

Impact of Intravenous Ascorbic Acid Infusion on Novel Biomarkers in Patients with Severe Sepsis

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Abstract

Objective: Severe sepsis is a leading cause of mortality and morbidity in the critically ill with no reliably effective treatments. The goal of this study was to determine whether intravenous ascorbic acid impacted novel biomarkers in sepsis.

Methods: This is a retrospective study of a phase I, randomized, double-blinded, placebo controlled safety trial of intravenous ascorbic acid in severe sepsis. In the safety trial, 24 patients were randomized to receive full ICU standard of care support plus intravenous ascorbic acid (50 or 200 mg/kg/24h) for 4 days or placebo. Novel biomarkers of sepsis such as circulating cell free DNA (cf-DNA), mitochondrial DNA (mtDNA), endogenous antimicrobial proteins (alpha-4-defensin [α 4D] and bactericidal permeability interacting protein [BPI]) and the red cell distribution width (RDW) were measured.

Results: Cf-DNA values were higher in non-survivors at baseline and remained elevated for 96 hours. MtDNA levels increased in the placebo group, but declined in the treatment groups without reaching statistical significance. RDW increased significantly only in the placebo group, while expression of the antimicrobial proteins increased significantly only in the treatment groups.

Conclusion: Ascorbic acid infusion may improve sepsis outcomes by reducing cf- and mtDNA levels while augmenting endogenous antimicrobial proteins and preserving RDW.

Keywords: Vitamin C infusion; Sepsis; Cell free DNA; Mitochondrial DNA; Red cell distribution width; Antimicrobial peptides

Introduction

Severe sepsis is a leading cause of death in the critically ill with more than 700,000 new cases diagnosed in the United States annually [1]. Although numerous advances continue to be made in both the identification and treatment of sepsis, the incidence of sepsis and its associated multiple organ failure continues to rise worldwide. This new reality has made sepsis a leading cause of morbidity and mortality in critically ill patients [1]. Despite enrollment of thousands of patients and the expenditure of millions of dollars in clinical trials, to date, the identification of reliable biomarkers or outcome predictors as well as cost-effective therapies remains elusive.

Intravenous ascorbic acid has recently been suggested as a potential treatment for severe sepsis. In our pre-clinical studies, we showed that parenteral infusion of ascorbic acid (200 mg/kg) could attenuate sepsis induced organ injury in wild type mice [2,3] and in vitamin C knockout mice (lacking L-gulonolactone oxidase, Gulo) [4]. In these studies parenteral ascorbic acid was effective at reducing mortality and sepsis-induced organ injury through pleiotropic mechanisms including down-regulation of pro-inflammatory mediator secretion, normalization of coagulopathy, improved alveolar fluid clearance and enhanced alveolar epithelial barrier function.

Other studies show that parenteral ascorbic acid prevents or even reverses excessive protein nitration in microvascular endothelial cells. Further, microvascular dysfunction characterized by refractory vasodilation, loss of endothelial barrier function, disseminated intravascular coagulation, edema, multi-organ failure and death in animal models of sepsis are effectively treated with ascorbic acid [5].

Surprisingly, few clinical trials that involve ascorbic acid use in critically ill patients have been performed. Sawyer et al showed that intravenous infusion of ascorbic acid (and other antioxidants) in patients with established ARDS exhibited a 50% reduction in mortality [6]. More recent studies show a therapeutic benefit of ascorbic acid when infused intravenously in surgical critically ill patients and in patients with extensive burns [7,8]. We recently completed a Phase I trial (ClinicalTrials.gov identifier NCT01434121) to determine whether intravenous ascorbic acid was safe to administer to critically ill patients with severe sepsis [9]. Further, we determined the extent to which ascorbic acid impacted organ failure scores and a priori selected blood biomarkers. Expectedly, the biomarkers we tested in the phase I trial we conducted differed from the mechanistic variables investigated in animal studies. In our Phase I trial observations, ascorbic acid infusion significantly reduced the pro-inflammatory biomarkers C-reactive protein and procalcitonin [9]. Unlike placebo patients, thrombomodulin in ascorbic acid infused patients exhibited no significant rise, suggesting attenuation of vascular endothelial injury. Moreover, patients receiving ascorbic acid exhibited significant

reductions in sequential organ failure assessment scores (SOFA scores) while placebo patients exhibited no such reduction [9].

Despite promising results, translation of a cost-effective therapy requires validation with other recently identified biomarkers as well as elucidation of the mechanism of action of intravenous ascorbic acid in humans with severe sepsis. Thus, we performed a retrospective analysis using serum and plasma that was obtained in the course of the Phase I trial along with limited patient laboratory data gathered at the time of the study. We sought to determine whether intravenous ascorbic acid impacted recently proposed novel biomarkers for sepsis that include circulating cell free DNA (cf-DNA), mitochondrial DNA (mtDNA), endogenous antimicrobial proteins (alpha-4-defensin [α 4D] and bactericidal permeability interacting protein [BPI]). Further, we measured changes in the red cell distribution width (RDW).

Methods

Phase I Trial

This study was approved by the VCU Institutional Review Board (IRB approval number: HM12903). Study groups in this trial were 1) Placebo: 5% dextrose and water; 2) Low dose ascorbic acid (Lo): 50 mg/kg/24 hours; and 3) High dose ascorbic acid (Hi): 200 mg/kg/24 hours. Ascorbic acid dosage was divided into 4 equal doses and administered over 30 minutes every 6 hours for 96 hours in 50 ml of 5% dextrose and water. All additional information regarding the trial has been published previously [9].

Blood samples

Blood samples were collected as described previously [9]. Briefly whole venous blood was drawn into sterile Vacutainer® tubes for serum and plasma. Serum samples were allowed to coagulate for 60 min at room temperature. Plasma and serum were separated by centrifugation. Plasma and serum were aliquoted and frozen at -70°C until use.

Quantification of Cell-Free DNA (cf-DNA)

The levels of cf-DNA in human serum were quantified as described previously [10]. Briefly, cf-DNA in serum was assessed using the Invitrogen Quant-iT PicoGreen dsDNA assay kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY). Fluorescence intensity was measured on a SpectraMax Gemini XPS microplate reader with excitation at 490 nm and emission at 525 nm, with 515 nm emission cutoff filter (Molecular Devices, Sunnyvale, CA).

Mitochondrial DNA quantification (mtDNA)

Cell free mitochondrial DNA was isolated from patient plasma using the QIAamp MinElute Virus Spin Kit (Qiagen) according to the manufacturer's protocol and quantified spectrophotometrically (Nano Drop 2000, Thermo Fisher, USA). Estimation of mtDNA levels was performed by quantitative real-time PCR (QPCR) essentially as described by Budnik et al [11]. Briefly two primer sets specific for the mitochondrial ribosomal 16S RNA were used: One pair amplified a 74-bp fragment that represented total mtDNA and included DNA released by apoptotic cells (mtDNA-74). The second primer pair amplified a 228-bp fragment that corresponded to mtDNA released by

non-apoptotic types of cell death (i.e. necrosis) or active secretion (mtDNA-228). The same specific forward primer was used for both mtDNA fragments while a different reverse primer was used for PCR. The sequences of the primers utilized are shown in Table 1. QPCR was performed as described below.

Name	Sequence 5' to 3'
MT 16S rRNA forward	CAGCCGCTATTAAGGTTTCG
MT 16S rRNA rev-74	CTGGATTACTCCGGTCTGAAC
MT 16S rRNA rev-228	CTCTGCCATCTTAACAAACC
DEFA4 forward (α 4D)	GGCAAGAGGTGATGAGGC
DEFA4 reverse (α 4D)	ACCAATGAGGCAGTTCCC
BPI forward	TCGAAACAAGATGAACAGCC
BPI reverse	AGACCATAGTTGATTCCAGCC
18S rRNA forward	GCAATAACAGGTCTGTGATGCC
18S rRNA reverse	CACGAATGGGGTTCAACG

Table 1: Sequence of primers for QPCR

Isolation of whole blood RNA

Intracellular RNA from whole blood was prepared with the PAXgene Blood RNA System (PreAnalytiX/QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions and essentially as described by Hindle et al [12]. Briefly, 2.5 ml venous blood samples were collected into PaxGene Blood RNA tubes, incubated for 2 h at room temperature to ensure complete cell lysis, frozen at -20°C for 24 h, then transferred to -80°C until batch processing. After equilibration to room temperature, cell lysates were pelleted, washed, treated with proteinase K to bring about protein digestion, and homogenized. Nucleic acids were bound to spin columns, washed, and treated with DNase I to remove trace amounts of DNA. Following additional washes, RNA was eluted, heat-denatured and chilled on ice. Spectrophotometry was performed to determine RNA concentrations, and RNA samples were stored at -80°C.

Real time polymerase chain reaction for quantification of mtDNA, alpha-4-defensin and bactericidal permeability interacting protein

Total blood RNA (1 μ g) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit. Complimentary DNA (cDNA) was diluted (1:500) and QPCR performed using POWER SYBR Green QPCR Master Mix as described previously [3]. Primers for α 4D and BPI were designed to anneal to sequences on separate exons or to span two exons. Cycling parameters were: 95°C, 10 min, 40 cycles of 95°C, 15 sec; 60°C, 1min. A dissociation profile was generated after each run to verify specificity of amplification. All PCR assays were performed in triplicate. No template controls and no reverse transcriptase controls were included. Human 18S rRNA was used as the housekeeping gene against which all the samples were normalized for differences in the amount of total RNA added to each cDNA reaction and for variation in the reverse transcriptase efficiency among the different cDNA reactions. Automated gene expression analysis was performed using the Comparative Quantitation module of MxPro QPCR Software (Agilent).

Determination of red cell distribution width (RDW)

Information regarding patient RDW was collected retrospectively from the patient database generated for the Phase I trial. RDW was measured as part of the complete blood count (CBC) at baseline and every 24 hours for up to 96 hours, using the Advia 2120i Hematology System (Siemens Corporation). RDW is reported as a coefficient of variation (percentage) of red blood cell volume. The normal reference range for RDW in the hospital laboratory is 11.3 – 14.7%.

Statistical Analysis

Statistical analysis was performed using SAS 9.3 and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE. Cell free DNA levels were compared using one-way ANOVA and the post hoc Tukey test to identify specific differences between groups. Changes in RDW values were analyzed by paired t test. Due to the variability among the pre-treatment values, mtDNA levels and QPCR data were transformed to exhibit log-normal distributions and analyzed by ratio t test. Statistical significance was confirmed at a p value of <0.05.

Results

Temporal changes in serum cf-DNA in severe sepsis patients

Serial serum samples and mortality data were available from the 24 patients enrolled in the study. Serum cf-DNA levels were determined at baseline (0h), 12h, 24h, 36h, 48h, 72h and 96h following enrollment in the study. As seen in Figure 1, serum cf-DNA values were higher in non-survivors than survivors at baseline and remained elevated for the duration of the study, reaching significance at 24h (p<0.05 ANOVA). Serum cf-DNA levels in survivors were essentially unchanged.

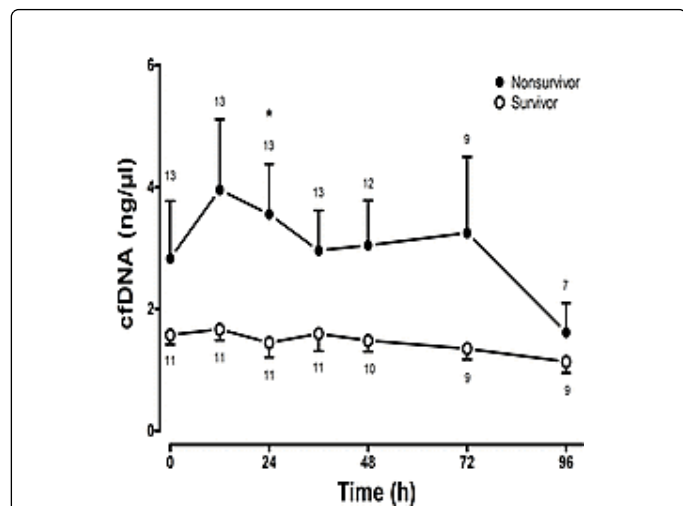


Figure 1: Temporal changes in serum cell free DNA (cf-DNA) in severe sepsis patients. (Survivors in the Phase I trial are shown in open circles (○), and non-survivors in closed circles (●). The number of patients for whom serum cf-DNA levels are available is indicated. Serum cf-DNA values were higher in non-survivors than survivors at baseline and remained elevated for the duration of the study, reaching significance at 24h (*, p<0.05, ANOVA). Data are expressed as mean ± SE.)

Next we examined whether treatment with intravenous ascorbic acid had an impact on serum cf-DNA levels. As seen in Figure 2, cf-DNA levels in the placebo group were elevated for up to 72 hours. In contrast, cf-DNA levels in patients receiving low dose ascorbic acid (50 mg/kg, Lo) trended downward after 24 h. Serum cf-DNA levels in patients receiving high dose ascorbic acid (200 mg/kg, Hi) were even lower and did not change over time. Although serum cf-DNA levels were lower in both treatment arms, these data did not reach statistical significance compared to the placebo group. Changes in the cf-DNA levels were reflected in the 28 day all-cause mortality between the three groups: placebo group - 63% mortality (5 of 8 patients died); Lo group - 38% mortality (3 of 8 patients died); and Hi group - 50% mortality (4 of 8 patients died). However, these changes were not statistically significant since this study was not powered to assess mortality.

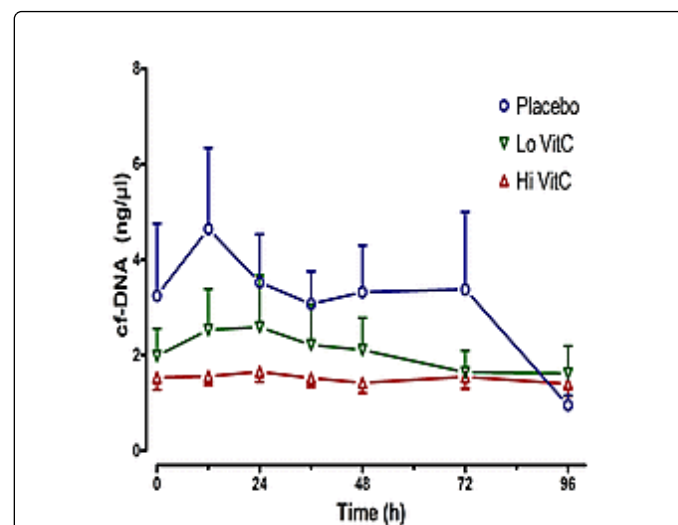
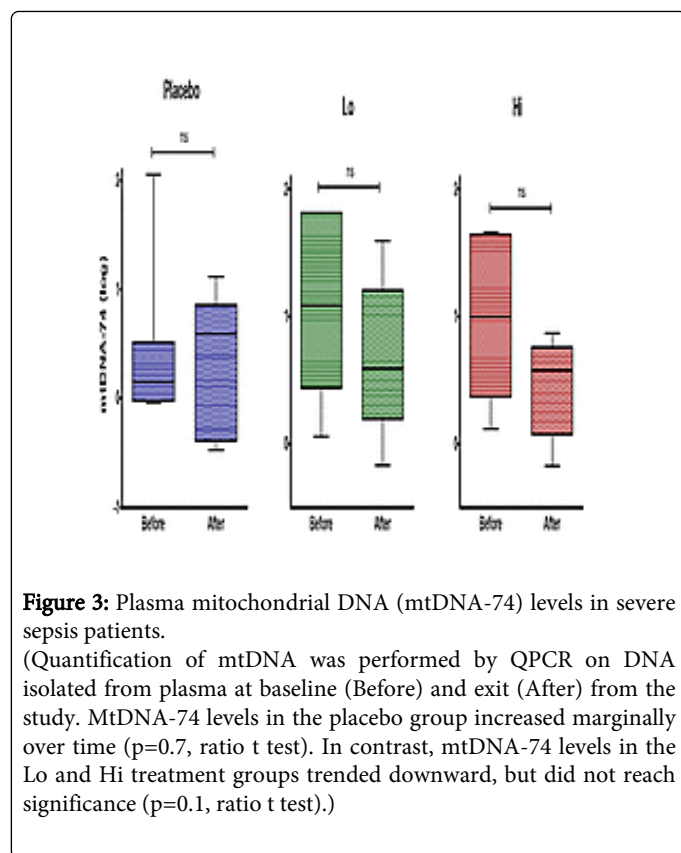


Figure 2: Impact of treatment with intravenous ascorbic acid on serum cf-DNA levels. (Serum cf-DNA levels of patients in the placebo (○), Lo (50mg/ml, ▽) and Hi (200mg/ml, Δ) groups are indicated. Patients in the placebo group exhibited highest levels of cf-DNA for up to 72 hours. Although serum cf-DNA levels were lower in both treatment arms, these data did not reach statistical significance (p>0.05, ANOVA). Data are expressed as mean ± SE.)

Plasma mitochondrial DNA in severe sepsis patients

We isolated DNA from plasma of septic patients enrolled in the Phase I trial and quantified levels of apoptotic and necrotic mitochondrial DNA (mtDNA). Quantification was performed on plasma at baseline (Before) and exit (After) from the study. Levels were normalized to mtDNA isolated from pooled plasma of 4 healthy volunteers. A majority of the mtDNA was apoptotic in origin (data not shown). Differences in baseline concentrations of mtDNA-74 were observed between the three groups (data not shown) but these were not statistically significant. Over the course of the study there was a trend toward a marginal increase in the mtDNA-74 levels in the placebo group (p=0.7) (Figure 3). In contrast, mtDNA-74 concentration in the Lo and Hi treatment groups trended downward, however, this decrease did not reach significance (p=0.1). Similar results were obtained with necrotic mtDNA levels (data not shown).

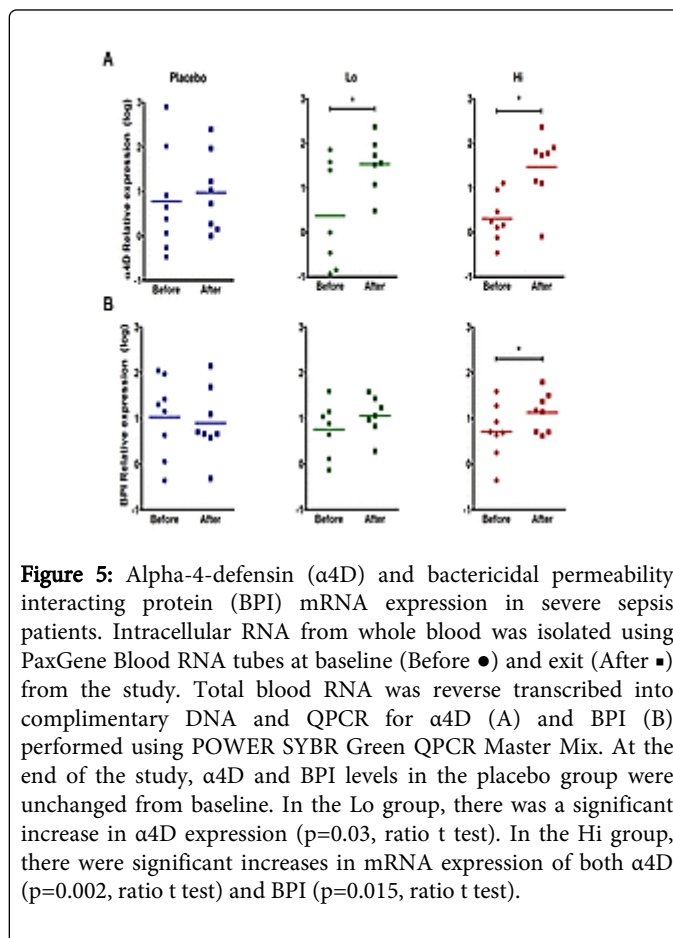
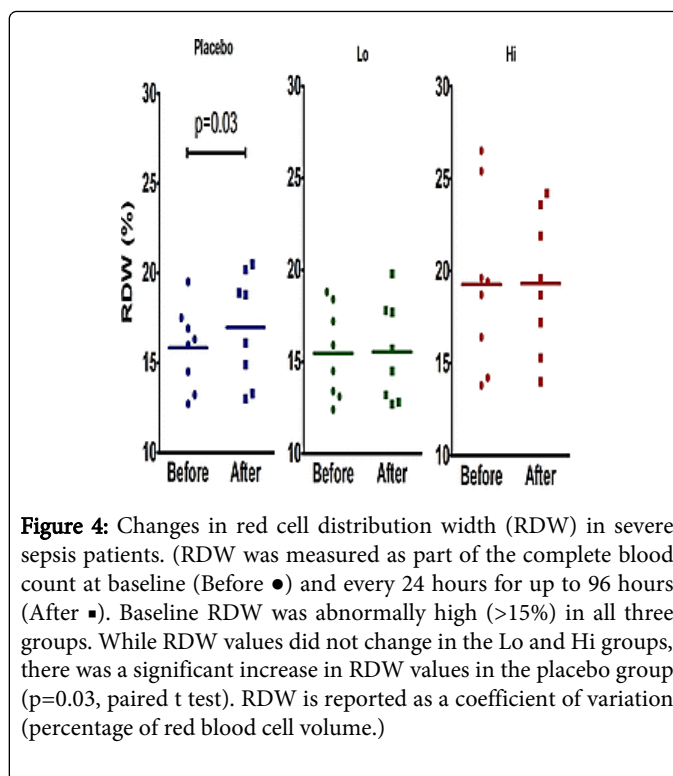


Changes in red cell distribution width in severe sepsis patients

Baseline RDWs, as well as elevations in RDW over time, have recently been implicated as independent prognostic factors of 28-day mortality and adverse outcomes in patients with severe sepsis and septic shock. We examined RDW in patients enrolled in the Phase I trial at baseline and at the end of the study. As seen in Figure 4, baseline RDW for these patients was abnormally high ($>15\%$) in all three groups. RDW levels remained unchanged in the Lo and Hi groups at the end of study and were not different from baseline. In contrast, there was a significant increase in RDW values in the placebo group (Figure 4, $p=0.03$). This suggests that even in this small cohort, any elevation in RDW is likely associated with adverse outcomes such as mortality. Moreover, infusion of ascorbic acid prevented any increase in RDW and thus potentially reduced adverse outcomes.

Infusion of ascorbic acid promotes neutrophil defensin expression

Defensins such as alpha-4-defensin ($\alpha 4D$) and bactericidal permeability interacting protein (BPI) are neutrophil encoded peptides with potent antimicrobial and endotoxin neutralizing activity. We isolated whole blood RNA from patients enrolled in the Phase I study (see Methods) to quantify changes in the expression of $\alpha 4D$ and BPI over the duration of the study. Relative changes in mRNA expression were compared to levels obtained from pooled blood RNA from 4 normal volunteers. We found no differences in the baseline mRNA expression of $\alpha 4D$ and BPI in patients in the three groups (Figure 5).



At the end of the study, α 4D and BPI levels in the placebo group were unchanged from that observed at baseline. In contrast, in the Lo group, there was a significant increase in α 4D expression by the end of the study (Figure 5A, $p=0.03$). Although BPI levels tended to increase in the Lo group, there was no significant difference between baseline and end of the study (Figure 5B, $p=0.1$). In the Hi group, there were significant increases in mRNA expression of both α 4D ($p=0.002$) and BPI ($p<0.05$). These results are indicative of augmented antimicrobial defenses in patients treated with ascorbic acid infusion.

Discussion

The primary goal of this study was to perform an analysis of novel serum and plasma biomarkers using limited patient laboratory data from a Phase I safety trial that examined intravenous ascorbic acid as a therapy for patients with severe sepsis (ClinicalTrials.gov identifier NCT01434121). The Phase I trial revealed that parenteral ascorbic acid was safe and well tolerated. Further, it reduced the extent of multiple organ failure and attenuated protein biomarkers of inflammation and endothelial injury such as C-reactive protein, procalcitonin and thrombomodulin. We sought to determine whether treatment with intravenous ascorbic acid impacted recently proposed genetic and bone marrow specific biomarkers for sepsis. These include circulating cf-DNA and mtDNA levels, endogenous antimicrobial proteins such as α 4D and BPI, as well as changes in the RDW.

Our study showed that serum cf-DNA values were higher in non-survivors than survivors at baseline (Figure 1). Additionally, cf-DNA levels remained elevated in non-survivors for the duration of the study when compared to survivors. When we compared cf-DNA levels between the three groups we found that patients in the placebo group demonstrated the highest levels of cf-DNA during the first 72 hours (Figure 2). Serum cf-DNA levels in patients receiving ascorbic acid (Lo or Hi ascorbic acid infusion) were relatively lower compared to the placebo group and did not change significantly with time. While these differences did not reach statistical significance, intriguingly, these trends in the cf-DNA levels mirrored the 28 day all-cause mortality between the three groups. Circulating cf-DNA as a biomarker in the critically ill has been explored in several recent studies [13-18]. Based on current evidence, the term cf-DNA implies free DNA fragments released into circulation from necrotic and apoptotic cells. Healthy individuals typically display very low levels of cf-DNA in circulation. In sepsis enhanced apoptosis [19] and cell necrosis [20] result in elevated cf-DNA levels [21]. A recent study by Dwivedi et al showed a very high discriminative capability of cf-DNA to predict mortality among ICU patients with severe sepsis [14]. Although our results agreed with these studies in that cf-DNA levels were higher in non-survivors than in survivors, we could not establish significant distinctions between the three groups being analyzed. A likely cause for this was the small number of patients in this Phase I trial. This was not unexpected since the trial was not designed to yield statistical significance for this parameter. Yet, the trends for higher cf-DNA levels in the placebo group remain in agreement with the mortality data observed in our study. Many different cell types release DNA including neutrophils [22], macrophages [23], and eosinophils [24]. In sepsis, neutrophil extracellular traps (NETs) are formed in response to pro-inflammatory stimuli such as lipopolysaccharide (LPS) and interleukin-8 (IL-8) [22,25] through expulsion of genomic DNA into web-like extracellular structures that ensnare and kill bacteria through their ability to display antimicrobial proteins such as neutrophil elastase and myeloperoxidase [26]. However, it has recently been

proposed that NETs hinder the flow of blood in the microcirculation, producing endothelial damage [22,27] and eventually tissue injury and organ damage [28]. We have previously shown in pre-clinical studies that ascorbic acid attenuates NET formation [10] and multiple organ dysfunction [4] in septic animals. The Phase I trial we have reported shows reduced multiple organ dysfunction in severely septic patients receiving intravenous ascorbic acid [9]. On this basis we can speculate that by reducing the release of NETs, ascorbic acid could attenuate multiple organ damage and mortality in sepsis.

Since cf-DNA is generated both by DNA of nuclear and mitochondrial in origin, we next quantified mtDNA levels using QPCR. We found variability in baseline levels of mtDNA between the three groups; however, these differences were not statistically significant. Over the course of the study, however, there was a trend towards a marginal increase in the mtDNA-74 concentrations in the placebo ($p=0.7$) group (Figure 3). Further, there was also a downward trend in the serum mtDNA-74 levels in the Lo and Hi groups. However, this decline did not reach significance ($p=0.1$). A likely cause for these results was the small number of patients in the Phase I trial, due to the fact that the Phase I trial was a safety trial and not designed to yield statistical significance for this parameter. Unlike cf-DNA, mtDNA is a critical activator of inflammation and the innate immune system. These free mitochondrial nucleotides act as an "alarmin" or Damage Associated Molecular Patterns (DAMPs) when released into the circulation [29,30]. Mitochondrial DAMPs express at least two molecular signatures, formyl peptides and mtDNA that act on formyl peptide receptors and Toll-like receptor 9 respectively [31]. These mediators activate neutrophils in circulation promoting non-specific organ attack and suppressing chemotactic responses to infective stimuli [29]. Recent evidence further suggests that in response to stimuli such as trauma [32] and microbial infection [33] cellular mtDNA levels decrease while circulating cell-free mtDNA levels are increased [34]. To that effect, Nakahira et al showed that mtDNA could serve as a viable plasma biomarker in medical ICU patients and that increased circulating mtDNA was associated with mortality [35]. Our study shows beneficial trends in total mtDNA levels in patients receiving intravenous ascorbic acid. While validating a potential biomarker was not the goal of the study, our data support the notion that mtDNA may serve as a viable biomarker in sepsis.

For over two decades the red cell distribution width (RDW), a quantitative measure of variability in the size of circulating erythrocytes, has been routinely reported as part of the automated complete blood count (CBC). Although the CBC remains among the most frequently ordered diagnostic tests in medicine, the use of this highly mechanized, relatively inexpensive evaluation of the cellular components of peripheral blood remains severely under-utilized as a prognosticating tool. Bazick et al found that the rate of sepsis was higher based on RDW levels, and that RDW was associated with significant risk for blood stream infection [36]. Jo et al recently showed that RDW of non-survivors was higher than that of survivors in severe sepsis and septic shock [37]. Further, these authors showed that RDW was seemingly related to the severity of sepsis and was an independent prognostic factor of 28-day mortality in these patients. Similarly Kim et al found that an increase in RDW from baseline after hospitalization was significantly associated with adverse clinical outcomes [38]. Finally Purtle et al showed that an elevated RDW at the time of discharge was a robust predictor of all-cause patient mortality in critically ill patients [39]. In our study we found that baseline RDWs for all patients were higher than normal (Figure 4). Importantly, RDWs increased only in the placebo group, but not in patients

receiving intravenous ascorbic acid. Purtle et al reported that a RDW exceeding 15.8% was associated with an odds ratio for mortality to be as high as 8.8 (95% CI) [39]. Remarkably, though patients in the Hi ascorbic acid treatment group had baseline RDW values approaching 20, and their RDW values remained unchanged over 96 hours, mortality and organ dysfunction score in this group was lower than the placebo group. Thus, despite the small numbers of patients in this study, our results point to temporal increases in RDW rather than baseline RDW as being important prognosticators of 28 day mortality in sepsis. The pathophysiologic basis to explain a RDW-associated mortality association is not clear, but may reflect the extent of systemic inflammation, since elevated RDW was found to be associated with enhanced biomarkers of inflammation (interleukin-6, CRP, tumor necrosis factor receptors) [40,41]. Additionally, increased oxidative stress may contribute to the RDW-mortality association since reactive oxygen species are known to activate redox-sensitive transcription factors such as nuclear factor- κ B (NF κ B) and stress kinases, which then increase pro-inflammatory mediator expression [42,43]. Since pro-inflammatory cytokines suppress red blood cell maturation and decrease the half-life of red blood cells, it is logical to conclude that pro-inflammatory responses could lead to elevations in RDW. Our pre-clinical studies have shown that ascorbic acid suppresses NF κ B activation and pro-inflammatory gene expression [2,4] in multiple tissues/cell types, thus providing a basis for preservation of RDW in the treatment groups.

Defensins are cationic host defense peptides and in higher species, form part of the complex immune system dedicated to protecting against infection. The α 4D, coded for by the DEFA4 gene is concentrated in human leukocytes and is considered to have the most potent antibacterial activity of the six known human defensins [44]. Human neutrophils also express BPI, which not only has antibacterial action but acts as a chemoattractant and an opsonin that binds to endotoxin (LPS), either to detoxify it or to present it to immune cells to induce an inflammatory response [45]. The promising nature of the action of BPI resulted in the use of a recombinant amino terminal fragment of BPI (rBPI23) as a therapeutic agent for neutralizing endotoxin-induced release of TNF- α p55 and p75 (soluble TNF- α receptors), IL-6, IL-8 and IL-10. Additionally, rBPI23 also prevented endotoxin-induced leucopenia, neutrophil degranulation and activation of coagulation systems [47]. More recently, another recombinant truncated form of BPI, rBPI21 was investigated as an adjunctive for the treatment of children with severe meningococcal sepsis [48]. While rBPI21 did not significantly improve the mortality rate, it had a substantial effect on morbidity with a significant decrease in the frequency of limb amputation in rBPI21-treated patients. In our study we found no differences in α 4D and BPI mRNA levels in the placebo group (Figure 5). In contrast, α 4D expression was increased in both the Lo and Hi ascorbic acid infused groups, while BPI was significantly induced in the Hi ascorbic acid infused group. These results are indicative of augmented antimicrobial defenses in patients treated with ascorbic acid infusion. Further, they are supportive of our pre-clinical and human data which show reduced bacterial burden, attenuated pro-inflammatory responses and reduced mortality following ascorbic acid infusion [2,4,9].

This study has a number of important limitations. First, although we observed significant changes in many of the biomarkers investigated here, the sample size is too small to make far-reaching conclusions on the effectiveness of the intervention. Only a small number of patients were recruited in the study due to limitations in

funding and the nature of the Phase I trial. We are currently funded to perform a larger, multi-center trial that will permit validation of these preliminary findings (ClinicalTrials.gov Identifier NCT02106975). Second, cf-DNA and mtDNA levels are likely to be influenced by pre-existing conditions. The increase in plasma cf-DNA concentrations may be due to increased release of these nucleotides from damaged cells or reduced clearance efficiency especially since these patients have impaired renal and hepatic function. Finally, the increase in cf-DNA could be due to release of DNA by certain strains of bacteria that facilitates their adherence and colonization to surfaces. Our study did not discriminate between endogenous cf-DNA and bacterial DNA in circulation.

This study also has certain advantages. Many previous reports quantified cf-DNA by using QPCR with a housekeeping gene such as β -globin. These studies were limited by detecting only a subset of cf-DNA since they would detect nuclear DNA which contains copies of the β -globin gene, but not mtDNA. Furthermore, the QPCR-based method would likely be influenced by the fragmentation grade of the DNA thus resulting in an underestimation of DNA levels. By using dye binding to DNA and fluorimetric measurement of the change in absorbance, we were able to measure the levels of cf-DNA, independent of the fragmentation grade of the DNA.

Conclusion

It is important to note that this study did not attempt to validate recently identified potential biomarkers of sepsis. Additionally, we believe that the data presented here adds to, rather than replaces, information gleaned from commonly measured physiologic parameters and biomarkers. The goal of the study was to examine potential mechanisms by which intravenous ascorbic acid could alter biomarkers of sepsis in order to attenuate the observed multiple organ dysfunction and mortality in a previously conducted Phase I safety trial [9]. Our work suggests that pharmacologic ascorbic acid repletion reduces the extent of multiple organ failure and attenuates mortality by multiple mechanisms involving potential reductions in circulating cf-DNA and mtDNA levels, augmentation of endogenous antimicrobial proteins such as α 4D and BPI as well as preservation of RDW. Unlike numerous single mediator approaches which have largely been unsuccessful, the data presented here suggests that infusion of ascorbic acid in pharmacological dosages to critically ill patients with sepsis may provide adjunctive therapy in the treatment of severe sepsis.

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Trial Registration

Primary phase I trial (ClinicalTrials.gov identifier NCT01434121).

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