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The role of free radicals in sepsis development

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1. ABSTRACT

Sepsis is a complex inflammatory syndrome with diverse etiology and wide spectrum of severity. Several researchers have linked reactive oxygen species (ROS) and reactive nitrogen species (RNS) with the onset, progression and outcome of sepsis, both in pre-clinical and in clinical studies. ROS/RNS are important signaling molecules but its overproduction must be avoided by organism, otherwise oxidative stress takes place. Even so, the use of antioxidant as treatment in sepsis constitutes a challenge, with both null and encouraging results. In this review, it will be summarized the role of free radicals in the onset, progression and outcome of sepsis, as well as its participation in organ failure and cardiovascular collapse. Experimental treatments that may interfere in oxidative stress in sepsis will also be contemplated.

2. INTRODUCTION

Sepsis is a complex inflammatory syndrome with diverse etiology and wide spectrum of severity, becoming the most common cause of mortality in intensive care units, mainly among elderly, immunocompromised and critical ill patients (1). Sepsis is diagnosed by signs of systemic inflammatory response in presence of an infectious process. Severe sepsis occurs when sepsis is accompanied by organ failure, hypoperfusion or hypotension. In case of persisting hypotension despite the adequate fluid resuscitation, sepsis is called shock septic (2).

Several molecular mechanisms of inflammation and cellular damage have been implicated in the pathogenesis of sepsis, septic shock, and multiple organ failure, including those related to the overt generation of

cytokines, eicosanoids and reactive oxygen species (ROS) and reactive nitrogen species (RNS) (3,4,5). The heterogeneity of sepsis may be caused by the type of infectious agent and genetic background of the host, leading researchers to question the prevailing theory that sepsis is caused by an uncontrolled inflammation (6).

ROS/RNS are important molecules involved in the maintenance of body homeostasis. In immune cells they can act as chemical weaponry and on vascular system regulate blood pressure (7,8). Moreover, ROS/RNS are important intra and intercellular messenger (9,10). ROS are signaling molecules that promotes NFkappaB translocation, adhesion molecules expression and release of pro-inflammatory cytokines (11). They can be generated by several pathways, such as enzymatic (NADPH oxidase, xanthine oxidase, myeloperoxidase, cyclooxygenase), non-enzymatic (Fenton reaction) and during mitochondrial electron transport chain activity. Healthy organisms are equipped with an arsenal to buffer ROS/RNS production. This arsenal is called antioxidant system and is composed by enzymatic (e.g. catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic (e.g. glutathione, bilirubin, vitamins, glutathione) defenses. When body produces more ROS/RNS than the buffering capacity, the oxidative stress takes place and can be detected by its by-products (lipid, protein and DNA oxidatively/nitrosatively modified).

Several mediators of sepsis progression, such as LPS, IL-1, IL-6 and TNF-alpha are able to increase free radicals generation (11,12). In addition, the release of some cytokines is stimulated by ROS signaling (11). Sepsis and endotoxemia have been described to induce imbalance between free radicals generation and its consumption by antioxidant defenses, thus creating a state of oxidative/nitrosative stress to the host. Plasma analyses revealed that non-enzymatic antioxidants, such as tocopherol, retinol, beta-carotene and lycopene were diminished in septic patients whereas thiobarbituric acid-reactive substances (TBARS), an index of lipoperoxidation, were increased (13). In addition, it was demonstrated that despite initiating in low levels, plasma total antioxidant capacity increased over time in non-survivors achieving supra normal levels (14). Pascual also have found higher values for plasma antioxidant capacity in septic shock patients than controls, whereas septic patients presented lower levels (15). This increase in non-enzymatic plasma antioxidant could be related to tissue damage and xanthine oxidase activity, since bilirubin and uric acid were the main contributors to the antioxidant potential in one study (15). Moreover, de Vega have shown direct correlation between plasma lipoperoxides and APACHE III score, and an inverse correlation between plasma total antioxidant capacity and APACHE III (16). These correlation analyses were all done using plasma markers of oxidative stress, which do not necessarily mirror the state of each independent organ.

In order to study the response of individual organs against sepsis challenge, animal models constitute important tools (17). In the late 80s and mainly in 90s researchers boosted studies about free radical participation in animal model of sepsis. At this time, some studies, mainly about respiratory failure and cardiovascular system started to arise, as well as the first proposals of antioxidant treatment (18). In line with this, our group systematically analyzed the major organs systems involved in the onset and progression of this syndrome using a well-accepted model of animal sepsis, namely cecal ligation and puncture (CLP) (19). Based on these results we also postulated an antioxidant treatment, which was able to reduce mortality rates in 22% (5). Animal models provide the possibility of analyzing organs independently and we have recently verified that biochemical markers of organ failure were strongly correlated with lipid oxidative damage and superoxide dismutase (SOD)/catalase (CAT) ratio activity in rats subjected to CLP. Moreover, both biochemical markers and oxidative damage were decreased with antioxidant administration, which suggest a link between both events (non-published results).

Inflammatory mediators in sepsis, such as LPS, TNF-alpha, IL-1beta and IL-6 are able to induce NADPH oxidase assembling, xanthine oxidase activity and mitochondria impairment, with consequent superoxide release from cells of immune system and vascular system. Thus, at the onset of sepsis the body can produce high amounts of ROS/RNS. In fact, our group and others researchers have shown that ROS production and oxidative damage occurs early in sepsis and may be used to predict mortality (19). Superoxide is a weak ROS but can activate NFkappaB translocation, endothelial apoptosis, endothelial nitric oxide synthase (eNOS) suppression, increased tissue factor expression and inflammatory process (20). Moreover, it can react with nitric oxide (NO) and generate peroxynitrite ($ONOO^-$) which is very harmful, since it can react with protein, lipids and DNA causing loss of function and energy depletion (21,22,23). Superoxide concentration is held in safe levels mainly by the action of SOD, which converts it to H_2O_2. Catalase and glutathione peroxidase (GPx) reduce H_2O_2 into H_20. In rats subjected to sepsis there is an imbalance between the H_2O_2 production and its consumption in favor of the former. The consequence is a "peroxide overload" and the possibility of hydroxyl generation through the Fenton reaction.

In this review, it will be summarized the role of free radicals in the onset, progression and outcome of sepsis, as well as its participation in organ failure and cardiovascular collapse. Experimental treatments that may interfere in the oxidative stress in sepsis will also be contemplated.

3. ROS AND OXIDATIVE DAMAGE IN SEPSIS - EARLY MEDIATORS AND LATE EFFECTORS

The increase in free radicals production occurs early in sepsis, since LPS induces oxidative burst and macrophages employ ROS to kill pathogens. This early steps may be decisive to the outcome of sepsis and several antioxidant protocols in preclinical models present effectiveness just when administered prior or soon after the sepsis induction (see table 1). This approach raise the

Table 1. Preclinical and clinical trials with antioxidant compounds.

Model	Antioxidant protocol	Main results	Ref
Endotoxemic pigs	Vitamine A, 30 min after	Decrease lung edema	[97]
Endotoxemic pigs	Vitamine A, 1h before	Improve cardiorespirtory function	[98]
Endotoxemic pigs	Vitamine A, before	Improve cardiac index, decrease endotoxin levels	[99]
CLP rats	Vitamine A, supplemented chow	Maintenance of WBC count	[100]
Endotoxemic mice	Antioxidant (polyphenols, vitamine C, E and trace elements) enriched diet, 7 days before	Improve survival and decrease plasma oxidative damage	[101]
Bacteremic rats (*S. pneumonia*)	Antioxidant (zinc) enriched diet, 3 days before up to 1 day after	Decrease Caspase-3 and 8 in cardiac tissue	[102]
CLP rats	Vitamine C (i.v.) immediately after CLP	Restore GSH/GSSG ratio, improve liver function	[103]
CLP rats	Vitamine E (i.p.), for 3 days before CLP	Restore GSH/GSSG ratio, improve liver function	[103]
Endotoxemic rats	TMG (water soluble vit E analog - i.v.), just before LPS injection	Improve survival, decrease pulmonary edema	[104]
CLP rats	Silymarin (oral), for 10 days before	Improve survival, decrease TNF, IL-1b, IL-6, LDH	[105]
CLP rats	NAC (oral), for 10 days before	Improve survival, decrease TNF, IL-1b, IL-6, LDH	[105]
Bacteremic rats (*K. pneumonia*)	NAC (i.v.), 1h after	Improve vascular function, decrease organ injury	[106]
Bacteremic rats (*E. coli*)	M40401 (SOD mimetic - i.v.), 3h after	In low mortality group (<66%) M40401 worsened survival whereas in high mortality group (>66%) M40401 improved survival	[26]
Endotoxemic rats	M40403 (SOD mimetic) - i.v.), 1 or 5h after	Reverse the fall in MAP, avoid adenochrome formation	[27]
CLP rats	NAC plus deferoxamine (subcutaneous), 3 hours after	Improve survival, avoid oxidative damage	[5]
ICU patients	NAC (i.v.)	Treatment initiated within the first 24h of internation improves survival. After this time, NAC worsened survival.	[24]
Septic patients	NAC (i.v.), treatment initiated within the firsts 12h after fullfilling sepsis criteria	Decrease NFkappaB activation, IL-8. Mortality was not described.	[25]

Abbreviations: CLP: cecal ligation and puncture; i.v.: intra venous; i.p.: intra peritoneal; GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; NAC: n-acetylcysteine

problem that virtually no patient can receive antioxidant treatment before the onset of sepsis, with the exception of the high-risk patients who are already in the hospital and can enter in a prophylactic protocol. Most patients arrive at the hospital fulfilling criteria for sepsis and antioxidant treatment only starts at this moment. Fortunately, free radicals also participate in the course of sepsis and some antioxidant treatments initiated after the sepsis onset succeed. Good examples in preclinical models are N-acetylcysteine (NAC) (24,25), SOD-mimetics (26,27) or those employed by our group, using a combination of NAC and deferoxamine (5). The late effects of ROS are closely linked with energy depletion, as we will discuss later herein.

3.1. Oxidative stress and mitochondrial damage, an energy-taking process

For many years, it was assumed that impairment of oxygen delivery to the tissue was the main cause of acidosis and organ failure during sepsis. However, endotoxemic animal models which have perfusion maintained at normal levels still presented acidosis (28). Some works have postulated that impairment in mitochondrial aerobic ATP production in multiple organ dysfunction is compatible with the cytopathic hypoxia hypothesis (29). In fact, investigation of mitochondrial structure after LPS insult revealed an increased oxidative damage, GSSG/GSH ratio and decreased oxidative phosphorylation relative to control group (30,31). Moreover, mitochondrial injury leads to the opening of permeability transition pore and organelle swelling, with the loss of electrochemical gradient and deficiency in ATP production (31). The pore also allows cytochrome c to be released into the cytosol where it interacts with the adaptor molecule Apaf-1 resulting in the activation of pro-caspase-9. Caspase-9 then cleaves and activates pro-caspases-3 and -7, which in turn are responsible for the biochemical and morphological changes characteristic of apoptosis (32,33). The close relationship between free radicals and mitochondrial dysfunction during sepsis becomes clearer considering that antioxidant treatment in rats subjected to CLP was able to avoid mitochondrial swelling and cytochrome c release (32).

Free radicals and RNS, mainly peroxynitrite, are able to cause single strand breakage to DNA (34). This kind of damage is a signal to Poly (ADP-Ribose) Polymerase (PARP) activation, which catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and then uses the latter to massive ribosylation of nuclear proteins. PARP activation causes energy depletion and can be related to cytopathic hypoxia discussed above. *In vitro* studies have shown that pharmacological inhibition of PARP improves the response of mitochondria and aortic ring against peroxynitrite challenge (35).

4. FREE RADICALS IN IMMUNOLOGIC RESPONSE – A DOUBLE EDGED SWORD

4.1. Free radicals as a bless

Macrophages and neutrophils are cells of the innate immune system, our first defense line against invading microorganism. For this purpose, they are equipped with a myriad of oxidant molecules, such as superoxide, hydroxyl, peroxynitrite and hypochlorous acid (7). Moreover, the hypochlorous acid reaction with alpha-amino acids yields reactive aldehydes, which can act as antimicrobial agent (36). The lack of this oxidant system can be deleterious to the host and this has been demonstrated in animal deficient in superoxide generation. Mice deficient in NADPH subunits $p47^{phox}$, $gp91^{phox}$ and chronic granulomatous disease patients, who also present a

null NADPH oxidase activity, are incapable to adequately perform the respiratory burst and bactericidal functions (37,38).

TNF-alpha, IL-1 and IL-6 are well known inducers of free radicals generation, as well as of antioxidant adaptation. SOD can be stimulated by LPS, TNF-alpha and IL-1 (39,40), providing an adaptive environment to avoid oxidative stress. Other molecules act in immune cells and promote either free radicals generation or antioxidant adaptation during sepsis. They can be exogenous molecules, such as lipoteichoic acid, flagelin or endogenous molecules, such as HMGB1, oxidized phospholipids and Activated Protein C (APC) (41,42,43).

In the course of sepsis, large amounts of damaged molecules are generated in organs and in plasma as a consequence of ROS and RNS generation, leading to widespread oxidative and nitrosative stress, respectively (19). Recently it has been published a protective role of oxidized phospholipids fatty acids. These molecules are able to avoid the LPS-induced inflammation and reduce mortality in endotoxemic mice, thus acting as a negative feedback in order to keep inflammatory process under control (42).

APC regulates the coagulation system by a proteolytic inactivation of activated forms of coagulation factors V and VIII. In addition to its anticoagulant activity, APC possesses anti-inflammatory properties and is known to inhibit the release of inflammatory cytokines, such as TNF-alpha in experimental animals challenged with endotoxin. Some of these activities are thought to be due to the ability of APC to inhibit NF-kappaB nuclear translocation, which is needed to start gene transcription. It has been suggested that APC action requires endothelial cell protein C receptor (EPCR) to exert its anti-inflammatory and antioxidant effects (44). However, APC showed EPCR-independent anti-inflammatory properties in human monocytes and this effect is believed to be mediated by APC intrinsic antioxidant activity (43,45).

4.2. And as a curse

So far we have discussed the important and necessary role of free radicals in immune system. However, if cells are excessively stimulated, they can contribute to injurious reactions through ROS/RNS, proteolytic enzymes and pro-inflammatory mediators. If this overwhelming inflammatory response is sustained, it can lead to progressive organ dysfunction.

Stimulated macrophages and monocytes are able to secrete HMGB1, a nuclear protein that mediates the delayed effects in sepsis and endotoxemia and exerts its effects in host cells through membrane receptor binding (46). Some of the characterized receptors for HMGB1 are RAGE, TLR-2 and TLR-4. HMGB1 causes epithelial cell barrier dysfunction, lung injury, fever and lethality, but not shock, and these effects are mediated in part by peroxynitrite generation (41).

The ability of macrophages to produce ROS can become a trouble to deal with. It happens because some ROS, such as H_2O_2, act as signaling molecules, and can act in the nearby cells, disseminating the inflammation process and leading to multiple organ failure. NF-kappaB is a nuclear factor involved with pro-inflammatory steps (stimulation of IL-1, IL-2, IL-6 and TNF-alpha) and its over stimulation leads to a "cytokine storm" and systemic inflammation. Moreover, NF-kappaB controls the expression of genes encoding chemokines (e. g. IL-8, MIP-1alpha, MCP1, RANTES, eotaxin, etc.), adhesion molecules (e. g. ICAM, VCAM, E-selectin), inducible enzymes (COX-2 and iNOS), growth factors, some of the acute phase proteins, and immune receptors, all playing critical roles during the control of inflammatory processes (47). Besides H_2O_2, neutrophils and macrophages are equipped with powerful enzymatic machinery to defeat invading pathogen and myeloperoxidase is part of this arsenal. This enzyme converts H_2O_2 plus chloride ions in hypochlorous acid (HOCl), a strong oxidant and microbicide agent. As side effect, HOCl can react with host molecules and participates in pathology progression. It is well documented the participation of neutrophils in Acute Respiratory Distress Syndrome (ARDS) (48) and atherosclerotic lesions (49). One of the postulated mechanisms is the formation of Advanced Glycation End Products (AGE), which arise from the reaction of proteins with glucose, glycolytic intermediates and other reactive aldehydes, that could be generated in lipid peroxidation and metal catalyzed protein oxidation (50). Recently, it was demonstrated that HOCl can react with free serine and generate N- (carboxymethyl)lysine (CML), a well characterized AGE (36). Besides causing protein malfunctioning, AGE can act as a signaling molecule, binding to receptors, such as Receptor for AGE (RAGE), galectin-3, Class A macrophage scavenger receptor (SR-A), and CD36 (51).

AGE can activate NF-kappaB and then promote increase in VCAM-1, VGEF, tissue factor, TNF-alpha and IL-8, apoptosis, procoagulant activity and platelet aggregation (52,53,54,55), which mirror some clinical conditions seen in atherosclerosis and diabetes (56,57).

5. FREE RADICALS IN CIRCULATORY DYSFUNCTION

Cardiovascular system plays an important role in sepsis, since it can define the progression in the most lethal form: septic shock. Because of this, several studies have focused on the role of free radicals on vessels (endothelial cells and smooth muscle), on heart collapse and on disseminated intravascular coagulopathy (DIC).

5.1. Nitric Oxide, vasodilatation and catecholamine oxidation by superoxide

Septic shock is defined by severe hypotension and decreased perfusion to critical organs despite the increasing levels of endogenous catecholamines and a further lack of response to both endogenous and exogenous administered catecholamines (2).

NOS can be stimulated by several mediators present in sepsis, such as LPS, TNF-alpha, HMGB1 among

others and contributes largely to hypotension seen in shock. The confirmation of its importance comes from iNOS knockout models of sepsis and endotoxemia. Animals lacking the inducible form were more resistant to pressure fall, had the survival improved after endotoxin challenge and presented inhibition of P-CAM and E-CAM expression (58,59).

Despite this, pharmacological interventions have not worked as expected. The main fault in this approach is the use of non-selective NOS inhibitor, which impairs the inducible as well as the constitutive form eNOS. Constitutive NOS is important to maintain blood flow, to avoid microvascular thrombosis, platelet aggregation and leukocyte adhesion (60,61). The design of new drugs, with specific inhibitory activity on the inducible form of NOS, is necessary to achieve better results. In this way, some comparative studies have arisen with advantages for selective inhibition drugs such as L-canavanine, aminoguanidine, isothiourea, S-methilisothiourea and recently, statins (61,62).

So far, the standard clinical approach is to keep pressure with the help of volume resuscitation and catecholamine administration. Catecholamines in circulation can undergo a superoxide-dependent deactivating process and have its vasoconstrictor activity broken down (3,4). Moreover, adrenochromes (the oxidized form of catecholamines) are believed to be cytotoxic (63,64). Based on these facts, Macarthur suggested a pharmacological approach by administering a SOD-mimetic which could avoid catecholamine oxidations as well as avoid other harmful effects of superoxide, such as the generation of peroxynitrite (3). In line with this, transgenic mice that overexpress CuZnSOD have shown decreased superoxide production in aortic rings cultured with LPS *ex vivo*, as well as improved carotid response to acetylcholine (65).

Curiously, our group found that early increased SOD activity is predictive of mortality in rats subjected to CLP model of sepsis (19). The origin of plasma SOD in our study is uncertain but it can come from damaged organs and works as a general organ failure marker, since tissue SOD are highly active in sepsis (19) and extracellular SOD seems to be diminished in endotoxemic mice and in smooth muscle cells treated with TNF-alpha (66,67).

5.2. Endothelial damage

Damage to vascular wall may cause unresponsiveness to vasoconstrictors, increased permeability to immune cells and pathogens being the main cause of respiratory failure, kidney damage and cardiovascular collapse (68). ROS and RNS participate actively of this process, acting either as a causative agent as a mediator (a signaling molecule). Moreover, at the end of the events, more ROS/RNS are generated as consequence.

One important mechanism of endothelial cell damage is the activation of PARS by peroxynitrite. This enzyme acts after DNA injury and its over activation causes massive ADP-ribosylation of nuclear proteins, leading to energy depletion and cellular injury (22). Szabó demonstrated that endothelial cells treated with peroxynitrite have a decrease in mitochondrial activity and a concomitant increase in PARS activity, which could be reversed with a pharmacological PARS inhibitor. In spite of this protection, the treatment was not able to avoid delayed apoptotic process initiated by peroxynitrite and the main cause seems to be the peroxynitrite-mediated DNA strand breakage, which can not be protected by PARS inhibitor (22). Other extra nuclear effects of peroxynitrite may be involved, such as lipid and protein oxidation and nitration, direct inhibition of mitochondrial respiration and inhibition of membrane pumps (68,69).

Recently, Boulos extended these findings treating human umbilical vein cells with serum from septic shock patients. The treatment caused a decrease in mitochondrial respiration and ATP levels compared with cells treated with serum from normal controls. Mitochondrial impairment could be reversed with both 3AB and L-NMMA, indicating a role for PARS and iNOS, respectively (69).

5.3. Coagulophaty

The hemostatic system is composed by plasmatic (coagulation cascade) and cellular constituents (platelets, leukocyte, endothelium) and has the objective of preserve intravascular integrity by achieving a balance between hemorrhage and thrombosis.

Coagulopathies are a common event in septic patients, being associated with organ failure and mortality in septic shocked patients (70) and in trauma patients (71). Even so, virtually all patients with sepsis show a procoagulant trend, since LPS, activated monocytes and neutrophils can trigger coagulatory process (72). Once the hemostasis is lost, DIC may evolve, resulting in widespread microvascular thrombosis (because of excessive clotting) and enhanced bleeding (because of depletion of clotting factors).

The systemic coagulation implies in vascular changes as vasodilation, increased capillary permeability and up-regulated cell adhesion, events that are mediated by the activation of several cell types including macrophages, neutrophils and endothelial cells (73).

Coagulation cascade can be didactically split in two pathways, namely extrinsic and intrinsic pathways. Extrinsic pathway is activated by the Tissue Factor (TF) that is released in the blood when endothelial damage happens. Intrinsic pathway can be activated by compounds already present in blood, and acts as an amplifier of coagulatory process. Both pathways converge to the activation of thrombin, which in turn, cleaves fibrinogen to generate the fibrin network (73). Free radicals can promote TF expression and activity in several cells, such as in aortic and pulmonary artery smooth muscle cells, endothelial cells, monocytes, isolated rabbit hearts subjected to ischemia followed by reperfusion. A variety of antioxidants are able to abolish the prothrombotic effects of free radicals but the site of free radical production remains to be elucidated (74). Some evidences point to NADPH oxidase,

since treatment with its inhibitor, diphenylene iodonium, was able to decrease TF expression in vascular smooth muscle cells stimulated by activated platelets, whereas xanthine oxidase and cyclooxygenase inhibitors have minimal effects (75).

Besides the role in early steps of coagulation, free radicals can act on thrombin activity and fibrinogen structure, with consequences for clottability and fibrinolysis. Shacter demonstrated that when the whole plasma is subjected to a radical generating system, fibrinogen becomes highly oxidized (76). Isolated fibrinogen subjected to a Fe^{2+}/ascorbate system presents impaired clottability that can be attributed to a decreased rate of fibrinopeptide A release. As consequence, fibrin polymerization goes slowly and the network is more branched and presents thinner fibers (77). Another relevant RNS in sepsis, peroxynitrite, was also tested and showed to impair fibrinocoagulation (78). Looking these data one could wonder that both ROS and RNS act in an anti-thrombotic way. Even so, we must pay attention in the consequences of the impaired fibrinocoagulation. Low rate of polymerization is associated with formation of branched and thinner fibers, which cause resistance to fibrinolysis (78). Taken together, these data suggest that ROS/RNS can trigger the coagulatory process and that the thrombus generated can not be easily removed. This scenario may contribute to DIC and organ failure seen in sepsis.

Another kind of posttranslational modification in plasma proteins is non-enzymatic glycation, which can be stimulated by oxidative stress, poor glycaemic control and action of MPO on plasma amino acids, all characteristics common in sepsis syndrome. In diabetics patients it was determined that fibrinogen presents higher levels of glycation than control subjects. The consequence is decreased lysis rate that can contribute to prothrombotic events seen in diabetes (79,80). Unfortunately, there are no studies investigating the role of fibrinogen glycation in sepsis so far. The lack of knowledge in this field has slowed the employment of anti-AGE drugs in preclinical studies.

6. IS THERE A PLACE TO ANTIOXIDANTS IN SEPSIS TREATMENT?

Since it became established that ROS and RNS participate in the onset of sepsis, as well in its progression and outcome, researchers have hypothesized that antioxidant therapy could restore the patient health. However, scientific literature presents several contradictory results, some of them summarized in the present review, which lead us to reason that ROS/RNS are a double-edged challenge. The most important pitfall in antioxidant treatments postulated to sepsis appears to be the short therapeutic window, as mentioned before (table 1). Furthermore, promising animal studies do not get support from clinical tests. Even so, some antioxidant therapies have succeeded in improving survival in clinical studies whereas others still await the chance to be tested.

6.1. SOD and SOD mimetic

Superoxide can react with catecholamines causing its deactivation and thus contributing to septic shock. Moreover, it can react with nitric oxide generating $ONOO^-$ which leads to protein nitration, DNA damage and contributes to multiple organ failure. To avoid these deleterious effects, SOD reduces superoxide to H_2O_2, which in turn is potentially more harmful than the very superoxide, since it can generate hydroxyl radical. Rats subjected to CLP presented increased superoxide generation and SOD activity as well, but catalase did not increase in a compensative manner (81). This imbalance between these enzymes could be responsible for the oxidative damage seen in this model. Conversely, some studies have shown good results with SOD and SOD mimetic administration (82). As showed by Macarthur, MK40403 (a synthetic and selective Mn-centered SOD mimetic) was very efficient to prevent catecholamines oxidation and to hold arterial pressure in rats challenged with LPS (3). Manganese-based metalloporphyrin complexes, such as Mn (III)tetrakis (4-benzoic acid)porphyrin (MnTBAP), and Mn (III)salen complexes, such as EUK-8 and EUK-134 are non-specific superoxide scavengers that scavenge multiple ROS. Thus, the use of SOD mimetic with broad antioxidant activity could circumvent H_2O_2 overload as consequence of SOD hyperactivity and has shown efficiency in some models of endotoxemia (83,84,85).

6.2. N-acetylcysteine and Deferoxamine

N-acetylcysteine (NAC) is a thiol compound that possesses antioxidant power against a variety of ROS (86) and acts as precursor in glutathione synthesis. Its beneficial effects seen *in vitro* and in cell culture suggest that thiol compounds would be useful in the treatment of free radicals associated pathologies. Endotoxemic rats treated with low doses of NAC (275mg/Kg in 48 h) presented improved survival rates and decreased circulating H_2O_2 whereas high doses of NAC (950mg/Kg in 48 h) worsened mortality and increased H_2O_2 levels (12). These deleterious effects are attributable to ability of thiol compounds undergo oxidation in presence of transitional metal and generate thiol-centered radicals which can, in turn, reduces Fe (III) to its catalytically active form Fe^{2+}. *In vitro* studies revealed that both GSH and NAC increase H_2O_2/Fe^{2+} mediated lipid peroxidation due to the formation of thyil radical (87).

As the transitional metals are important in these pro-oxidant effects of thiol antioxidants, we reasoned that an iron chelator would improve the beneficial effects. Thus, in a well-characterized rat CLP model, with mortality rate of 95% in 5 days, we administered NAC (20mg/Kg each 6 h for a total of 3 days) plus deferoxamine (20mg/Kg each 12 h for a total of 3 days) 3 hours after the surgery. NAC or deferoxamine alone were not effective but when administered concomitantly, mortality decreased to 40% (5). Not surprisingly this association has been effective in several models of inflammation, such as acute hepatic failure (88), acute lung injury (48) and in a model of lung damage after coal dust inhalation (89).

6.3. Carbonyl trap and AGE breakers

Aminoguanidine (AMG) is a highly nucleophilic molecule that reacts with alpha-oxoaldehydes, thus acting as an aldehyde scavenger, avoiding the generation of a heterogeneous class of molecules called AGE. AMG has been employed mainly in diabetes models with some success in avoiding nephrophaties, vascular and lens degeneration (90). The role of AGE in sepsis has been underestimated but some studies start to describe the importance of these molecules, as well as of its receptors (RAGE) (91,92). Besides the role of AMG as anti-AGE, it is also an iNOS inhibitor and metal scavenger. Thus, it may help to maintain vascular tone and avoid metal-catalyzed oxidative damage (90). However, AMG has been shown weak clinical effects and has been rising safety concerns in diabetic patients (93,94), which suggests that new drugs must be developed. In this line, pyridoxamine has arisen as an alternative. Pyridoxamine is one of the three forms of vitamin B6, which is necessary to cysteine synthesis (the rate-limiting precursor for GSH synthesis and have anti-AGE activity (90). This molecule has been effective in both animal model of diabetes and in clinical trial with diabetic nefropathy patients (95,96). Despite this optimistic view about anti-AGE agents, few studies have investigated them in sepsis syndrome.

7. PERSPECTIVES

Several good revisions about sepsis and free radicals have been published recently but we feel that researchers have not discussed some important points concerning the role of free radicals in coagulation, as well as the role of AGE in sepsis. The main goal of this review was to enlighten the reader about some points that, in our opinion, have been overlooked. The role of ROS/RNS in the sepsis coagulopathy and AGEs in sepsis signaling are examples of incipient knowledge that can provide information to the development of new therapies. Moreover, antioxidant treatments are still waiting for clinical trials.

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