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Invited Review

Critical Role for Oxidative Stress, Platelets, and Coagulation in Capillary Blood Flow Impairment in Sepsis

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ABSTRACT

Sepsis is a complex multifaceted response to a local infectious insult. One important facet is the circulatory system dysfunction, which includes capillary bed plugging. This review addresses the mechanisms of capillary plugging and highlights our recent discoveries on the roles of NO, ROS, and activated coagulation in platelet adhesion and blood flow stoppage in septic mouse capillaries. We show that sepsis increases platelet adhesion, fibrin deposition and flow stoppage in capillaries, and that NADPH oxidase-derived ROS, rather than NO, play a detrimental role in this adhesion/stoppage. P-selectin and activated coagulation are required for adhesion/stoppage. Further, platelet adhesion in capillaries (i) strongly predicts capillary flow stoppage, and (ii) may explain why severe sepsis is associated with a drop in platelet count in systemic blood. Significantly, we also show that a single bolus of the antioxidant ascorbate (injected intravenously at clinically relevant dose of 10 mg/kg) inhibits adhesion/stoppage. Our data suggest that eNOS-derived NO at the platelet-endothelial interface is anti-adhesive and required for the inhibitory effect of ascorbate. Because of the critical role of ROS in capillary plugging, ascorbate bolus administration may be beneficial to septic patients whose survival depends on restoring microvascular perfusion.

Key words: systemic inflammation, capillary bed, platelets, microthrombi, reactive oxygen species, nitric oxide, ascorbate

Abbreviations used: AP1, Activator protein 1; BH_4, Tetrahydrobiopterin; CLP, Cecal ligation and perforation; DIC, Disseminated intravascular coagulation; eNOS, Endothelial nitric oxide synthase; FIP, Feces injection into the peritoneum; INFγ, Interferon γ; iNOS, Inducible nitric oxide synthase; IRF1, Interferon regulatory factor 1; Jak2, Janus kinase 2; JNK, c-Jun N-terminal kinase; LPS, Lipopolysaccharide; NADPH, Nicotinamide adenine dinucleotide phosphate; nNOS, Neuronal nitric oxide synthase; NO, Nitric oxide; PAI-1, Plasminogen activator inhibitor 1; PP2A, Protein phosphatase type 2A; PSGL-1, P-selectin glycoprotein ligand 1; ROS, Reactive oxygen species; SNAP, S-nitroso-N-acetyl-penicillamine; Stat1, Signal transducer and activator of transcription 1; TF, Tissue factor; TNFα, Tumor necrosis factor α

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INTRODUCTION

Sepsis is often defined as a systemic inflammatory response to a local infection. It is one of the top ten causes of death in North America [46,47] with a mortality rate near 40% [46]. The incidence of sepsis has become more frequent as the population ages, the use of invasive procedures increases, and antibiotic resistance emerges. Despite many clinical trials, the only treatment that has reached regulatory approval is activated protein C (drotrecogin alpha) [16]. However, the efficacy of this drug has been challenged and the 2008 Clinical Practice Guidelines [30] downgraded the recommendations for its use in patients with multiple organ failure to a weak recommendation. Clearly, this apparent lack of progress demonstrates a fundamental lack of understanding of sepsis and the mechanisms by which it

leads to multiple organ failure and death. Further research of the mechanism of organ failure in sepsis is critical in developing appropriate novel therapies for septic patients.

Sepsis is characterized by a number of circulatory disorders, including decreased systemic vascular resistance, hypotension, impaired oxygen utilization, lactic acidosis, and maldistribution of blood flow in the microcirculation [9,60,79]. Based on an intravital microscopic approach, we first reported impaired capillary blood flow (i.e., plugged capillaries) in skeletal muscle in septic rats [35]. Capillary flow impairment has since been reported in various animal models of sepsis and similar derangements in the microcirculation have been seen in septic patients [7,8,69,71]. The impairment leads to tissue hypoxia due to increased diffusion distance for oxygen [22]. It has been proposed that hypoxia is the initial and important step in the progression to organ failure [22]. Thus, in early sepsis, tissue (i.e., mitochondria) is capable of extracting O_2 but O_2 delivery is impaired [22]. Tissue hypoxia in multiple organs may explain why capillary blood flow impairment is a strong predictor of death and one-third of severe sepsis patients die of organ failure even when shock is prevented [68,69]. The aim of this review is to examine the role of nitric oxide, reactive oxygen species, and platelets/coagulation pathway in the capillary blood flow impairment, and to assess the effect of the antioxidant ascorbate as a potential treatment against the septic impairment.

MECHANISM(S) OF SEPTIC IMPAIRMENT OF CAPILLARY BLOOD FLOW

Role of NO and ROS

Numerous studies have shown that sepsis attenuates arteriolar diameter response to vasoconstrictors [5,85,86] and vasodilators [4,21]. NO overproduction by iNOS has been implicated in sepsis-induced impairment of arteriolar responsiveness [4,28,85] and capillary blood flow [6]. Pharmacological studies of the role of NOS isoforms have been confounded by the lack of specificity of the NOS inhibitors employed [21], including aminoguanidine used by Bateman *et al.* [6]. To address this issue, our laboratory has used a mouse model of sepsis, which permits employing NOS knockouts (eNOS, nNOS or iNOS). We discovered that, in septic skeletal muscle, iNOS knockout prevents arteriolar hypocontractility to norepinephrine and to angiotensin II [85,86], while nNOS knockout prevents both reduced arteriolar hypodilation to acetylcholine [40] and reduced propagation of vasoconstriction along the arteriole [49]. However, we also showed that impaired capillary blood flow in septic skeletal muscle (Figure 1) is not affected by the knockout of any of the three NOS isozymes (Figure 2). Rather, knockout of gp91phox (a subunit of NADPH oxidase) significantly reduces this impairment [77] (Figure 2),

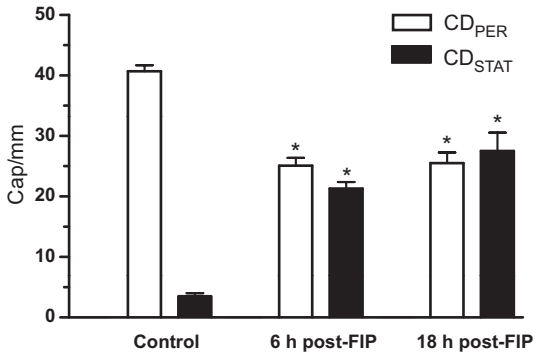

Figure 1. Sepsis impairs blood flow in capillaries in mouse hindlimb skeletal muscle. Sepsis was induced by feces injection into the peritoneum (FIP). Densities of capillaries per millimeter of test line (Cap/mm), with moving red blood cells (CD_{PER}) and with stationary red blood cells (CD_{STAT}), were measured in control mice and in septic mice at 6 and 18 hours post-FIP. Sepsis impaired capillary blood flow within six hours. Data are shown as mean ± standard error (SE). *Significant difference from control, n = 5–32 mice/group, $p < 0.05$. Figure is from [77].

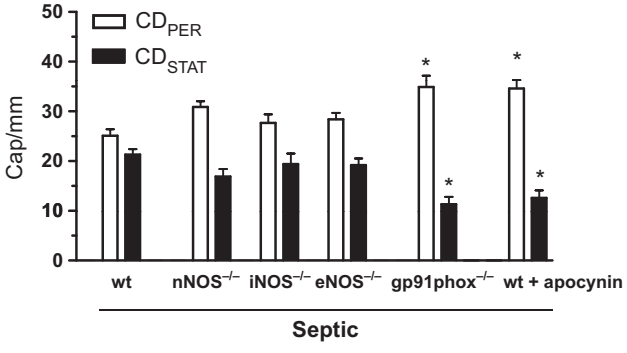

Figure 2. Effect of nNOS, iNOS, eNOS and gp91phox (NADPH oxidase subunit) knockout on capillary blood flow at six to seven hours post-FIP. Septic impairment of capillary flow was NOS-independent but NADPH oxidase-dependent. Intravenous injection of the NADPH oxidase inhibitor apocynin in wild type (wt) mice at six hours restored capillary blood flow at seven hours post-FIP. Data are shown as mean ± SE. *Significant difference from wt group, n = 6–37 mice/group, $p < 0.05$. Figure is adapted from [77].

demonstrating that the impairment is oxidant-dependent. This discovery indicates that prevention of arteriolar hypocontractility (i.e., by iNOS knockout) or hypodilation (by nNOS knockout) does not necessarily lead to improved capillary blood flow. Rather, NADPH oxidase-derived ROS plays a major role in the capillary flow impairment. Our data suggest that other mechanisms, independent of arteriolar function, contribute to the impairment (e.g., reduced red blood cell deformability [6] and/or increased adhesion of platelets to the endothelium [12]).

Role of Platelets/Coagulation Pathway

Since DIC is a common clinical condition in sepsis [37], platelet-endothelial interaction, platelet aggregation, and

microthrombi formation in capillaries could plug capillaries and thus explain the blood flow impairment. The process of DIC is initiated by TF, leading to eventual formation of thrombin, fibrin, and intravascular thrombi [37,64]. There is also inhibition of the fibrinolytic pathway in DIC [37,38]. Lipopolysaccharide (an initiating factor in sepsis) and inflammatory cytokines (e.g., TNFα) increase TF expression at the cell membrane of monocytes and endothelial cells [18,39,42,74]. LPS and cytokines also increase expression of P-selectin at the endothelial cell surface [19,41,44], whereas PSGL-1 at the platelet surface functions as a major ligand for P-selectin to initiate platelet adhesion in endotoxemia [10]. Further, endotoxemia potentiates platelet aggregation [87]. Thus, platelet activation, aggregation, and platelet-endothelial adhesion in sepsis could contribute critically to microthrombi formation in capillaries and their plugging.

Endothelial/platelet-derived ROS enhance platelet activation and adhesion and promote coagulation during inflammation [13,25,33,39,61]. We showed that sepsis increases ROS production in mouse skeletal muscle tissue [85,86] and that LPS+INFγ activates NADPH oxidase to produce superoxide in cultured microvascular endothelial cells derived from this muscle [83]. ROS can increase both platelet adhesion to the endothelium [33] and platelet aggregation [73], promote translocation and upregulation of P-selectin at the surface of platelets and endothelial cells [39,62], and upregulation of TF [26,39]. The resulting formation of TF and factor VII complex promotes generation of thrombin which, in turn, activates ROS production increase via NADPH oxidase [26]. It has been proposed that, through this positive feedback, ROS drive the thrombogenic cycle within the vasculature [26]. Knockout of gp91phox was shown to prevent P-selectin-dependent platelet-endothelial adhesion [70], while pharmacological inhibition of NADPH oxidase attenuated platelet aggregation [73] and thrombin-induced TF mRNA expression [25].

Since the ROS-sensitive pro-thrombogenic process could occur in the septic capillary to precipitate microthrombus formation and subsequent capillary plugging, we used the mouse skeletal muscle model to examine the effect of sepsis on platelet adhesion in capillaries. Accordingly, we showed that sepsis increases platelet adhesion in capillaries, NADPH oxidase-dependently, with negligible adherence of leukocytes in these microvessels (Figure 3). Although we also observed platelet adhesion is septic venules, we never saw flow stoppage in these blood vessels [70]. The capillary blood flow stoppage in sepsis was significantly reduced by platelet depletion [70]. Given the importance of P-selectin in the platelet-endothelial interaction, we also showed that intravenous injection of the P-selectin blocking antibody inhibits increased platelet adhesion and flow impairment in

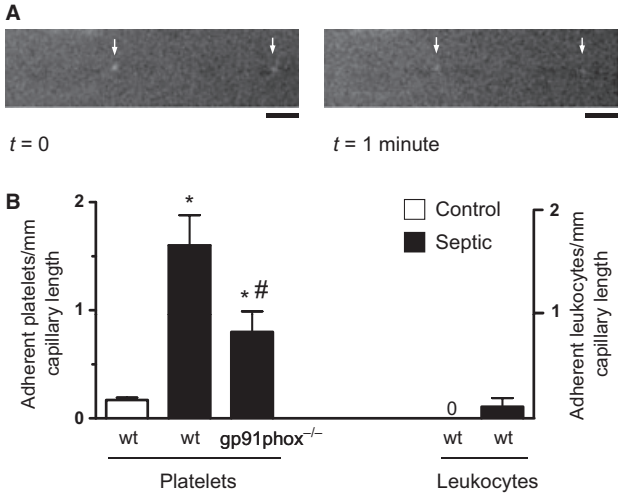

Figure 3. Panel **A**: An example of adhering platelets in a capillary of septic mouse skeletal muscle at seven hours post-FIP. At seven hours, a 0.35 × 0.47 mm microscopic field of view of the muscle surface was fluorescently illuminated and video-recorded for 10 seconds while focusing up and down (recording R1). One minute later, the same field was again fluorescently illuminated and recorded for 10 seconds, focusing up and down (R2). Within the next five minutes, the field was recorded under bright light illumination for 10 seconds (R3). During an off-line analysis of R1 (frame-by-frame), fluorescently labeled stationary ''platelets'' were noted. For each of these platelets, the best-focused frame from R1 was used to determine if the ''fluorescing platelet'' was a single platelet or an aggregate of several platelets. Next, recording R2 was also frame-by-frame analyzed to view the previously noted stationary platelets/aggregates and to establish whether they remained stationary over the one minute period. These remaining stationary platelets were then identified as adhering platelets. Using recording R3, the total capillary length within the field of view was measured. The total count of adhering platelets per field was then divided by the total capillary length to compute ''adherent platelets/mm capillary length.'' The left part of Panel **A** shows two adhering platelets (white arrows) in a septic capillary from recording R1. The right part shows the same two adhering platelets in this capillary from recording R2. Each bar represents 25 μm. In the majority of experiments in control and septic mice, platelets adhered singly rather than in aggregates. Panel **B**: Sepsis at six to seven hours post-FIP increases platelet adhesion in capillaries NADPH oxidase-dependently. Leukocyte adhesion (readily identifiable from R1 and R2) in both control and septic capillaries is negligible. Data are shown as mean ± SE. For platelet adhesion, * difference from wild type control, # difference from wild type septic group, $n = 7–9$ mice/group, $p < 0.05$. There was no difference in leukocyte adhesion between control and septic capillaries, $n = 4$ and 6 mice for control and septic groups, respectively, $p > 0.05$. Figure in Panel **B** is adapted from [70]; details of platelet adhesion quantification are provided in [70].

septic capillaries [70] (Figure 4). Finally, we demonstrated that sepsis increases both fibrin deposition (Figure 5) and thrombogenic potential in septic capillaries [70] and that inhibitors of the coagulation pathway (eptifibatide, antithrombin) inhibit increased platelet adhesion and flow impairment in septic capillaries [70] (Figure 5). The latter finding is consistent with the reported inhibitory effect of activated protein C (anticoagulant/anti-inflammatory

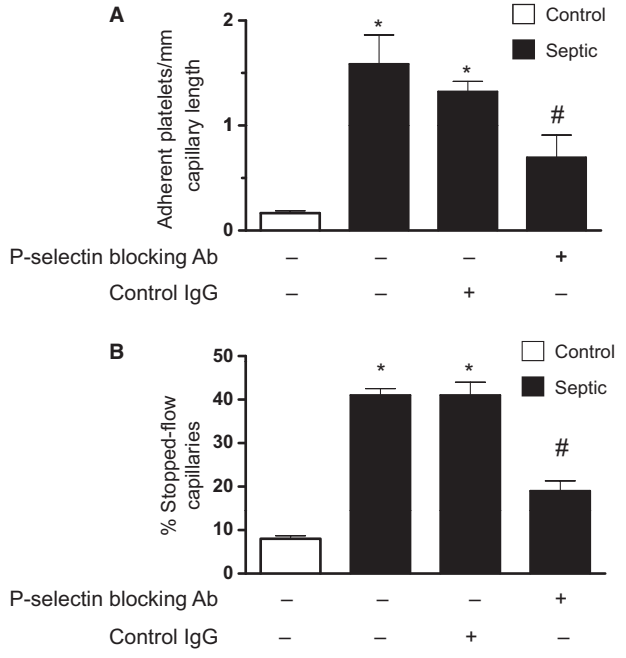

Figure 4. P-selectin blocking antibody prevents septic platelet adhesion (Panel **A**) and blood flow impairment in capillaries (Panel **B**). IgG is immunoglobulin. Impairment (i.e., % stopped flow capillaries) was computed as $100 \times CD_{STAT}/(CD_{STAT} + CD_{PER})$. Data are shown as mean ± SE. Panel **A**: * difference from control, # difference from vehicle-treated septic group, $n = 4–7$ mice/group, $p < 0.05$. Panel **B**: * difference from control, # difference from vehicle-treated septic group, $n = 6–18$ mice/group, $p < 0.05$. Figure is adapted from [70].

agent) against LPS-induced impairment of capillary bed perfusion in a hamster skinfold model of sepsis [27].

Based on our findings and the literature, Figure 6 shows a proposed mechanism of plugging of capillaries in sepsis. Clearly, because of the complexity of cellular and systemic responses to sepsis, this mechanism does not preclude other contributing mechanisms. For example, circulating platelet microaggregates in sepsis could plug capillaries because endotoxemia promotes platelet aggregation [87]. To this end, we observed that platelets adhere mostly singly (Figure 3A), rather than in aggregates, in capillaries of septic skeletal muscle [70]. The proposed mechanism (Figure 6) includes involvement of endothelial P-selectin (as opposed to platelet P-selectin) in platelet-endothelial adhesion. This is because it has been shown that endotoxemia markedly increases P-selectin expression within the microvascular endothelium but it does not alter platelet P-selectin expression [41]. The platelet-endothelial adhesion, however, may not be limited to the presence of P-selectin. Others have proposed that fibrinogen binding to adhesion molecules on endothelial cells can also sustain binding of platelets to these cells [48]. This and other mechanisms are plausible because we found that the

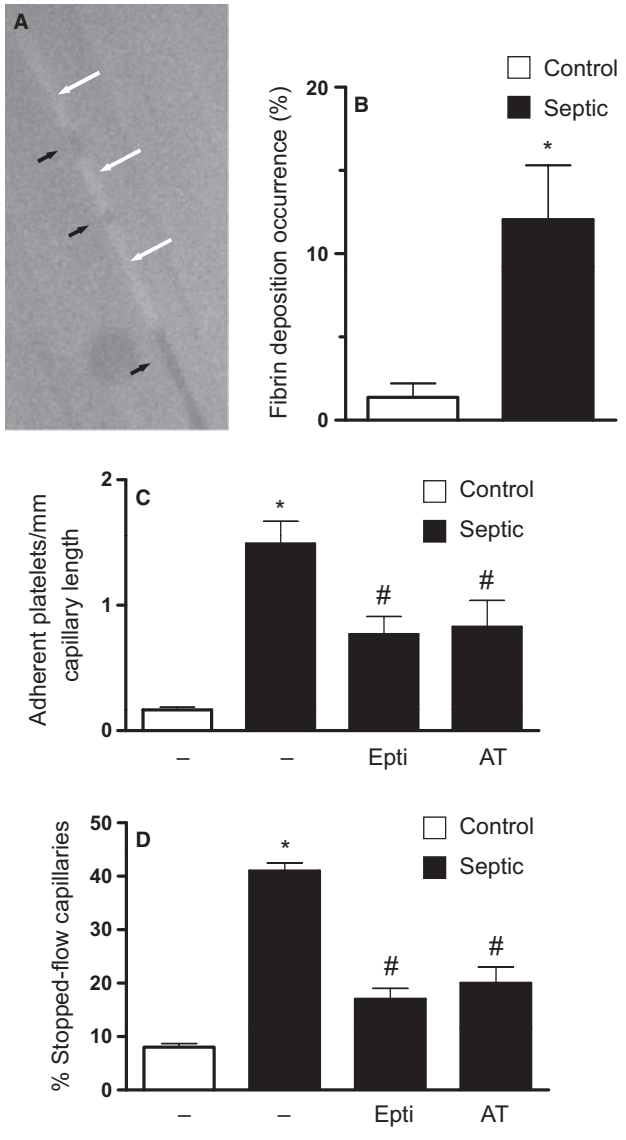

Figure 5. Sepsis increases the occurrence of fibrin in capillaries (Panels **A**, **B**). Platelet adhesion and blood flow stoppage requires activated coagulation pathway (Panels **C** and **D**). Panel **A**: fibrin depositions (white arrows) were visualized with fluorescently labeled antifibrin antibody (antibody does not bind to fibrinogen [66]). Fibrin depositions were seen only in capillaries with stationary red blood cells (black arrows). Panel **B**: Sepsis increased fibrin deposition in capillaries defined as the length of fibrin-containing capillaries seen under fluorescent illumination divided by the total length of capillaries seen under bright light illumination (this ratio was expressed in %). * Difference from control, $n = 2$ and 4 mice in control and septic groups, $p < 0.05$. Panel **C**: anticoagulant eptifibatide (Epti, glycoprotein IIb/IIIa inhibitor) and antithrombin (AT, thrombin inactivator) inhibit platelet adhesion in septic capillaries. * Difference from control, # difference from vehicle-treated septic group, $n = 5–9$ mice/group, $p < 0.05$. Panel **D**: eptifibatide and antithrombin inhibit blood flow impairment in septic capillaries. * Difference from control, # difference from vehicle-treated septic group, $n = 5–18$ mice/group, $p < 0.05$. Data are shown as mean ± SE. Figure is adapted from [70].

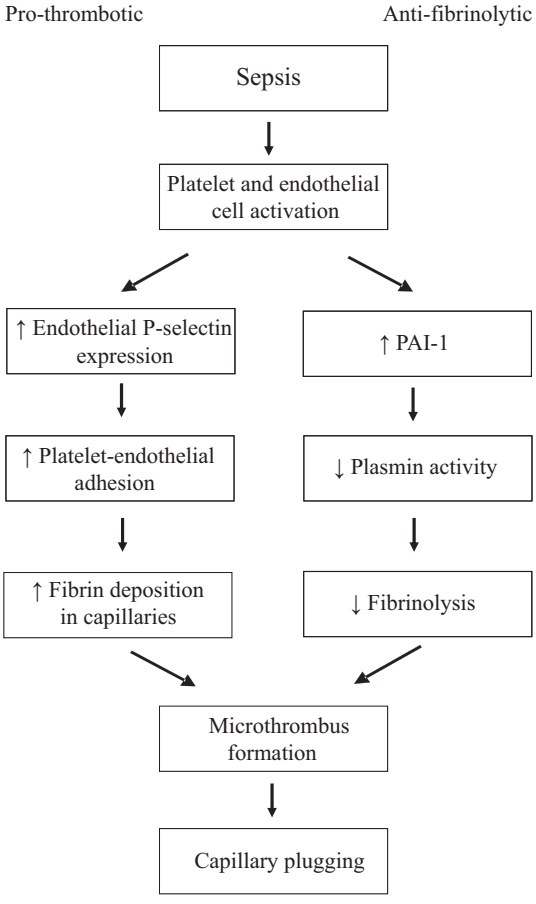

Figure 6. A proposed mechanism of plugging of capillaries in sepsis. Sepsis leads to activation of both prothrombotic and antifibrinolytic pathways [38] in the microcirculation, precipitating microthrombi formation and capillary plugging. PAI-1 is plasminogen activator inhibitor 1.

treatment with P-selectin blocking antibody does not completely restore platelet adhesion back to the control level (Figure 4A). Finally, circulating microparticles in sepsis (recently reviewed by Meziani *et al* [51]) need to be considered since their procoagulant function could also contribute to microthrombus formation and capillary plugging.

Significance of Systemic Platelet Count in Sepsis

In critically ill patients, a 30% drop in platelet count independently predicts death [54]. In our septic mouse model, a similar drop in platelet count (from 850 to 610 $\times 10^9$/L) occurred at six hours of sepsis [70]. Since the life span of platelets is longer than six hours [57], we carried out the following order-of-magnitude computation to see if the "missing" platelets (i.e., 240 $\times 10^9$/L) could be accounted for by their trapping in the septic microvasculature. For a 20 g mouse having 1.7 mL total blood volume [67], the 240 $\times 10^9$/L drop translates to the reduction of $\sim$ 400 $\times 10^6$ platelets in systemic blood. Taking the average capillary length volume density to be 3,100 mm per mm^3 of skeletal muscle [31], the specific gravity of muscle 1.01 $\times 10^{-3}$ g/mm^3 [14], and taking the density and specific gravity to be those of the entire mouse, the total length of capillaries could be estimated as 61.4 $\times 10^6$ mm (= 3,100 $\times$ 20/[1.01 $\times 10^{-3}$]). If the increase in sepsis-induced platelet adherence were 1.6 platelets per mm of capillary length as shown in Figure 3 (most likely an underestimate of actual adherence, [70]), then sepsis would increase platelet trapping in capillaries by $\sim$ 100 $\times 10^6$ platelets. This is the same order of magnitude as the 400 $\times 10^6$ platelets missing from systemic blood, indicating that platelet trapping in sepsis is likely an important component of the platelet count drop.

Recently, we reported a strong linear correlation (R^2 = 0.9) between blood flow stoppage and platelet adhesion in capillaries of septic mice [70]. This correlation underscores the significance of activated platelets/coagulation pathway in capillary flow impairment and it suggests that the death-predicting 30% drop in platelet count [54] reflects both platelet trapping in capillaries and their subsequent plugging in sepsis.

BENEFICIAL EFFECTS OF ASCORBATE AGAINST SEPSIS

Septic patients show signs of oxidative stress (e.g., lipid peroxidation) and decreased levels of plasma ascorbate [20]. Although other antioxidants are also affected by sepsis, there is a strong correlation between survival and the concentration of ascorbate [20,23]. In the following, we summarize our work on the effect of this particular antioxidant in rodent models of sepsis.

Protective Effect of Ascorbate in the Septic Vasculature

Consistent with the protective effect of NADPH oxidase knockout, and the restorative effect of apocynin injection (Figure 2) we recently showed in the mouse model of sepsis (i.e., feces injection into peritoneum) that an early intravenous injection of ascorbate bolus (i.e., at the onset of sepsis) prevents the development of platelet adhesion and plugging of capillaries at six hours of sepsis (Figures 7A,B) [70]. Furthermore, we showed that ascorbate bolus injection delayed to six hours reverses this adhesion and plugging at seven hours of sepsis (Figures 7C,D) [70] and that this beneficial effect of delayed ascorbate lasts at least 12 hours post-injection [77]. We used a clinically relevant dose of 10 mg/kg [77] in these experiments. The route of ascorbate administration is important here, because ascorbate given intravenously yields much higher

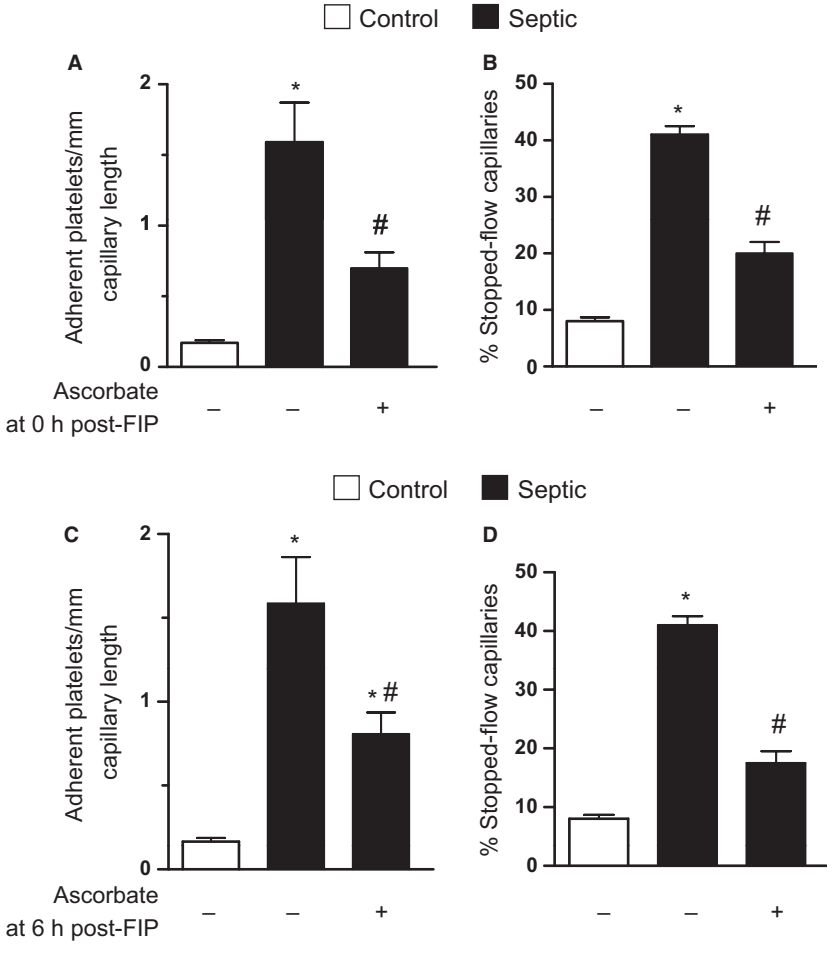

Figure 7. An early injection of ascorbate bolus (10 mg/kg) at the onset of sepsis prevents platelet adhesion (Panel **A**) and flow stoppage (Panel **B**) in septic capillaries at six to seven hours of sepsis, whereas injection delayed to six hours reverses platelet adhesion (Panel **C**) and flow stoppage (Panel **D**) at seven hours. Data are shown as mean ± SE. * Difference from control, # difference from vehicle-treated septic group, $n = 6–9$ (Panel **A**), 6–18 (Panel **B**), 9–17 (Panel **C**) and 9–18 (Panel **D**) mice/group, $p < 0.05$. Figure is adapted from [70].

(∼ 100 ×) ascorbate levels in plasma than when given orally [63,78].

These recent findings agree with our previous reports of beneficial effects of ascorbate in sepsis. Using a rat model of sepsis (cecal ligation and perforation), we also showed that the early ascorbate bolus (76 mg/kg), or bolus delayed to 6–24 hours post-CLP, reduces the capillary blood flow impairment at 24 and 48 hours post-CLP [2,76]. In the mouse CLP model, we showed that the early ascorbate prevents the increase in plasma protein carbonyl (a measure of oxidative stress) and nitrite/nitrate levels, the increase in iNOS protein expression in skeletal muscle, and the reduction in arteriolar contractility to norepinephrine and to angiotensin II at six hours post-CLP [85]. The latter effect is corroborated by the report of the protective effect of ascorbate against vascular hypodilation in human endotoxemia [53]. Finally, we also showed in CLP mice [50] that both early and delayed (23 hours post-CLP) ascorbate boluses inhibit the reduced arteriolar conducted vasoconstriction and the increased nNOS enzymatic activity in the cremaster muscle at 24 hours.

Using cultured microvascular endothelial cells (mouse skeletal muscle origin) stimulated with LPS+INFγ (*in vitro* model of sepsis), we also studied how ascorbate affects signaling in these cells [80]. Ascorbate pretreatment of cells inhibited (i) increased superoxide production, (ii) upregulation of NADPH oxidase subunit p47phox, (iii) activation of JNK and Jak2 phosphorylation, and (iv) activation of AP1, Stat1 and IRF1 nuclear transcription factors involved in iNOS induction [82–84]. Using this *in vitro* model, ascorbate's inhibition of sepsis-induced oxidant production by NADPH oxidase was also found to be critical in preventing (i) protein phosphatase type 2A activation, (ii) PP2A-dependent dephosphorylation and redistribution of occludin, and (iii) disruption of the endothelial barrier [24].

Mechanism of Protection by Ascorbate in Septic Capillaries

We have reported that the protection/reversal by ascorbate against sepsis-induced platelet adhesion and blood flow stoppage in capillaries occurs in skeletal muscle of wild type mice (Figure 7) but not in skeletal muscle of eNOS$^{-/-}$

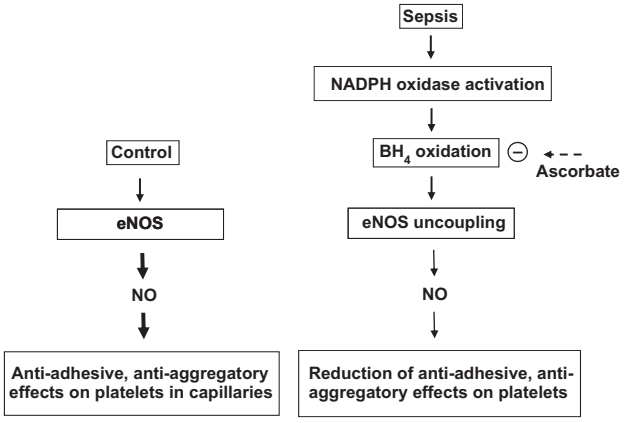

Figure 8. Proposed mechanism of protection by ascorbate against sepsis-induced blood flow stoppage in capillaries [77]. Under control conditions, endothelial nitric oxide synthase (eNOS)-derived NO in the microvasculature has anti-aggregatory and anti-adhesive effects on platelets in capillaries. Sepsis activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to increase reactive oxygen species production and oxidation of the eNOS cofactor tetrahydrobiopterin (BH_4). Oxidized BH_4 uncouples eNOS in platelets and endothelial cells and reduces/eliminates NO synthesis, promoting platelet adhesion/aggregation in capillaries and cessation of blood flow therein. Ascorbate prevents BH_4 oxidation, eNOS uncoupling, and cessation of blood flow in capillaries.

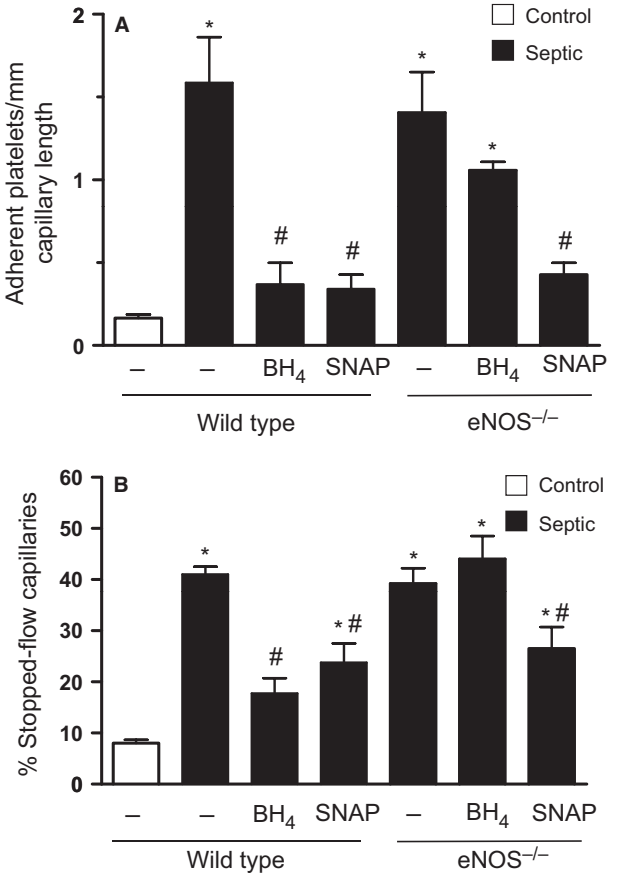

Figure 9. Effect of BH_4 and NO donor S-nitroso-N-acetyl-penicillamine (SNAP) on platelet adhesion and capillary blood flow impairment in wild type and $eNOS^{-/-}$ mice. At six hours of sepsis, a bolus of BH_4 flooded the surface of mouse hindlimb skeletal muscle and platelet adhesion and impairment were determined at seven hours. Alternatively, a bolus of SNAP flooded the surface, and platelet adhesion and impairment were determined 15 minutes later (i.e., when the temporary SNAP-induced vasodilation had ended). Panel **A**: BH_4 reversed platelet adhesion in septic capillaries of wild type but not $eNOS^{-/-}$ mice. SNAP reversed platelet adhesion in both types of mice. * Difference from control, # difference from the appropriate vehicle-treated septic group, $n = 4–10$ mice/group, $p < 0.05$. Panel **B**: BH_4 reversed septic blood flow impairment in wild type but not in $eNOS^{-/-}$ mice whereas SNAP reversed septic impairment in both types of mice. * Difference from control, # difference from the appropriate vehicle-treated septic group, $n = 5–18$, $p < 0.05$. Data are shown as mean ± SE. Figure is adapted from [70].

mice [70]. This indicates that eNOS-derived NO is required for the beneficial effect of ascorbate. There is evidence that low levels of endothelial/platelet-derived NO from eNOS are protective against platelet adhesion/aggregation [29,43,56]. NO prevents P-selectin protein expression [3], while inhibition of NO leads to P-selectin-dependent platelet adhesion [3,52] and increased platelet aggregability [75]. To explain the beneficial effect of ascorbate, we offer the following hypothesis [77] (Figure 8): since ROS have been shown to decrease the concentration of the eNOS cofactor BH_4 in endothelial cells [36], we propose that sepsis-induced ROS oxidize BH_4, which then cannot function as a cofactor of eNOS [17,34]. eNOS becomes uncoupled [1] and stops producing the protective levels of NO. (The locally produced NO from eNOS in endothelial cells/platelets in capillaries may play a critical anti-aggregatory and anti-adhesive role here [17,65,75]). In the hypothesized mechanism, ascorbate prevents BH_4 oxidation [53], increases BH_4 content in platelets and endothelial cells, elevates their eNOS-derived NO [17,32,72], reduces platelet adhesion and aggregability [65,75], and thus restores capillary blood flow.

To test this hypothesis, we reasoned that, if the eNOS-dependent inhibition of impairment by ascorbate was due to an increase in endogenous BH_4 concentration, then exogenous supplementation of this cofactor at six hours of sepsis should inhibit capillary flow impairment in wild type but not $eNOS^{-/-}$ mice. Our intravital experiments indeed showed that applying BH_4 to the surface of septic skeletal muscle reversed platelet adhesion and blood flow stoppage in capillaries of wild type but not $eNOS^{-/-}$ mice (Figure 9) [70]. This effect of local BH_4 agrees with the reported inhibitory effect of intra-arterially injected BH_4 against LPS-induced endothelial dysfunction in humans [53]. Finally, we also tested if exogenous BH_4 stimulates eNOS activity to increase NO production and thus inhibits capillary blood flow impairment in sepsis [12]. Consistently, the

NO donor SNAP applied on the septic muscle surface reversed platelet adhesion and blood flow stoppage even when the temporary vasodilatory effect of SNAP had ended (Figure 9) [70]. (Note: Since the lack of eNOS-derived NO may contribute to septic capillary flow impairment, genetic deletion of eNOS in sepsis would not be expected to provide a beneficial effect [77]).

In septic skeletal muscle, nNOS enzymatic activity dominates over the activity of other NOS isoforms [49] (nNOS here is situated mainly in skeletal myocytes [55]). Intriguingly, nNOS knockout does not affect the septic blood flow impairment in skeletal muscle (Figure 2), suggesting that nNOS-derived NO overproduction in myocytes does not protect against platelet adhesion/flow stoppage here. To reconcile this lack of effect with the observed beneficial effect of local exogenous NO (Figure 9) and with the proposed beneficial effect of eNOS-derived NO (Figure 8), we speculate that the myocyte nNOS-derived NO reacts with other molecules (e.g., superoxide to form peroxynitrite [33]) before reaching the platelet-endothelial interface in capillaries to exert its anti-adhesive effect there.

Effect of Ascorbate in Sepsis, Clinical Considerations

The various clinical considerations for ascorbate use in sepsis, including potential risks, have been recently reviewed by Wilson [80]. Regarding clinically relevant parameters measured in the rat CLP model of sepsis, we showed that ascorbate prevents blood pressure drop and fever [2,76]. In our mouse CLP model, survival is 9% at 24 hours post-CLP. Early injection of ascorbate increases survival to 65% at 24 hours post-CLP [86]. In our mouse FIP model, survival is 19% at 24 hours post-FIP. Injection of ascorbate delayed to six hours post-FIP increases survival to 50% at 24 hours post-FIP [77].

Clinically, administration of ascorbate and α-tocopherol reduce the incidence of organ failure and duration of hospitalization in critically ill patients [59,80]. However, the use of other antioxidants in septic patients does not always offer similar results [15]. This is because ROS may be required in defending against infection, including killing of bacteria and upregulation of anti-inflammatory cytokines [45]. The beneficial effect of ascorbate bolus injection [77,86] could be due to ascorbate's ability to accumulate in microvascular endothelial cells (up to 16 mM) [81] and due to the body's ability to relatively quickly excrete through the kidneys any excess of ascorbate [2,77]. Thus, accumulated ascorbate in the endothelial cells could specifically protect the microvasculature from oxidative injury, including all increased BH_4 content in platelets and endothelial cells (i.e., by preventing BH_4 oxidation), increased eNOS-derived NO in these cells [17,32,72], reduced P-selectin protein expression [3,11], and reduced platelet adhesion [58,65,75].

SUMMARY AND CONCLUSION

This review examined the role of NO, ROS, platelets, and coagulation pathway in septic impairment of capillary blood flow and discussed the effect of ascorbate as a potential treatment against this impairment. Our work shows that NADPH oxidase-derived ROS, rather than NO, play a detrimental role in sepsis-induced platelet adhesion and blood flow stoppage in capillaries. P-selectin and activated coagulation pathway are required for this adhesion and stoppage. Significantly, a single intravenous bolus injection of the antioxidant ascorbate prevents as well as reverses adhesion/stoppage. Our work also indicates that local NO at the platelet-endothelial interface, produced via eNOS in endothelial cells and/or platelets, dislodges platelets from the capillary wall and it is critical in ascorbate's inhibitory effect against adhesion/stoppage in septic capillaries. In conjunction with the beneficial effect at the capillary level, it has been shown that ascorbate also protects against sepsis-induced dysfunction at the arteriolar level (it prevents arteriolar hyporesponsiveness to vasoactive agents and impaired arteriolar conducted response).

In conclusion, microvascular dysfunction is an important facet of sepsis that may lead to tissue hypoxia and organ failure. An intravenous injection of the antioxidant ascorbate may be beneficial as an adjuvant treatment in septic patients whose survival depends on restoring microvascular perfusion/function.

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