inner membrane potential, inhibition of oxidative phosphorylation resulting in cellular energy failure. It is also postulated that the impaired oxygen consumption by cells known as 'cytopathic hypoxia' is responsible for the oxidative stress in sepsis patients.[35] Importantly, cellular energetic failure due to mitochondrial dysfunction is associated with cytopathic hypoxia observed in critically ill septic patients. Subsequently, enhanced mitochondrial biogenesis is known to improve the survival of sepsis patients.[32] The pathophysiological effects of mitochondrial dysfunction occur due to cellular adenosine triphosphate (ATP) depletion, excessive production of ROS, release of pro-apoptotic proteins and imbalance in calcium homeostasis. Additionally, the presence of highly reactive form free radicals such as O_2^- and $ONOO^-$ inhibits the function of cytochrome C oxidase membrane complex and triggers the opening of mitochondrial permeability transition pore leading to drop in mitochondrial membrane potential. Reduced membrane potential hampers the ATP production and augments the generation of free radicals. Subsequently, failure to produce ATP through oxidative phosphorylation diverts the metabolic pathway towards aerobic glycolysis, to convert pyruvate into lactate, an alternative source of ATP, which is known as 'Warburg' effect.[36] Increased amounts of lactate are seen as a marker of stress and are often associated with increased organ dysfunction and mortality in sepsis patients.[36] The percentage of monocytes with loss of mitochondrial membrane potential is increased in sepsis patients. Also, peripheral blood mononuclear cells in septic patients showed reduction in the activities of complex I, III and IV of electron transport chain, which is associated with reduced oxygen consumption by cells.[32] Other than mitochondrial dysfunction-mediated host cell damage, reactive oxidants can directly attack endothelial cells leading to deterioration of endothelium. Endothelial damage can further contribute to enhanced vascular permeability, decreased colloidal osmotic plasma pressure and hypotension.[37, 38] Further, oxidation-induced activation of cyclic guanosine phosphate-dependent protein kinase-1 α results in dilation of blood vessels and decreased cardiac output.[39] Altogether, this process leads to lowered organ perfusion causing ischemia and multi-organ failure. As mentioned before, oxidative stress-induced mitochondrial dysfunction leads to lower levels of ATP and higher amounts of lactate. Since cardiac muscles require constant supply of ATP for ventricular contraction, lower ATP levels contribute to reduced cardiac output further potentiating hypotension and reduced organ perfusion.[40] The markers of oxidative stress include decreased levels of reduced glutathione, increased protein carbonylation and increased SOD/CAT ratio, leading to accumulation of H_2O_2 in cells.[32]

In addition, it is reported that mitochondrial ROS can induce the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF.[41] Inflammatory cytokines such as TNFα, IL-1α, IL-1β and IL-6 produced by activated innate immune cells are also known to contribute to sepsis severity. The expression of pro-inflammatory cytokines during inflammation is largely regulated by the transcription factor nuclear factor (NF)-κB.[42, 43] Upon recognition of stimuli, NF-κB associated IκB in cytosol is phosphorylated, ubiquitinylated and degraded. NF-κB translocates into the nucleus and binds to the specific binding sequence in the promoter region of its target genes.[43] As shown in figure 2, one of the crucial genes transcribed by NF-κB, which plays central role in redox regulation is nitric oxide synthase 2 (NOS2). An inducible isoform of NOS expressed in variety of cells during inflammatory conditions. In addition, ROS may also regulate the functions of NF-κB, pre-dominantly by up-regulating its activity either by activating kinases (IKKs), which phosphorylates and degrade IκB or by inhibiting phosphatases, which maintains IKKs in active state.[44, 45] In addition to the host-derived pro-inflammatory cytokines, sepsis is frequently associated with endotoxemia, which is accumulation of large amount Gram-negative endotoxin (lipopolysaccharide) in blood.[46, 47] Lipopolysaccharide upon binding to its receptor further augments the secretion of pro-inflammatory cytokines through activation of NF-κB pathway in innate immune cells (Fig. 2). Interestingly, pre-treatment with leptin, an adipocyte-derived protein before induction of endotoxemia in rats blunted the production of pro-inflammatory cytokines IL1β, IL6 and TNFα with increased survival.[48] Based on the aforementioned aspects, antioxidant therapy can be useful for treatment of sepsis. Some of the most promising antioxidant therapies that require future studies with detailed analysis are described below.

Pharmacological modulators of cellular energy sensing targeting mitochondrial dysfunction

AMP activated protein kinase (AMPK): Loss of cellular homeostasis due to mitochondrial dysfunction occurs because of ATP depletion and increased ROS production, therefore conservation of ATP molecules might prevent metabolic impairment and organ injury.[24] AMPK is a key molecular sensor of cellular energy. Its activation leads to suppression of energy-consuming anabolic processes

Fig. 2: Overview of lipopolysaccharide (LPS)-induced signaling pathway

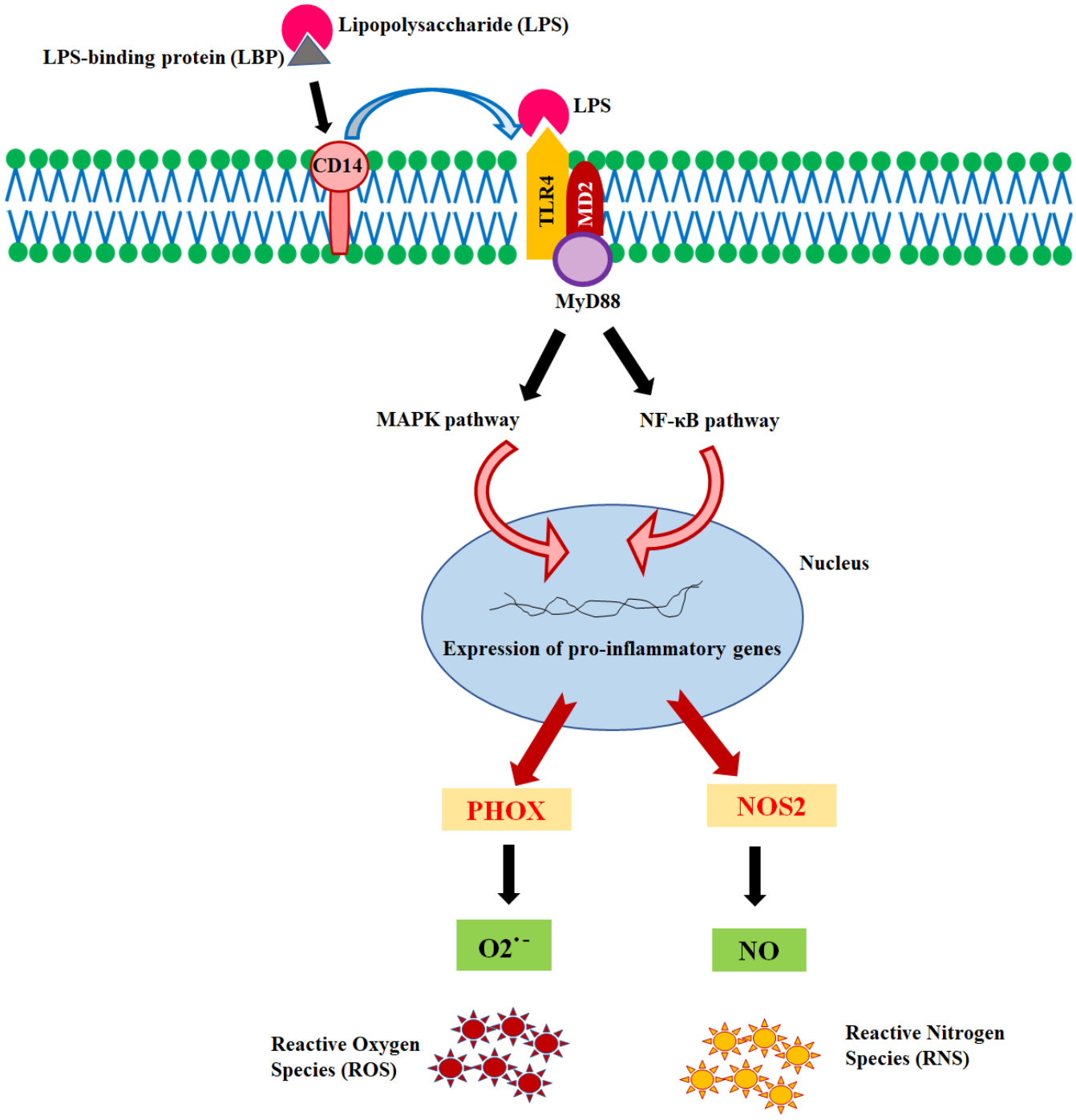

Figure 2: Overview of the Lipopolysaccharide (LPS) -induced signaling pathway. LPS from the Gram-negative cell membrane can bind to the LPS-binding protein (LBP) which is present as an acute phase protein in the plasma. LPS-LBP complex binds to the CD14 receptor present on the mammalian cell membrane. The LPS-CD14 complex then interacts with the TLR4-MD2 complex, leading to downstream signal transduction via the adaptor protein MyD88. This interaction results in the activation of Mitogen Activated Protein Kinase (MAPK) and the Nuclear Factor kappa B (NF-κB) pathways, leading to expression of pro-inflammatory genes like Phagocyte Oxidase (PHOX) and Nitric Oxide Synthase 2 (NOS2). These are the key enzymes involved in the production of ROS and RNS.

and enhances energy-producing catabolic processes with refueling of cellular ATP.[49] Specific agonist of AMPK, AICAR, prevents cecal ligation puncture (CLP) and lipopolysaccharide (LPS)-induced acute kidney injury and suppresses neutrophil activation and cytokine induction.[49] Septic shock patients pre-treated with AMPK-activator, metformin, showed improved survival compared to the non-treated group.[50] AMPK can also be activated indirectly through activation of sirtuin-1. Accordingly, treatment with sirt-1 activators impede oxidative stress, cytokine response, mitochondrial dysfunction-mediated liver, kidney, heart and brain damages in CLP model of sepsis in rodents.[24]

Thiamine: ATP synthesis in mitochondria is dependent on availability of acetyl-CoA, produced via glycolytic pathway by pyruvate dehydrogenase complex. Activity of pyruvate dehydrogenase complex is further dependent on the availability of thiamine pyrophosphate derived from thiamine.[51] Septic patients with organ dysfunction are associated with thiamine deficiency.[52] Supplementation of thiamine-deficient septic patients with thiamine lowers the serum lactate levels.[53] Administration of septic patients with a cocktail of thiamine (1500 mg every 6h for 4 days), ascorbic acid (200 mg every 12h for 4 days) and hydrocortisone (50 mg every 6h for 7 days) drastically reduced organ dysfunction and mortality.[54] Although thiamine deficiency negatively regulates the outcome of sepsis, it is still not clear whether individual components govern the beneficial effects or they act synergistically.[24]

Hydrogen sulphide (H_2S): H_2S has broad range of effects, varying from immune modulation to hemodynamic stabilization and mitochondrial homeostasis. It preserves mitochondrial homeostasis by boosting ATP production, upregulating antioxidants and quenching free radicals.[24] Although LPS-induced sepsis and septic shock patients show increased amounts of plasma H_2S, knockout mice of one of the major H_2S producing enzymes do not show significant improvement in survival during sepsis.[24, 55] Importantly, the potential protective effects of low dose of H_2S can be impaired by vascular hemodynamic destabilization and immunosuppressive effects at higher concentration of H_2S.[24]

Antioxidant molecules

Mitochondrial respiration not only augments ATP production but also induces generation of ROS. Consequently, optimal therapy comprising stimulation of mitochondrial functions to promote ATP synthesis in combination with antioxidant therapy to suppress oxidative stress may preserve mitochondrial function during sepsis.[24]

Alpha-lipoic acid: It is a naturally occurring, water- and fat-soluble dithiol compound.[24] Alpha-lipoic acid and its reduced form are scavengers of free radicals and can further increase the anti-oxidant capacity by reducing the oxidized form of glutathione and stimulating the activity of SOD and CAT in septic rats.[56] However, no studies with alpha-lipoic acid on human sepsis patients have been conducted till date.[24]

Ascorbic acid: It is a water-soluble vitamin and an essential cofactor for copper and iron containing enzymes with immunomodulatory activity. Its antioxidant properties are mainly attributable to its functions in NADPH oxidase-mediated redox signaling, which reduces free radicals.[57] Among the critically ill patients, levels of ascorbic acid are significantly lesser in patients with organ failure than in patients without organ failure.[58] Treatment of septic patients with ascorbic acid is demonstrated to be safe and involves reduction in levels of PCT, CRP and SOFA score. However, before implementing ascorbic acid into clinical treatment guidelines, further trials are warranted to confirm these results and design the optimal treatment regimen.

Glutathione (GSH): GSH is synthesized from the amino acids cysteine, glutamate and glycine and contains a thiol group, which can scavenge free radicals.[59] It is the most abundant mitochondrial antioxidant, which prevents mitochondrial dysfunction and protects mitochondrial DNA from oxidative damage.[60] The levels of GSH can be increased by administration of its synthetic precursor N-acetyl cysteine (NAC). Both GSH and NAC can increase the plasma levels of GSH and reduce the oxidative stress and organ damage in septic shock patients.[61] However, administration of NAC 24h after the onset of sepsis is associated with cardiovascular instability due to formation of harmful metabolites from NAC. Although the protecting effects of GSH and NAC are well understood, its side effects need to be addressed further.

Selenium: Selenium is an essential nutrient incorporated into selenoproteins and is essentially involved in antioxidant defense. Human plasma contains twenty-five different selenoproteins, which are actively involved in redox signaling such as glutathione peroxidase.[62] Sepsis is associated with reduced plasma levels of selenium and selenoproteins.[63] Reduced selenium and glutathione

peroxidase levels demonstrate corresponding increase in organ failure and mortality in critically ill patients.[64] A meta-analysis of randomized clinical trials of 72 septic patients shows reduced mortality upon selenium administration, although length of stay in ICU and incidences of pneumonia were not affected.[65]

Nitric oxide synthase and generation of NO during sepsis

NO is a critical molecule involved in the host immune responses and inflammation. NO being a pleiotropic molecule, governs a broad spectrum of processes, ranging from regulation of immune responses to cell-cell interaction and microbicidal functions. NO regulates the production of cytokines, differentiation, proliferation and apoptosis of immune cells.[66] In addition, NO plays important roles during inflammation by mediating cell-cell interactions and regulating the expression of adhesion markers required for recruitment of leukocytes to the site of inflammation.[67] Considering the diversity of functions that NO has on different cell types, it can either have cytotoxic or protective roles in the host. NO can be synthesized by three different isoforms of NO synthases (NOS) which can be broadly categorized as constitutively expressed and calcium-dependent: neuronal NOS (NOS1) and endothelial NOS (NOS3). On the other hand, inducible NOS (NOS2) is calcium independent.

The regulatory roles of different isoforms of *Nos* have been addressed during sepsis. Kinetic expression of *Nos* isoforms in the lung tissue of sheep infected with live *Pseudomonas aeruginosa* revealed lack of induction of NOS1, early and transient induction of NOS3 followed by sustained induction of NOS2.[69] In a rat model of CLP-induced sepsis, a significant increase in the NOS2-derived NO inhibited the activity of NOS3 irreversibly.[70] In the *Salmonella* Typhimurium-peritonitis-induced model of sepsis in mice, it is observed that NOS2 is the sole isoform of *Nos* induced in the peritoneal cells and is required for the initiation and generation of efficient innate immune responses via early induction of cytokines and chemokines during sepsis. Profound increase in the expression of NOS2 is seen during infectious and inflammatory conditions and the protective roles of NOS2 in different models of sepsis are reported.[71] Additionally, NOS2-drived NO is required for the infiltration of neutrophils to the site of infection, leading to lower spread of infection and increased survival of the mice.[35] Interestingly, neutrophils from septic patients are also known to express higher transcripts of *Nos2*.[72] However, the roles of neutrophils during sepsis are not clear. In case of neonates, neutropenia and lower neutrophil storage pool in the bone marrow is associated with higher incidences of sepsis.[73] The lack of neutrophil responses at the site of infection results in higher lung tissue damage during peritonitis-induced septic shock in rabbits.[74] In addition, defects in the migratory ability of neutrophils correlates with sepsis severity, culminating in higher mortality.[75] On the contrary, uncontrolled neutrophil activation during sepsis may lead to multi-organ failure due to inflammation-induced host tissue damage.[76] Moreover, neutrophil-specific expression of NOS2 during CLP-induced sepsis in mice contributes to acute lung injury due to modulation of neutrophil-endothelial interactions, trans-endothelial neutrophil migration and pulmonary neutrophil infiltration.[77] The above studies demonstrate the importance and diversity in the roles of neutrophils during sepsis. During sepsis, increased amount of NO lowers the neutrophil migration by inhibiting the process of rolling and adherence to the endothelium of blood vessels.[78] On the other hand, NO is also reported to increase IL8 amounts, a potent chemoattractant and an activator of neutrophils.[79] These results illustrate that the functions of NOS2 may vary depending on the cellular location, temporal expression and type of inflammatory responses. During *S.* Typhimurium-peritonitis-induced sepsis in mice, it is seen that complete abrogation of NOS2-derived NO leads to higher liver tissue damage and drop in myocardial function. Organ protective functions of NOS2-derived NO during sepsis is further established with reduced liver damage and improved myocardial functions upon exogenous NO supplementation.[14] In the field of sepsis, abundant literature centers around NO and sepsis, however, the specific roles of NO in the pathophysiology of sepsis are controversial. Inhibition of NO, although, improved the hemodynamic parameters, it failed to improve the mortality index, whereas NO donors improved the microcirculatory defects but lowered the blood pressure of septic patients.[80] NO donors, such as linsidomine or molsidomine (SIN-1), are known to improve the mesenteric blood flow, cardiac index and myocardial dysfunction in fluid-resuscitated dogs during early phase of endotoxic shock.[44] Cardiomyocytes show higher expression of NOS2 with increased amounts of released NO during sepsis.[81] During *S.* Typhimurium peritonitis-induced sepsis, the lack of NOS2 leads to a significant drop in percent of ejection fraction and fractional shortening of heart functions, which was improved upon exogenous NO supplementation.[14] Possibly, the targeted increase in the availability of NO during the onset of sepsis

has beneficial effects on microvascular dysfunction.[82]

NO plays a central and a pleiotropic role in maintaining the microcirculatory integrity and function. Distraught leukocyte trafficking, activation of coagulation and endothelial damage altogether contribute to microcirculatory dysfunction during sepsis.[82] The increase in plasma levels of nitrite and nitrate in sepsis patients has been observed and is implicated in destabilization of vascular hemodynamics.[83-85] Importantly, cytokine-mediated vasodilation in humans is not due to NOS2 but due to the induction of tetrahydrobiopterin leading to the activation of NOS3.[86] It would be relevant to mention the failure of clinical trials for the treatment of sepsis, using LNMA, the NOS inhibitor.[87, 88] Later, efforts were made to improve the deranged microcirculation during sepsis by increasing the availability of NO by exogenous NO donors. Intravenous supplementation of septic shock patients with NO donors, such as nitroglycerine leads to improvements in microcirculatory defects.[89] Overall, the application of NO donor during sepsis, despite lowering the blood pressure increases the organ blood flow and tissue oxygenation. The resultant low blood pressure due to NO can be treated efficiently since in intensive care units of hospitals, patients are continuously monitored for their hemodynamic parameters. Therefore, further clinical trials of sepsis patients with NO donors may be performed.[80] Interestingly, a combination of NO donor to substitute for the physiological concentration of NOS3-derived NO in blood vessels and NOS2 specific inhibitor to prevent the excessive production of NO can be examined together.[82] Accordingly, in *S.* Typhimurium infection-induced sepsis supplementation of *Nos2*$^{-/-}$ mice, but not wild type C57BL/6 mice with NO donor leads to improved survival and lesser liver damage.[14]

Highlights and summary

In conclusion, oxidative stress, due to increased amounts of ROS and NO, plays complex roles in the pathogenesis of sepsis. In addition to their rapid reactivity with cellular components, these reactive molecules can also participate in signaling and initiate a variety of inflammatory pathways. Increased amounts of ROS and the subsequent mitochondrial dysfunction can altogether lower the levels of ATP, culminating into cellular apoptosis and multi organ failure. Although antioxidant therapy has proven to be beneficial in treating sepsis and shock patients, further studies are required to evaluate whether the use of antioxidants can be incorporated into the current treatment regime for sepsis and its advanced forms.[24,29] It may be favorable to administer antioxidants that can protect the mitochondria from oxidative stress and prevent organ damage caused by mitochondrial dysfunction. However, despite the promising results obtained with the use of antioxidant therapy in sepsis patients, very limited data is available presently with respect to mitochondria-specific antioxidants like ubiquinone, mitoquinone and mito-tempo.[24]

The levels of NO during sepsis are critical basically because of two reasons: First, NO regulates systemic blood circulation by regulating smooth muscle relaxation and vasodilation. Hence, excess of NO during sepsis results in hypotension and septic shock. On the contrary, NO is also crucial for maintenance of microcirculation leading to efficient tissue oxygenation and nutrient supply to tissues and organs. Secondly, NO exhibits anti-microbial effects and lowers microbial growth during infections. Therefore, during sepsis, NO exhibits heterogeneous effects and acts like a double-edged sword. Excessive production of NO can lead to hypotension, whereas lower NO levels can result in poor organ perfusion and greater sensitivity to infections. Careful examining of the intrinsic levels of NO before and after treatment, as well as the stage of sepsis patients need to be considered before the application of NO-modulating agents. However, due to its very short half-life, direct NO measurements have always been a challenge. This has prompted the use of indirect markers of NO bioavailability, which include NOS substrates like L-arginine and homoarginine.[90] Studies in different *in vivo* models with various parameters are necessary before the concept of antioxidant therapy or NO-modulating agents can be extended to clinical trials with sepsis patients. Overall, future trials with antioxidants and NO modulating agents will need to consider the diverse and complex roles these molecules play.

Competing interests

The authors declare that they have no competing interests.

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