Mechanism of action of vitamin C in sepsis: Ascorbate modulates redox signaling in endothelium

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Abstract.

Circulating levels of vitamin C (ascorbate) are low in patients with sepsis. Parenteral administration of ascorbate raises plasma and tissue concentrations of the vitamin and may decrease morbidity. In animal models of sepsis, intravenous ascorbate injection increases survival and protects several microvascular functions, namely, capillary blood flow, microvascular permeability barrier, and arteriolar responsiveness to vasoconstrictors and vasodilators. The effects of parenteral ascorbate on microvascular function are both rapid and persistent. Ascorbate quickly accumulates in microvascular endothelial cells, scavenges reactive oxygen species, and acts through tetrahydrobiopterin to stimulate nitric oxide production by endothelial nitric oxide synthase. A major reason for the long duration of the improvement in microvascular function is that cells retain high levels of ascorbate, which alter redox-sensitive signaling pathways to diminish septic induction of NADPH oxidase and inducible nitric oxide synthase. These observations are consistent with the hypothesis that microvascular function in sepsis may be improved by parenteral administration of ascorbate as an adjuvant therapy.

1. Introduction

Vitamin C (ascorbic acid) dissociates at physiological pH to form ascorbate, the redox state of the vitamin which is found most abundantly in cells [1]. It is well known that ascorbate acts physiologically as a reductant and enzyme cofactor. The purpose of the present review is to examine recent evidence that ascorbate modulates the intracellular mechanisms that cause microvascular dysfunction in critical illnesses such as sepsis.

The clinical syndrome of sepsis is not a single homogeneous disease process but a generic term for a large group of diseases [2]. Sepsis may develop as a consequence of surgery, pneumonia, soft-tissue infection associated with malignancy or peripheral vascular disease, or many other events. Sepsis syndromes range from the systemic inflammatory response syndrome to severe sepsis (acute organ dysfunction secondary to infection) and septic shock (severe sepsis plus hypotension not reversed with fluid resuscitation) [2,3]. These syndromes are the major causes of death in critical care units worldwide. The mainstays of treatment include fluid resuscitation to restore mean circulating filling pressure, antibiotic therapy and source control to remove the sepsis-inducing insult, vasopressor or combined inotropic-vasopressor therapy to prevent shock, institution of glycemic control, prophylaxis for deep vein thrombosis, and stress ulcer prophylaxis to prevent upper gastrointestinal bleeding [3]. Nevertheless, despite best medical and surgical managements, mortality remains high.

In sepsis, patients respond to whole bacteria, bacterial products such as endotoxin [e.g., *Escherichia coli* lipopolysaccharide (LPS)], and intracellular products released from injured tissues [2]. The responses include changes in microvascular function that comprise: (i) decreased density of perfused capillaries and elevated proportion of nonperfused capillaries; (ii) increased microvascular permeability (i.e., loss of barrier function) that leads to edema formation and hyperdemia; and (iii) arteriolar hyporesponsiveness to vasoconstrictors and vasodilators [4–16]. If these changes occurred only in small, localized regions of injured tissue, they might benefit the patient by lessening hemorrhage from disrupted blood vessels, delivering antimicrobial mediators and phagocytic cells to the site of injury, or preventing dissemination of toxic substances [2]. But the widespread, systemic occurrence of these changes in sepsis is recognized as microvascular dysfunction because it leads to tissue hypoxia, mitochondrial dysfunction, and ATP depletion that precipitate organ failure, even in fluid-resuscitated patients

with adequate arterial blood oxygenation and cardiac output [17]. Indeed, microvascular dysfunction is a significant predictor of death, and one-third of severe sepsis patients die of organ failure [10]. The therapeutic efficacy of antibiotics is confounded by the increasing number of infections due to multidrug resistant bacteria. Furthermore, the pathogens that are killed by antibiotics may release large amounts of toxic products (e.g., LPS) that continue to injure the patient [18]. Therefore, septic patients may benefit from adjuvant therapy that targets microvascular dysfunction.

2. Vitamin C levels in critically ill patients and relevant experimental models

Subnormal ascorbate concentrations in plasma and leukocytes are common features of the critically ill in general and of patients with sepsis in particular [19–25]. Furthermore, plasma ascorbate correlates inversely with multiple organ failure [19] and directly with survival [21].

One reason for ascorbate depletion in hospitalized, critically ill patients may be low levels of the vitamin in parenteral nutrition solutions, because of the degradation of ascorbate and dehydroascorbic acid (DHA) that occurs during preparation and storage [26,27]. Another cause of vitamin C depletion is an increased requirement for ascorbate [22,28]. The amount of vitamin C provided in standard parenteral nutrition multivitamin preparations (nominally 200 mg/day) is not adequate to normalize plasma vitamin C levels in critically ill patients, even when administered for 7 days [29]. The basis for the increased requirement may be oxidation of ascorbate by excess reactive oxygen species (ROS). By acting as a ROS scavenger and enzyme cofactor, ascorbate becomes oxidized to ascorbate free radical, which then dismutates to form DHA.

As depicted in Fig. 1, ascorbate is transported into endothelial cells by the specific sodium-dependent vitamin C transporter 2 (SVCT2), while DHA is taken up through facilitative glucose transporters (GLUTs) and then reduced to ascorbate. Ascorbate efflux from endothelial cells can be stimulated by calcium-dependent mechanisms, but these cells normally retain intracellular concentrations of ascorbate that are much higher than the extracellular levels [1,30–33]. Overall, these transport systems cause endothelial cells to rapidly accumulate millimolar levels of ascorbate that either alters intracellular function or is released in regulated ways to the extracellular fluid.

Inflammatory cytokines (tumor necrosis factor-alpha, interleukin-1beta) inhibit ascorbate uptake in endothelial cell cultures that spontaneously express SVCT2 [34]. This action may deplete intracellular ascorbate from the endothelium during sepsis. A second reason why intracellular ascorbate may be depleted is the poor control of plasma glucose, which leads to episodes of hyperglycemia in septic patients [3]. Acute hyperglycemia causes ascorbate deficiency in endothelial cells and impairs endothelium-dependent vasodilation in healthy human subjects [35]. These effects are consequences of the competitive inhibition by glucose of DHA uptake into
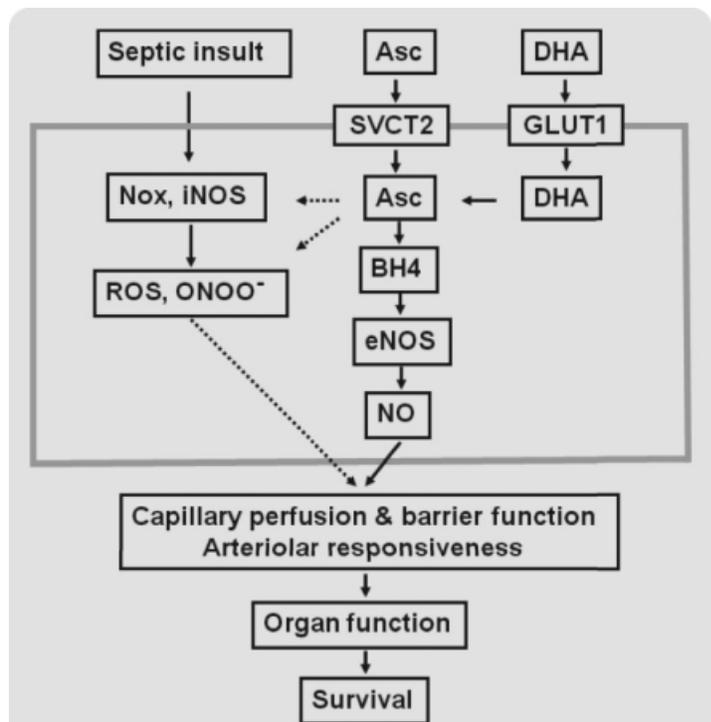

Fig. 1. Intracellular ascorbate modulates the effects of septic insult on microvascular endothelial cell function. The largest rectangle represents a microvascular endothelial cell, in which arrows with solid lines indicate stimulation and those with dotted lines indicate inhibition. Septic insult increases NADPH oxidase (Nox) and inducible nitric oxide synthase (iNOS) activities, which elevate reactive oxygen species (ROS) and peroxynitrite (ONOO⁻) levels. ROS and ONOO⁻ impair capillary blood flow, capillary barrier function, and arteriolar responsiveness to vasoconstrictors and vasodilators. Ascorbate (Asc) and dehydroascorbic acid (DHA) enter the cell through sodium-dependent vitamin C transporter 2 (SVCT2) and glucose transporter 1 (GLUT1), respectively, and DHA becomes reduced to Asc. Intracellular Asc rapidly scavenges ROS and ONOO⁻, while stimulating tetrahydrobiopterin (BH4)-dependent endothelial nitric oxide synthase (eNOS), to increase the local concentration of nitric oxide (NO). Asc also decreases Nox activity, prevents induction of the enzyme's p47phox subunit, and blocks induction of iNOS.

endothelial cells, since the impairment of vasodilation can be reversed by intravenous ascorbate (2 g bolus [ref. 36]; 3 mg/min infusion [ref. 37]). A third potential cause of intracellular depletion of ascorbate is that excessive ROS may oxidize ascorbate to DHA and then oxidize the latter irreversibly.

LPS raises ascorbate concentration in the adrenal gland, heart, kidney, and liver [38]. This phenomenon apparently does not require SVCT2, because there is no interaction between the effects of LPS and SVCT2 deficiency (SVCT2+/− heterozygote mice) on ascorbate concentration
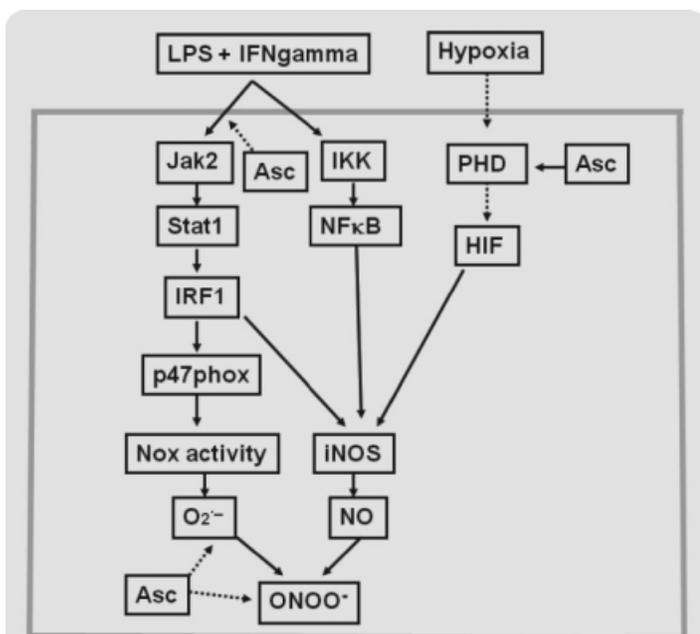
Fig. 2. Intracellular ascorbate (Asc) modulates redox-sensitive signaling pathways in microvascular endothelial cells during sepsis. The largest rectangle represents a microvascular endothelial cell, in which arrows with solid lines indicate stimulation and those with dotted lines indicate inhibition. Lipopolysaccharide (LPS) and cytokines such as interferon-gamma (IFNgamma) stimulate signaling pathways that trigger the expression of NADPH oxidase (Nox) subunit p47phox, inducible nitric oxide synthase (iNOS), and other inflammatory mediators. Hypoxia increases hypoxia-inducible factor 1 (HIF-1), by inhibiting HIF-1 prolyl-hydroxylase (PHD), and thereby induces the expression of sepsis-associated genes. Asc inhibits the activation of Jak2-Stat1-IRF1 pathway and increases the activity of PHD. Asc further regulates local nitric oxide (NO) concentration by scavenging superoxide (O_2^-) and peroxynitrite ($ONOO^-$). IKK; NFκB. Ascorbate does not affect the IKB kinase (IKK) and nuclear factor-κB that also mediate iNos induction in microvascular endothelial cells exposed to LPS and IFN gamma.

in these organs [38]. In most cell types that have been studied, the uptake and reduction of extracellular DHA to ascorbate is not impaired by LPS. On the contrary, LPS and nitric oxide donors upregulate the expression of GLUT1 in endothelial cell cultures [39,40]. Septic insults accelerate the rate at which extracellular DHA is taken up and reduced to ascorbate in multiple cell types [38,41] (although not in all, since septic insult inhibits DHA uptake in cultured astrocytes [42]).

Endothelial cells respond to LPS with increased expression of glucose-6-phosphate dehydrogenase, the key enzyme of the pentose cycle (hexose monophosphate shunt) that produces NADPH [43]. Induction by LPS of glucose-6-phosphate dehydrogenase may increase the supply of reducing equivalents from NADPH for conversion of DHA to ascorbate.

In tissue regions with nonperfused capillaries, hypoxia may inhibit hypoxia-inducible factor (HIF) prolyl-hydroxylase (PHD) and consequently increase the expression of HIFs (Fig. 2). HIF-1 increases the expression of the transporters GLUT1 and GLUT3, glycolytic enzymes, and several genes involved in inflammation [44,45]. Hypoxia stimulates DHA uptake through GLUT1 [46]. The elevated reducing power associated with hypoxia may then increase the capacity for reduction of DHA to ascorbate inside the cells.

3. Clinical trials of vitamin C in critically ill patients

As detailed later, sepsis is associated with increased production of ROS and peroxynitrite that deplete antioxidant molecules and oxidize proteins and lipids. ROS also alter redox-sensitive activation and expression of proteins that alter capillary blood flow distribution, capillary permeability (i.e., capillary barrier function), and arteriolar responsiveness to vasoconstrictors and vasodilators (Figs. 1 and 2). Therefore, patients with sepsis may benefit from adjuvant therapy that prevents the increase of ROS, particularly at intracellular signaling sites. Parenteral ascorbate may be an intervention that confers this benefit.

Administering ascorbate parenterally rather than orally increases its effects on plasma ascorbate concentration and microvascular function [1]. For instance, when oral and intravenous routes of ascorbate administration (500 mg/day for 30 days) are compared in sedentary men, only intravenous ascorbate improves endothelium-dependent arteriolar function as indicated by flow-mediated vasodilation [47].

Parenteral administration of ascorbate may decrease morbidity and mortality in critically ill patients who are septic or at risk of becoming septic. In a randomized, double-blind, placebo-controlled trial with 216 critically ill patients, 28-day mortality was decreased in the patients who received combined ascorbate and vitamin E by intravenous infusion compared with those who did not [48]. A second randomized trial with 595 critically ill surgical patients found that a combination of ascorbate (1,000 mg q8h by intravenous injection) and vitamin E (1,000 IU q8h by naso- or orogastric tube), begun within 24 h of traumatic injury or major surgery, decreased relative risk of pulmonary edema and multiple organ failure [49]. These two trials were not designed to distinguish between the actions of ascorbate and vitamin E. However, a third randomized trial observed decreased morbidity for severely burned patients who received a very high dose of ascorbate (1,584 mg/kg/day) parenterally [50]. Of particular relevance to microvascular barrier function, ascorbate treatment was associated with significant reductions in edema formation, fluid resuscitation volume, and respiratory dysfunction [50].

4. Effects of vitamin C on survival in experimental sepsis

Animal models of sepsis syndromes provide fundamental information about the potential benefit and mechanism of

action of ascorbate. Prior depletion of ascorbate decreases survival in mice injected with pathogenic bacteria [51]. Consistently, parenteral administration of ascorbate prevents hypotension and edema in LPS-injected animals [5,6,11] and it improves capillary blood flow, arteriolar responsiveness, arterial blood pressure, liver function, and survival in experimental sepsis [4,12–15,52].

Among the most clinically relevant models of polymicrobial sepsis are cecal ligation and puncture (CLP) and feces injection into peritoneum (FIP). Similar to the changes observed in septic patients, CLP in animals increases oxidative stress markers and decreases ascorbate concentration in plasma and tissue [4,12,14]. Injection of ascorbate (200 mg/kg, i.v.) increases survival in CLP mice [15]. Survival rates at 24 h post-CLP are 9% and 65% in the vehicle-injected and ascorbate-injected mice, respectively. The protective effect is not attributable to inhibition of bacterial replication at the infectious nidus, because the number of bacterial colony-forming units in peritoneal lavage fluid after CLP does not differ between vehicle- and ascorbate-injected mice [15]. In FIP mice, 24-h survival is 19% after saline vehicle injection but 50% after intravenous ascorbate injection (10 mg/kg, i.v.) [13].

5. Capillary perfusion deficit

5.1. Rapid response to ascorbate

Intravenous ascorbate injection may protect several microvascular functions, namely, capillary blood flow, microvascular permeability barrier, and arteriolar responsiveness to vasoconstrictors and vasodilators. Intravenous injection of ascorbate prevents and reverses the maldistribution of blood flow in capillaries of septic models. The effect of parenteral ascorbate is both rapid and persistent. This section discusses the mechanisms underlying the onset of the response to ascorbate.

Systemic inflammation causes stoppage of blood flow in some capillaries. In clinical sepsis, the pattern of capillary blood flow distribution improves in survivors but fails to improve in nonsurvivors [10]. Improved capillary blood flow during fluid resuscitation is associated with prevention of organ failure independently of changes in global hemodynamics [53]. Similar to clinical sepsis, CLP and FIP decrease the density of perfused capillaries and increase the proportion of nonperfused capillaries in skeletal muscles of mice and rats, despite administration of fluid for volume resuscitation to prevent shock [4,12,13].

In critically ill patients, vasodilators transiently increase the proportion of perfused capillaries [54]. Whether vasodilation by ascorbate occurs and is a direct cause of restoration of capillary blood flow in clinical sepsis are not known with certainty. However, no increase in flow velocity (measured as red blood cell velocity) is detectable in capillaries after injection of ascorbate that restores the number of perfused capillaries to normal in septic mouse skeletal muscle [13]. Therefore, the evidence from experimental sepsis studies is that restoration of capillary blood flow is not achieved through a vasodilatory effect of ascorbate. Instead, the reason why blood flow stops in some capillaries may be deficiency of nitric oxide in endothelial cells and platelets. Indeed, nitric oxide appears essential for keeping microvessels patent, and local application of a nitric oxide donor restores capillary blood flow to normal in septic mice (6 h post-FIP) [13].

The decreased availability of nitric oxide inside septic endothelial cells and platelets may be attributable to ROS. Septic insult increases the activity of NADPH oxidases that synthesize ROS in blood vessels and microvascular endothelial cell cultures [16,33,55]. Indeed, NADPH oxidase activity is the principal source for stimulated production of superoxide in microvascular endothelial cells incubated with a septic insult (a combination of LPS and interferon-gamma [IFN-gamma]; LPS + IFNgamma) [33,55]. Accelerated production of superoxide is detectable within 2 h of the cells' initial exposure to LPS + IFNgamma [33]. NADPH oxidase-derived ROS impair capillary blood flow during sepsis, since either knocking out the gp91phox (Nox2) subunit of NADPH oxidase or pharmacologically inhibiting the enzyme is sufficient to correct the maldistribution of blood flow caused by FIP in mice [13]. ROS oxidize tetrahydrobiopterin, which in its reduced form is a cofactor for enzymatic synthesis of nitric oxide. The loss of tetrahydrobiopterin (due to its oxidation) uncouples endothelial nitric oxide synthase (eNOS) in endothelial cells and platelets, so that this enzyme synthesizes superoxide rather than nitric oxide [56]. Local application of tetrahydrobiopterin restores capillary blood flow during sepsis in wild-type mice but not in eNOS$^{-/-}$ mice [13]. These observations support the hypothesis that tetrahydrobiopterin stimulates eNOS activity to increase nitric oxide production and thus reverses the maldistribution of capillary blood flow in sepsis.

Compared with vehicle injection, bolus intravenous ascorbate injection at 0, 1, 6, or 24 h after the onset of septic insult improves the distribution of capillary blood flow in CLP rat skeletal muscle [4,12]. For example, injection of ascorbate (10 mg/kg) at 6 h after the onset of septic insult reverses the maldistribution of blood flow within 10 min [13]. Ascorbate's rapid improvement of blood flow distribution during sepsis is eNOS-dependent because it occurs in wild-type, neuronal nitric oxide synthase knockout (nNOS$^{-/-}$) and inducible nitric oxide synthase knockout (iNOS$^{-/-}$) mice but not in eNOS$^{-/-}$ mice [13]. The stimulatory effect of ascorbate on nitric oxide levels in endothelial cells is attributable to multiple mechanisms. First, as shown in Fig. 1, ascorbate prevents and reverses tetrahydrobiopterin oxidation, increases tetrahydrobiopterin content, and elevates tetrahydrobiopterin-dependent synthesis of nitric oxide by eNOS, which are actions that N-acetylcysteine cannot do [7,57,58]. Second, ascorbate scavenges superoxide and other ROS that otherwise react with nitric oxide [55] (Fig. 2).

Blood flow stoppage in septic capillaries may result from interactions between leukocytes, platelets, and capillary endothelial cells. ROS activate intracellular redox signaling pathways to increase adhesion of leukocytes and platelets to endothelium [59]. Consistent with this fact, platelet adhesion is stimulated and inhibited, respectively, by locally

generated superoxide and nitric oxide during experimental sepsis [59]. Endothelial- and platelet-derived ROS also enhance platelet aggregation [60]. It is possible that formation of blood clots in microvessels after platelet adhesion and aggregation may contribute to blood flow stoppage during systemic inflammation. Intravenous injection of 2 g ascorbate enhances the inhibition of platelet aggregation by a nitric oxide donor in patients who are prothrombotic because of chronic heart failure [61]. The mechanism underlying this effect on cell adhesion may involve ascorbate inhibiting the expression and activation of NADPH oxidase, thereby preventing local scarcity of nitric oxide [13,33]. It seems likely that ascorbate has a similar antiaggregation effect in patients who are prothrombotic because of sepsis.

5.2. Persistent response to ascorbate

The capillary perfusion deficit in experimental sepsis can be mitigated for at least 12 and 47 h by ascorbate doses of 10 and 76 mg/kg, respectively [4,12,13]. Thus, microvascular effects of parenteral ascorbate persist for many hours after plasma ascorbate returns to baseline [13]. One reason for the long duration of this effect is that cells retain high concentrations of intracellular ascorbate that persist longer than does extracellular ascorbate [33]. A second reason why the microvascular response to ascorbate endures is that the vitamin alters gene expression, as discussed later.

Cells maintained under standard culture conditions often contain no ascorbate because ascorbate and DHA are either omitted from the medium or inadvertently destroyed during the preparation and storage of culture media and sera. In ascorbate-free microvascular endothelial cells, LPS + INF-gamma rapidly increases the activity of NADPH oxidase [33,55]. Endothelial NADPH oxidase synthesizes intracellular superoxide, which reacts to form other ROS (e.g., dismutation of superoxide produces hydrogen peroxide) that then induce prolonged redox signaling effects [62]. Either LPS + INF-gamma or exogenous hydrogen peroxide stimulates Jak2/Stat1/IRF1 signaling and increases expression of NADPH oxidase subunit proteins [33,55]. Thus, septic insult initiates a feed-forward mechanism to increase NADPH oxidase-derived ROS production. Incubation of microvascular endothelial cells with ascorbate raises intracellular ascorbate concentration and prevents the induction by LPS + IFN-gamma or hydrogen peroxide of endothelial NADPH oxidase activity [33]. Ascorbate also inhibits the induction of the enzyme's p47phox subunit [33]. The latter effect is mediated by the Jak2/Stat1/IRF1 signaling pathway because ascorbate prevents activation of this pathway by LPS + IFNgamma or hydrogen peroxide [33] (Fig. 2). Selectivity is shown by the fact that ascorbate inhibits superoxide synthesis by NADPH oxidase in endothelial cells [33] but not in neutrophil leukocytes [63–66].

The prolonged effect of ascorbate on microvascular function may also involve suppression of gene expression, which is dependent on HIF-1 (Fig. 2). Ascorbate acts through the PHD cofactor, iron, to increase the enzyme's activity and thereby inhibit the induction and stabilization of HIF-1alpha by hypoxia [44]. Furthermore, inhibition by ascorbate of endothelial NADPH oxidase [33] and scavenging of oxidants by ascorbate may preserve PHD activity. This is because oxidants, such as NADPH oxidase-derived ROS, inhibit PHD activity [67]. The lowering of HIF-1 levels by ascorbate inhibits expression of HIF-1 sensitive genes, such as GLUT1 and iNOS [14,15,45,55].

Activation of coagulation during sepsis is another potential cause of capillary blood flow impairment, which may be modulated gradually by ascorbate. ROS promote expression of adhesion molecules and tissue factors at the surface of platelets and endothelial cells [60]. Subsequent formation of tissue factors and factor VII complex leads to generation of thrombin that activates NADPH oxidase. This positive feedback mechanism may stimulate formation of microthrombi [60] and its abrogation may be an important mechanism by which ascorbate's improvement of blood flow distribution is sustained long enough to increase survival. Injection of a tissue factor pathway inhibitor increases survival in a CLP model of sepsis [68], which is an effect similar to that achieved by injection of ascorbate [15].

Another potential role for ascorbate is suggested by the observation that superoxide stimulates expression of cell surface intercellular adhesion molecule 1 (ICAM-1) in microvascular endothelial cells [62]. ICAM-1 mediates adhesion of leukocytes to the endothelium and may thereby impair the microcirculation. Since ascorbate inhibits superoxide production in microvascular endothelial cells exposed to septic insult [33], further research is warranted to determine if the vitamin prevents leukocyte plugging of microvessels.

6. Vitamin C and increase in endothelial permeability in sepsis

Increased permeability of the endothelium occurs in multiple organs during sepsis, leading to plasma extravasation and edema formation. This causes respiratory dysfunction, blood volume decrease, and disease progression to septic shock. Parenteral administration of ascorbate decreases edema formation in patients with severe burn injury [50] as well as in burn-injured or LPS-injected animals [5,69,70]. Ascorbate also attenuates the increase in endothelial permeability caused by LPS in vitro [71].

One reason for the loss of barrier function in sepsis may be endothelial cell apoptosis [2]. Therefore, the role of ascorbate in both preventing apoptosis in endothelial cells and stimulating their proliferation may be beneficial [72–75].

Another action of ascorbate on endothelial permeability may involve nitric oxide, superoxide, and peroxynitrite. Basal nitric oxide production by eNOS is necessary for maintenance of the endothelial barrier function (i.e., to keep the endothelium's paracellular permeability to plasma proteins low) [76]. The protective effect of nitric oxide is diminished during the inflammatory response because of simultaneous production of superoxide. Nitric oxide reacts with superoxide to form peroxynitrite, which causes lipid peroxidation, oxidation of sulfhydryl groups, and nitration of tyrosine residues

in proteins. In particular, nitration of protein phosphatase type 2 and cytoskeletal proteins by peroxynitrite appears to be a key step in the development of microvascular barrier dysfunction [77,78]. The principal sources of the superoxide are likely endothelial NADPH oxidase and uncoupled eNOS and iNOS. Evidence for the role of iNOS is that genetic or pharmacological interventions that inhibit this enzyme also decrease microvascular leakage in experimental sepsis [79]. By scavenging superoxide, inhibiting protein expression of p47phox and iNOS, and preventing superoxide synthesis by uncoupled eNOS and iNOS, ascorbate decreases the formation of peroxynitrite. Additionally, ascorbate reduces the oxidation products formed by reaction of peroxynitrite with cell proteins [80]. These actions of ascorbate may account for its effectiveness in preventing edema in critically ill patients and experimental models [5,50,69,71].

The mechanism underlying the septic induction of iNOS and its abrogation by ascorbate has been elucidated. The oxidants that arise from NADPH oxidase activity (e.g., hydrogen peroxide formed by dismutation of superoxide) enhance the induction of iNOS in septic blood vessels and endothelial cells [15,33,77]. iNOS synthesizes abundant nitric oxide, which in turn reacts with superoxide, resulting in excessive production of peroxynitrite. Ascorbate prevents the induction of iNOS by septic insults in blood vessels *in vivo* and endothelial cells in culture [14,15,81]. Ascorbate's suppression of NADPH oxidase mediates, at least in part, this inhibition of iNOS expression [55]. Upon stimulation by LPS + IFNgamma, NADPH oxidase produces ROS that activate the JNK-AP1 and Jak2-IRF1 signaling pathways of iNOS induction, and ascorbate prevents this activation [55].

7. Arteriolar hyporesponsiveness to vasoconstrictors

Hypotension in septic patients may be caused by impairment of myocardial function and by loss of arteriolar responsiveness to vasoconstrictors. Parenteral ascorbate may counter the latter problem, because infusion of ascorbate reverses arteriolar hyporesponsiveness to vasoconstrictors (norepinephrine, angiotensin, vasopressin) in human subjects who have inflammatory disease or have been injected with LPS [9,82].

Comparable results have been obtained in animal models of sepsis. For example, increased heterogeneity of capillary blood flow is followed by the development of arterial hypotension in CLP rats [12]. Arteriolar vasoconstriction and arterial blood pressure responses to norepinephrine and angiotensin II are inhibited in mice at 6 h post-CLP [14,15]. Intravenous ascorbate and iNOS gene deficiency (iNOS$^{-/-}$ mice) are equally effective in preventing the CLP-induced impairment of arteriolar responsiveness [13–15]. Arteriolar responsiveness and arterial blood pressure are higher in CLP rats injected intravenously with ascorbate, compared with those injected with vehicle, when these parameters are measured at 18–24 h postinjection [4,12].

8. Arteriolar hyporesponsiveness to vasodilators

Endothelial cells regulate arteriolar responsiveness to vasodilators through eNOS-derived nitric oxide and prostaglandin endoperoxide H2 synthase-1 (PGHS)-derived prostacyclin [83]. Nitric oxide enters arteriolar smooth muscle cells and activates soluble guanylyl cyclase, thereby raising intracellular cGMP. Prostacyclin stimulates adenylyl cyclase to raise intracellular cAMP. Both cGMP and cAMP then mediate smooth muscle relaxation. However, septic insult increases the production of superoxide, which reacts with nitric oxide to form peroxynitrite that inactivates endothelial PGHS, which can then no longer synthesize prostacyclin. Superoxide and other NADPH oxidase-derived oxidants (i.e., hydrogen peroxide and peroxynitrite) may also decrease the effective cellular level of nitric oxide below that required for guanylyl cyclase activation [83]. Thus arteriolar responsiveness to vasodilators is inhibited by LPS infusion in human subjects and by CLP-induced sepsis in animals [7,8,16].

Infusion of ascorbate or tetrahydrobiopterin prevents inhibition by LPS of endothelium-dependent vasodilation responses (assessed as changes in forearm blood flow) to acetylcholine in healthy human subjects [7,8]. This effect of ascorbate is associated with a marked increase in plasma tetrahydrobiopterin concentration [7]. Ascorbate may maintain normal levels of eNOS-derived nitric oxide and PGHS-derived prostacyclin by suppressing NADPH oxidase expression, scavenging ROS, and enhancing tetrahydrobiopterin levels within the endothelial cells of arterioles.

Parenteral ascorbate remarkably enhances arteriolar responsiveness to vasodilators in several diseases. For example, when either *N*-acetylcysteine (48 mg/min) or ascorbate (18 mg/min) is infused intra-arterially in human subjects with essential hypertension, only the ascorbate treatment enhances vasodilation by acetylcholine [58]. Recently, the topic of responsiveness to vasodilators in clinical sepsis has become controversial. Kienbaum et al. [84] reported that the acetylcholine-induced decrease in forearm vascular resistance (forearm blood flow/mean arterial pressure) did not differ between septic patients and controls. However, since the septic patients had lower vascular resistance initially, the decrease in vascular resistance caused by acetylcholine infusion may have been less in these patients. In a study of healthy human subjects before and during experimental endotoxemia, arteriolar hyporesponsiveness to acetylcholine was found 4–6 h after LPS administration, at the time when circulating cytokines are at their highest [8]. Therefore, ascorbate-sensitive arteriolar hyporesponsiveness to vasodilators may vary with time or disease severity during sepsis syndrome progression.

9. Unresolved issues meriting further exploration

There are no studies that compare ascorbate and DHA for efficacy in treating sepsis. Maximal uptake rates are higher

for DHA than ascorbate in most mammalian cell types, when studied under glucose-free conditions [1]. But glucose inhibits DHA uptake into most cells, including endothelial cells [35], and hyperglycemia that often occurs in sepsis [3] may decrease the cellular uptake and therapeutic efficacy of administered DHA.

The safety of parenteral ascorbate requires further investigation. A study of intravenous ascorbate in patients with advanced malignancies reported that injection of 1.5 g ascorbate/kg body weight three times weekly is well tolerated [85]. However, ascorbate is metabolized to oxalate, which accumulates as nephrotoxic calcium oxalate crystals (nephrolithiasis) in the kidneys of susceptible individuals, as reported in a recent case study [86]. Another concern is that ascorbate donates electrons to transition metals (e.g., iron), which then catalyze the synthesis of hydrogen peroxide. Repeated intravenous injections of 750–7,500 mg/day of ascorbate for 6 days do not induce pro-oxidant changes in the plasma in healthy volunteers [87]. But in surgical patients, intravenous injection of 2 g ascorbate at 2 h before major surgery increases oxidative modification of plasma lipids in the venous blood samples obtained during the ischemic phase of surgery [88].

10. Conclusion

Further study is needed to determine definitively the safety and efficacy of ascorbate in patients with sepsis. Nevertheless, current evidence supports the hypothesis that microvascular function may be improved in sepsis by parenteral administration of ascorbate as an adjuvant therapy.

Acknowledgement

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