Citrulline: A potential immunomodulator in sepsis

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Background. Sepsis leads to a complex systemic response of cytokines (both pro- and anti-inflammatory) and more recently recognized adipokine mediators. Endothelial nitric oxide (NO) may be a key component in regulating this response, but the pharmacologic manipulation of endothelial NO via L-arginine supplementation or inhibitors has provided inconsistent clinical data related to outcomes. These failures are related to the metabolism of L-arginine in the liver, toxicity of L-arginine, and asymmetric dimethylarginine inhibition, all of which may explain the “arginine paradox.” L-citrulline (CIT) offers a potentially valuable means of supplementing arginine and therefore impacting favorably NO availability. The goal of this study was to determine whether CIT supplementation altered the systemic response of mediators and cytokines in a rat model of sepsis with varying degrees of severity.

Methods. Sepsis was induced with 2 models of cecal ligation and puncture (CLP) of varying severity in Wistar rats. CIT supplementation was provided to half the animals as 8% CIT-supplemented feed for 3 weeks. Baseline mediator levels were assessed in the Wistar rats followed by comparison of the following groups at days 0, 1, and 3: sham-operated; CLP 8-mm (localized); and CLP 12-mm (extensive). The following analyses were performed in the groups: interleukin-6 (IL-6), IL-8, IL-10, resistin, and adiponectin levels (enzyme-linked immunosorbent assay performed in duplicate). L-arginine and CIT were measured with high-performance liquid chromatography combined with mass spectrometry.

Results. Ninety-eight Wistar rats were evaluated, and survival was similar in both sepsis models with and without CIT. Serum IL-6 levels were lower in the CIT/CLP 8-mm group compared to the standard rat chow (STD)/CLP 8-mm group (41 vs 117 pg/mL; \( P = .011 \)) on postoperative day 3. Serum IL-8 and IL-10 responses were similar across all groups. Serum resistin levels were lower in the CIT/CLP 12-mm group compared to the STD/CLP 12-mm group in the more severe sepsis model on day 3 (19 vs 38 ng/mL; \( P < .0001 \)). The levels of serum L-arginine were greater in the CIT-supplemented animals compared to STD rodent diet animals before surgical insult (86.3 vs 294.0 \( \mu \)M; \( P = .004 \)). Adiponectin was not affected by CIT supplementation.

Conclusion. CIT may decrease the proinflammatory mediator response (IL-6 and resistin) without impairing the secretion of anti-inflammatory mediators (IL-10 and adiponectin) and thereby provide a safe means of immunomodulation that preserves the anti-inflammatory mediator response. (Surgery 2011;150:744-51.)

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Sepsis leads to a complex systemic response of cytokines (both pro- and anti-inflammatory) and more recently recognized adipokine mediators, and is a clinical syndrome with high mortality. Mortality and morbidity may be explained by an overreactive, proinflammatory host immune response and the diminished availability of endothelial-derived nitric oxide (NO). Healthy endothelium is essential to all organ systems within the body with its active role in vascular homeostasis (vasodilatation, inhibition of platelet aggregation, and leukocyte adhesion). Endothelial-derived NO is synthesized from endothelial NO synthase (eNOS), and the cofactors that are thought to promote and stabilize the active...
form of eNOS are arginine, tetrahydrobiopterin (BH4), and heme. While eNOS has the positive effects mentioned above, inducible NO synthase (iNOS) may aggravate endothelial dysfunction and tissue injury by outstripping the supply of arginine, resulting in the production of superoxides. Both in vitro and in vivo studies have reported that the administration of L-arginine increases bioactivity of endothelial-derived NO, but because of its limited bioavailability it has provided inconsistent clinical data related to outcomes. These failures are thought to be related to the metabolism of L-arginine in the liver, the toxicity of L-arginine, and asymmetric dimethylarginine (ADMA) inhibition, all of which may explain the “arginine paradox.” Alternatively, the nonessential amino acid L-citrulline (CIT) does not encounter this same fate because it is converted to L-argininosuccinate and subsequently to L-arginine in the kidney. CIT may be able to serve clinically as a precursor for enhanced L-arginine function. There has been limited investigation of the impact of CIT administration in sepsis; however, some data suggest that oral CIT may lead to greater increases in plasma L-arginine concentrations than the administration of L-arginine itself.

In addition to the proinflammatory (−6 and −8) and anti-inflammatory (−10) interleukins, various new endocrine mediators (eg, resistin and adiponectin) may provide additional targets for immunomodulation. Resistin was reported originally in a murine model and was felt to be an adipose tissue–specific hormone. Subsequent work has determined that the majority of resistin is derived from macrophages rather than adipocytes. Resistin promotes a proinflammatory response by modulating the enhancement of activated B cells by nuclear factor kappa-light chain (NF-κB). A recent study has identified resistin as a marker for acute inflammatory response and possibly a prognostic marker in critically ill patients without sepsis. Adiponectin is an adipocyte-derived hormone that is secreted into circulating blood and regulates systemic metabolism. Adiponectin improves whole-body insulin sensitivity in animal models and diet-induced obesity and stimulates fatty acid oxidation and glucose uptake in skeletal muscle by increasing peripheral insulin sensitivity. Few studies have evaluated adiponectin as a potential acute phase mediator, which would be important because it is also thought to regulate biologic processes, such as apoptosis, proliferation, migration, and inflammation.

The goal of this study was to determine whether CIT supplementation alters L-arginine.

**Fig 1.** Mean serum L-arginine values (μM ± SD) comparing standard rodent diet (STD) to citrulline-supplemented diet (CIT) in sham, 8-mm cecal ligation and puncture (CLP 8mm), and 12-mm cecal ligation and puncture (CLP 12mm) models of sepsis. POD1, Postoperative day 1; POD3, postoperative day 3.
levels and a panel of systemic response mediators in a rat model of sepsis with varying degrees of severity.\textsuperscript{15}

METHODS

Approval of the Institutional Animal Care and Use Committee (IACUC) was obtained before this study. We used 2 separate models of severity by the technique of cecal ligation and puncture (CLP) in 350-g Wistar rats (Charles River Laboratories, Wilmington, MA). Animals were acclimated and had access to water and rodent chow ad libitum. Isoflurane anesthesia 5% was used for induction and 3% for maintenance during procedures. Postoperative analgesia consisted of buprenorphine 0.1 mg/kg every 8 hours. The CIT groups were provided a diet supplemented with 8% citrulline (Frontier Distributing, Inc, Oxford, MI) for 3 weeks before the induction of sepsis and postoperatively. The nontreated groups were provided a standard rodent diet (STD) pre- and postoperatively. The study design allocated animals to 6 groups: sham operated/CIT; sham operated/STD; CLP 8-mm (localized)/CIT; CLP 8-mm (localized)/STD; CLP 12-mm (extensive)/CIT; and CLP 12-mm (extensive)/STD.

Two models of varying degrees of treated sepsis severity were performed. The operative procedure was performed via a 2-cm incision on the anterior abdominal wall for cecal extraction and manipulation. The 8-mm model used cecal ligation 8 mm proximal to the tip of the cecum with 2-0 polyglycolic acid suture. A single puncture through the wall of the cecum was performed using a 19-gauge needle. Cecal content was extracted through the puncture holes and smeared onto the cecum. The 12-mm model was performed in a similar fashion, but 3 through and through puncture holes were made in the cecum, followed by extracting cecal content and creating a cecal slurry with 2 mL of normal saline for application into the abdominal cavity. The incision was then closed in 2 layers using 4-0 polypropylene (Prolene; Ethicon, Inc, Somerville, NJ) and 3-0 polyglactin (Vicryl; Ethicon, Inc, Somerville, NJ) sutures. The sham animals underwent a similar laparotomy and cecal manipulation without ligation, puncture, or application of cecal contents. In order to more closely mimic the clinical situation, we treated the animals with imipenim (14 mg/kg) and normal saline resuscitation (20 mL/kg) subcutaneously on induction and every 8 hours until animals were killed.

Cardiac puncture and exsanguination was performed under 5% isoflurane anesthesia before the

Fig 2. Mean serum L-citrulline values (\textmu M ± SD) comparing standard rodent diet (STD) to citrulline-supplemented diet (CIT) in sham, 8-mm cecal ligation and puncture (CLP 8mm), and 12-mm cecal ligation and puncture (CLP 12mm) models of sepsis. POD1, Postoperative day 1; POD3, postoperative day 3.
lethal injection of pentobarbital (1 mL/10 lbs). Whole blood was collected in 5-mL vials containing ethylenediaminetetraacetic acid, centrifuged at 3,000 rpm for 10 minutes, and serum was aliquoted and stored at –80°C. The mediator panel used in the study included IL-6, IL-8, IL-10, resistin, and adiponectin. All assays were performed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocols (BioVentor LLC, Candler, NC) in duplicate. CIT and L-arginine levels were measured using high-performance liquid chromatography combined with mass spectrometry. All levels were assessed at day 0 (baseline) and postoperative days 1 (POD1) and 3 (POD3). Parametric 2-sample t and Mann–Whitney U tests were used for statistical analysis. Continuous data are presented as the mean ± standard deviation.

RESULTS

Ninety-eight Wistar rats (n = 7 per group) were evaluated, and survival was 90% in both sepsis models with and without CIT. The levels of L-arginine were greater in the CIT-supplemented animals compared to STD diet animals before operative insult (86.3 vs 294.0 μM; P = .004). This statistical difference was maintained through POD1 (100.1 vs 206.3 μM; P = .004) and POD3 (85 vs 222.9 μM; P = .003) in the sham/CIT model and but only reached statistical significance on POD3 in the CLP8/CIT model (81.6 vs 199.2 μM; P = .016). The more severe model showed similar trends (Fig 1). The levels of CIT were greater on POD1 (5.9 vs 31.9 μM; P = .004) and POD3 (5.9 vs 22.7 μM; P = .004) in the sham/CIT model. Interestingly, in both models of sepsis, CIT levels were lower in the CLP8/CIT group on POD1 (8.4 vs 4.1 μM; P = .006) and on POD1 and POD3 in the CLP12/CIT group (3.8 vs 0.4 μM; P = .003; and 3.5 vs 0.5 μM; P = .003; Fig 2).

The response of the inflammatory mediators varied by the marker studied over time, with an impact only on the proinflammatory mediators. We found no difference in either the pro- or anti-inflammatory mediator response related to the 2 sepsis models; however, IL-6 levels were lower in the CLP8/CIT group compared to the CLP8/STD group (41 vs 117 pg/mL; P = .011) on POD3.

![Image](image-url)
Resistin levels were lower in the CLP12/CIT group compared to the CLP12/STD group on POD3 (19 vs 38 ng/mL; \( P < .0001 \); Fig 4). Conversely, there were no differences in the anti-inflammatory mediators IL-10 and adiponectin, regardless of CLP or CIT supplementation (Tables I and II).

DISCUSSION

Sepsis can be defined as an overreactive systemic response to an infection with often detrimental effects. Attempts to suppress the sepsis response have been limited to treatment of the septic focus and support of the patient’s physiology while awaiting physiologic recovery. Better understanding of the molecular pathogenesis of sepsis has led to theories related to immunomodulation as a means of controlling this response. There are current data to support the concept that L-arginine availability plays a key role in maintaining the balance of oxidants and antioxidants necessary for eNOS in the healthy state. In sepsis, plasma and intracellular muscle levels of L-arginine are decreased, compared to healthy subjects. Arginine is a semi-essential amino acid that is primarily synthesized endogenously in the proximal renal tubule by conversion of CIT to arginine, and under normal circumstances contributes to about 10% to 15% of whole-body arginine production. In sepsis, de novo production of arginine is decreased—in addition to increasing iNOS production—resulting in the outstripping of arginine availability. This response results in the production of reactive oxygen species (ROS) and oxidative stress, which promotes a proinflammatory response (IL-6, IL-8, tumor necrosis factor-\( \alpha \), and resistin). By providing enough arginine for eNOS production via CIT supplementation, oxidative stress and the production of proinflammatory cytokines may be decreased.

Our data are unique in 4 ways. First, these data show the ability of oral CIT supplementation to enhance L-arginine levels in the Wistar rat. The amino acid levels decrease initially and then recover partially in the septic animals compared to the nonseptic animals, likely because of the impaired ability to maintain dietary consumption and ongoing use related to the insult. Second, we showed that

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**Fig 4.** Mean serum resistin values (ng/mL ± SD) comparing standard rodent diet (STD) to citrulline-supplemented diet (CIT) in sham, 8-mm cecal ligation and puncture (CLP 8mm), and 12-mm cecal ligation and puncture (CLP 12mm) models of sepsis. POD1, Postoperative day 1; POD3, postoperative day 3.
Table I. Serum values for cytokines, mediators, and amino acids at baseline, postoperative day 1, and postoperative day 3 for sham-operated groups

<table>
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<tr>
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<td></td>
<td></td>
<td>CIT</td>
<td>STD</td>
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<td>CIT</td>
<td>STD</td>
<td>P value</td>
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<td>STD</td>
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<td></td>
<td></td>
<td>465 (437)</td>
<td>207 (175)</td>
<td>NS</td>
<td>163 (130)</td>
<td>66 (28)</td>
<td>NS</td>
<td>23 (9)</td>
<td>276 (404)</td>
<td>NS</td>
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<tr>
<td>IL-8 (pg/mL)</td>
<td></td>
<td>32 (33)</td>
<td>18 (18)</td>
<td>NS</td>
<td>31 (17)</td>
<td>26 (19)</td>
<td>NS</td>
<td>55 (49)</td>
<td>104 (52)</td>
<td>NS</td>
<td></td>
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<td>IL-10 (pg/mL)</td>
<td></td>
<td>58 (43)</td>
<td>368 (447)</td>
<td>NS</td>
<td>23 (17)</td>
<td>41 (17)</td>
<td>NS</td>
<td>91 (69)</td>
<td>75 (36)</td>
<td>NS</td>
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<td>Resistin (ng/mL)</td>
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<td>27 (13)</td>
<td>34 (6)</td>
<td>NS</td>
<td>26 (4)</td>
<td>32 (12)</td>
<td>NS</td>
<td>18 (4)</td>
<td>25 (4)</td>
<td>NS</td>
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<tr>
<td>Adiponectin (ng/mL)</td>
<td></td>
<td>620 (252)</td>
<td>1252 (520)</td>
<td>NS</td>
<td>1033 (296)</td>
<td>899 (349)</td>
<td>NS</td>
<td>971 (177)</td>
<td>994 (102)</td>
<td>NS</td>
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| LC/MS         | L-arginine (µM) | 294.0 (90.1)| 86.3 (12.8)| .004     | 206.5 (46.7)| 100.1 (29.5)| .004     | 222.9 (44.3)| 85.0 (21.9)| .003     |          |          |          |
|               | L-citrulline (µM) | 30.9 (25.9)| 8.5 (5.5)  | NS       | 31.9 (13.3)| 5.9 (2.5)  | .004     | 22.7 (15.4)| 5.9 (1.4)  | .004     |          |          |          |

Values in parentheses are standard deviations.
CIT, Citrulline-supplemented diet; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LC/MS, liquid chromatography/mass spectrometry; NS, not significant; STD, standard rodent diet.

Table II. Serum values for cytokines, mediators, and amino acids on postoperative days 1 and 3 for 8- and 12-mm cecal puncture and ligation sepsis models

<table>
<thead>
<tr>
<th>ELISA</th>
<th>CLP 8-mm</th>
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<td></td>
<td>Day 1</td>
<td>Day 3</td>
<td>P value</td>
<td>Day 1</td>
<td>Day 3</td>
<td>P value</td>
<td>Day 1</td>
<td>Day 3</td>
<td>P value</td>
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<td>Day 3</td>
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<td>IL-6 (pg/mL)</td>
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<td></td>
<td></td>
<td>43 (10)</td>
<td>150 (107)</td>
<td>NS</td>
<td>42 (14)</td>
<td>118 (59)</td>
<td>.011</td>
<td>75 (70)</td>
<td>81 (88)</td>
<td>NS</td>
<td>57 (24)</td>
<td>92 (84)</td>
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<tr>
<td>IL-8 (pg/mL)</td>
<td></td>
<td>246 (180)</td>
<td>299 (161)</td>
<td>NS</td>
<td>45 (27)</td>
<td>56 (60)</td>
<td>NS</td>
<td>366 (167)</td>
<td>326 (200)</td>
<td>NS</td>
<td>85 (60)</td>
<td>103 (54)</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
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<td>155 (153)</td>
<td>43 (25)</td>
<td>NS</td>
<td>90 (58)</td>
<td>118 (165)</td>
<td>NS</td>
<td>250 (159)</td>
<td>127 (55)</td>
<td>NS</td>
<td>66 (36)</td>
<td>101 (114)</td>
</tr>
<tr>
<td>Resistin (ng/mL)</td>
<td></td>
<td>48 (7)</td>
<td>55 (12)</td>
<td>NS</td>
<td>20 (4)</td>
<td>19 (2)</td>
<td>NS</td>
<td>58 (6)</td>
<td>47 (12)</td>
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<td>19 (5)</td>
<td>38 (8)</td>
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<tr>
<td>Adiponectin (ng/mL)</td>
<td></td>
<td>1118 (161)</td>
<td>1067 (174)</td>
<td>NS</td>
<td>658 (195)</td>
<td>715 (116)</td>
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<td>939 (176)</td>
<td>599 (316)</td>
<td>NS</td>
<td>601 (292)</td>
<td>903 (304)</td>
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| LC/MS         | L-arginine (µM) | 124.6 (65.4)| 75.1 (15.9)| NS       | 199.2 (50.3)| 81.6 (26.9)| .016     | 59.6 (13.8)| 60.6 (8.2)| NS       | 141.9 (47.8)| 88.0 (36.0)| NS       |
|               | L-citrulline (µM) | 4.1 (5.4) | 4.0 (2.6)| NS       | 0.5 (0.1) | 7.2 (1.6) | .006     | 0.4 (0.1) | 3.8 (1.4) | .003     | 0.5 (0.1) | 3.5 (1.5) | .001     |

Values in parentheses are standard deviations.
CIT, Citrulline-supplemented diet; CLP 8-mm, 8-mm cecal ligation and puncture; CLP 12-mm, 12-mm cecal ligation and puncture; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LC/MS, liquid chromatography/mass spectrometry; NS, not significant; STD, standard rodent diet.
CIT supplementation impacted the proinflammatory mediator response by decreasing IL-6 and resistin levels for both degrees of sepsis. Third, we found similar levels over time and across the degrees of sepsis for the anti-inflammatory mediators adiponectin and IL-10. Finally, unlike the sham animals, where the CIT levels remained increased throughout the study, in the septic models, there was a decrease in serum CIT but a consistent increase in serum arginine. This preliminary finding may be indicative of CIT consumption during sepsis to support the availability of arginine. Because we relied on oral intake, the septic animals may not have been able to consume sufficient CIT to keep up with this consumption.

These findings may have been related to the high survival rate in our treatment model. The clinical signs of sepsis for both models were most prominent in the first 24 to 48 hours with lethargy, piloerection, edema, exudate around the eyes, loss of interest in surroundings, and most importantly decreased oral intake. Because these animals were treated with imipenem and fluid resuscitation, clinical improvement was noted, and oral intake resumed before POD3. This observation correlated with our identification of lower serum levels of IL-6 and resistin on POD 3 in CIT-supplemented groups. There is also a possibility that our treatment protocol of fluid resuscitation and antibiotics may have contributed to the response of the anti-inflammatory mediators.

A weakness of our study is the reliance on ad libitum consumption of the CIT-supplemented rodent diet during the septic phase. This assumption is confirmed by the decrease in L-arginine levels on POD1 and recovery at POD3 as the animals' clinical condition improved. The sham animals consumed consistently the diet after surgery and did not experience a similar decrease in serum amino acid levels. Therefore, the benefits of preinsult supplementation may have waned by decreased bioavailability of CIT and a subsequent decrease in L-arginine. It is likely that these amino acids were also consumed at greater rates during the septic phase. Support of CIT administration during sepsis by either intravenous or gavage feeding may have further enhanced the benefits of CIT. A second weakness is that function of NO was not measured directly in the study. There is a significant body of work to support the impact of CIT on NO function; however, the correlation of NO and markers of inflammation will provide a greater understanding of the underlying mechanisms of organ dysfunction in sepsis. Finally, we did not measure the metabolic pathways for the administered CIT, which is a focus of our ongoing work.

REFERENCES
DISCUSSION

Dr Daniel Deziel (Chicago, IL): I would like to thank Dr Asgeirsson and his colleagues. The authors point out that a major challenge in sepsis is lack of a biologically effective arginine and that this prevents nitric oxide regulation of several functions. They hypothesize that citruline might provide a more stable source of arginine and thus improve outcomes in septic shock.

Using a rat model, they found a significant decrease in interleukin (IL)-6 and resistin at 2 points in each of the experimental groups. They conclude that citruline may preferentially reduce proinflammatory cytokines but not antiinflammatory cytokines.

So, I have a few questions. You looked at several possible mediators. I am curious why you did not choose to assess IL-1β levels, because this cytokine is often involved in gram-negative sepsis. Second, do you have any evidence that citruline supplementation actually affected either arginine or nitric oxide levels in these rats?

Because there was no difference in outcomes between the groups that were treated with citruline or without, what do you think is the potential impact of this? Have you looked at this in other models, or as a way perhaps to look at a postinjury model, rather than as a preinjury supplementation? That would be more akin to the clinical situation.

Dr Theodor Asgeirsson (Los Angeles, CA): The first question was about IL-1β, when we were choosing our panel of mediators to look at, we wanted to accentuate the data that we had. For example, IL-1β, which has been looked at previously. What struck us is that IL-1β, especially in a pediatric sepsis population, has not been shown to change with clinical improvement.

So it is a later phase reactant response than some of the more acute mediators that have been looked at. So we thought that the value of that for this study was not there. So we chose IL-6 and resistin as the acute inflammatory mediators.

But I think it is likely a worthwhile endeavor to look at the whole gamut of mediators in the citruline response, and that is probably what we are going to aim for in our next series of studies about this.

The second question was about measurements of nitric oxide and whether we did affect that. It is very hard to measure nitric oxide because of its half-life, and there are multiple indirect ways to measure nitric oxide. We did do a panel of measuring nitrate and nitrite, and that did not show the same trends as the full inflammatory mediators did.

In addition to nitrates, which is very intriguing, is the arginine AVMA ratio, which can be an indirect measurement of nitric oxide function. We did measure that, which is a pretty complex procedure, because we have to use mass spectrometry and high-performance liquid chromatography. We did see trends related to the proinflammatory trends, both in the localized sepsis model and in the severe sepsis model. We have currently an abstract in the makings for that data, which is additional to this.

Finally, the models that we worked with, this was essentially a rat survival model, so we kept them alive for the whole procedure. So with relations to outcomes, the only thing that we could do was look at the rats objectively during these 72 hours and see if the citruline group behaved differently than the other. We could not, on those kind of measurements, see any difference.

In addition to doing serum samples, we took heart, lung, and kidneys, and we are currently in the phase of analyzing that data and see if we can see true end-organ changes in function in relation to citruline supplementation.