Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat

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Armour, John, Karel Tyml, Darcy Lidington, and John X. Wilson. Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. *J Appl Physiol* 90: 795–803, 2001.—Septic patients have low plasma ascorbate concentrations and compromised microvascular perfusion. The purpose of the present experiments was to determine whether ascorbate improves capillary function in volume-resuscitated sepsis. Cecal ligation and perforation (CLP) was performed on male Sprague-Dawley rats. The concentration of ascorbate in plasma and urine, mean arterial blood pressure, and density of continuously perfused capillaries in the extensor digitorum longus muscle were measured 24 h after surgery. CLP caused a 50% decrease (from 56 ± 4 to 29 ± 2 μM) in plasma ascorbate concentration, 1,000% increase (from 46 ± 13 to 450 ± 93 μM) in urine ascorbate concentration, 20% decrease (from 115 ± 2 to 91 ± 2 mmHg) in mean arterial pressure, and 30% decrease (from 24 ± 1 to 17 ± 1 capillaries/mm) in the density of perfused capillaries, compared with time-matched controls. A bolus of intravenous ascorbate (7.6 mg/100 g body wt) administered immediately after the CLP procedure increased plasma ascorbate concentration and restored both blood pressure and density of perfused capillaries to control levels. In vitro experiments showed that ascorbate (100 μM) inhibited replication of bacteria and prevented hydrogen peroxide injury to cultured microvascular endothelial cells. These results indicate that ascorbate is lost in the urine during sepsis and that a bolus of ascorbate can prevent microvascular dysfunction in the skeletal muscle of septic animals. Our study supports the view that ascorbate may be beneficial for patients with septic syndrome.

vitamin C; blood pressure; antioxidant; capillary; septicemia

THE MAJORITY OF DEATHS AMONG critically ill patients requiring intensive care are attributable to sepsis, systemic inflammatory response syndrome, and acute respiratory distress syndrome (8, 16). These pathologies are associated with severe oxidative stress and cardiovascular symptoms, particularly systemic hypotension, maldistribution of blood flow in organs, and impaired microvascular control of tissue oxygenation. For instance, microvascular permeability increases in skeletal muscle of patients with severe sepsis (27). Microvascular dysfunction may compromise tissue nutrient flow and contribute to the development of multiple organ dysfunction syndrome.

Septic impairment of the microcirculation can be studied in animal models. For instance, the capillary blood flow distribution (21, 32) and the vasodilator response to acetylcholine (41) in hindlimb muscle are impaired in the rat cecal ligation and perforation (CLP) model of volume-resuscitated sepsis. This dysfunction may be caused by an excessive production of oxidants. Oxidative stress is detectable in skeletal muscle soon after the CLP procedure, with inhibition of mitochondrial respiration and stimulation of hydrogen peroxide production becoming evident within 12 h (22). Furthermore, CLP decreases the activities of the antioxidant enzyme Mn superoxide dismutase, catalase, and glutathione peroxidase in muscle (22). Conversely, survival is improved in CLP rats treated with superoxide dismutase and catalase (34). It is not known whether antioxidants can preserve microvascular function after CLP.

The most abundant endogenous antioxidant in the aqueous phase is ascorbate, which is the reduced form of vitamin C. Circulating levels of ascorbate are decreased in patients with sepsis or septic syndrome (11, 24). This may be important for the development of septic syndrome because ascorbate has direct bacteriostatic effects (36, 46) and is also required for the bactericidal activity of neutrophils (15). Additionally, ascorbate is essential for normal endothelial function (23).

The purpose of the present experiments was to determine whether acute administration of ascorbate improves functional capillary density in an animal model of volume-resuscitated sepsis. We characterized the changes in ascorbate concentration occurring after CLP and determined that bolus infusion of ascorbate can maintain normal microvascular blood flow in skeletal muscle of septic rats.

MATERIALS AND METHODS

Animal preparation. The experimental protocol was approved by the University of Western Ontario Council on

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Animal Care. Male Sprague-Dawley rats (300–350 g) were divided into control and sepsis groups. Sepsis was induced by the CLP procedure (41). Briefly, rats were anesthetized with a mixture of halothane (1–2%) and oxygen (remainder) throughout the procedure. An intravenous catheter (PE-50) was inserted into the jugular vein for the administration of ascorbate and the saline vehicle. A right carotid artery catheter (PE-50) was placed in the ascending aorta to permit withdrawal of blood samples and measurement of blood pressure. Both catheters were tunneled through a posterior neck incision and attached to a swivel harness to allow free movement of the rat in its cage. In the CLP group, a midline laparotomy was performed. The cecum was isolated and ligated distal to the ileocecal valve to maintain bowel integrity. The cecum was punctured twice with an 18-gauge needle and returned to the peritoneal cavity, and then the abdominal incision was closed with sutures. Control rats were only catheterized to permit infusion, because sepsis was defined in the present study as the outcome of the laparotomy and CLP procedures.

Experimental protocol. The animals were allowed to acclimatize for 30 min after surgery, and arterial blood pressure was measured. Subsequently, the animals were infused intravenously for 30 min with 3 ml of ascorbate (7.6 or 76 mg/100 g body wt) solution or heparinized saline vehicle. These doses of ascorbate were selected because they had been evaluated previously in a rat model of skeletal muscle ischemia-reperfusion injury (19). We expected that the low dose would confer a beneficial effect, and we included the high dose to evaluate possible toxic actions. At the end of this rapid infusion (i.e., 1 h after surgery), a blood sample (0.7–0.8 ml) was collected and centrifuged (4°C, 10 min) to obtain plasma for measurement of hemoglobin, ascorbate, and urate levels. Next, the carotid catheter was infused with a mixture of saline (10 ml·kg^{-1}·h^{-1}), fentanyl (analgesic; 10 μg·kg^{-1}·h^{-1}), and heparin (0.5 U/h) via an infusion pump for an additional 23 h.

Twenty-four hours after surgery, the rats were removed from the infusion pump, and blood pressure was measured for 30 min. A second blood sample (0.25 ml) was collected for analysis of blood gases, pH, bicarbonate, lactate, and hemoglobin. Then a final blood sample (0.7–0.8 ml) was collected for measurement of plasma hemoglobin, ascorbate, and urate concentrations.

The effects of CLP and ascorbate on functional capillary density in skeletal muscle were determined by intravital videomicroscopy. The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (65 mg/kg; Somnotol, MTC Pharmaceuticals), with subsequent injections every 30–60 min to maintain the anesthetized state. Body weight was recorded, and a thermocouple probe (Tele-Thermometer, Yellow Springs Instruments) was inserted rectally to measure core body temperature. Rats were placed on the stage of an intravital microscope (Leitz, ELR) that was equipped with a heating pad (K20C, American Medical System) and lamp to maintain core body temperature at 37°C. The extensor digitorum longus (EDL) muscle was exposed, and its surface was covered by a plastic coverslip. The muscle surface was epi-illuminated with a Schott SL 1500 fiber-optic light source. The microcirculation was allowed to stabilize for 30 min before recordings were made. The surface temperature of the muscle remained at the physiological temperature of 32°C throughout the recording period. Capillary blood flow was visualized at the final magnification of ×240 by using a closed-circuit television system (Panasonic MV5410 monitor, MTI camera, Panasonic Omnivision VHS recorder). Five fields of view of the microcirculation were selected systematically for examination in each rat. Each field was recorded for 1 min. Subsequently, the videotaped records were analyzed along three lines per field to identify capillaries perfused continuously with red blood cells, intermittently perfused (defined as capillaries exhibiting transitions between high velocity and cessation of blood flow), or not perfused (defined as capillaries with stationary red blood cells) during the 1-min observation period. Results were expressed as the number of capillaries per millimeter of test line. Additionally, red blood cell velocity in capillaries was measured by using the video flying-spot technique (41). At the end of the videomicroscopic recording period, the rats were euthanized, and bladder urine was collected.

Blood gases, pH, bicarbonate, and hemoglobin levels were measured by using an Acid Base Lab 3 blood analysis instrument. Blood lactate concentration was measured by using a Yellow Springs Instruments model 2300 STAT Plus analyzer. Plasma hemoglobin concentration was measured spectrophotometrically by using a commercial kit (Sigma Diagnostics).

To measure the concentrations of ascorbate and urate, 3,4-dihydroxybenzylamine (100 μM final concentration) was added to the samples as an internal standard to monitor recovery. Next, the samples were extracted with methanol, and the extracts were stored at −80°C. Ascorbate, urate, and 3,4-dihydroxybenzylamine concentrations were determined by using a HPLC-based assay with electrochemical detection (44). We assumed that the amount of 3,4-dihydroxybenzylamine lost during handling procedures (i.e., extraction, storage, HPLC assay) was proportional to the amount of ascorbate and urate lost and that the percent recovery of this internal standard could be used to estimate the recovery of these other reductants. The recovery of 3,4-dihydroxybenzylamine was calculated by comparing the amount in the sample to an external standard curve and was found to be 93 ± 3% for plasma ($n = 37$) and 104 ± 7% for urine ($n = 19$). Ascorbate and urate concentrations were calculated by interpolation on an external standard curve and corrected for percent recovery of 3,4-dihydroxybenzylamine.

Protection by ascorbate after CLP could occur on a number of levels. Two potential mechanisms are *1*) inhibition of bacterial replication at the site of infection, and *2*) antioxidant defense at the level of the endothelium. We carried out two in vitro experiments to evaluate these potential mechanisms. First, the effect of ascorbate on the replication of fecal bacteria was determined in vitro. Miller's Luria LB agar plates were pretreated by adding 1 ml of cold ascorbate solutions (200 and 2,000 μM) to the plates and incubating them at 4°C 2 h before excess fluid was removed. Fecal samples obtained from the rat cecum were suspended in deionized water at a concentration of 8 mg/ml. These suspensions were incubated with 0, 100, or 1,000 μM ascorbate for 30 min (37°C). Subsequently, 40-μl aliquots of the suspensions were added to the plates and incubated for 8 h before the number of bacterial colonies was counted.

We also evaluated the ability of intracellular ascorbate to protect microvascular endothelial cells against oxidative stress in vitro. Cultures of microvascular endothelial cells were prepared from hindlimb muscle of adult male rats according to the procedure of Wilson et al. (44). Cultures were used for experiments at *passage 7*. These cultures tested positively for coagulation factor VIII antigen expression and *Griffonia simplicifolia* lectin I isolectin B4 binding, as is characteristic of microvascular endothelial cells. The cells did not synthesize ascorbate de novo, but they accumulated ascorbate when it was added to the culture medium (44).

To assess the antioxidant effect of intracellular ascorbate, endothelial cells were loaded by incubation with a physiological concentration of ascorbate (100 μM). The ascorbate was dissolved in culture medium containing homocysteine (final concentration, 80 μM), a reductant that slows ascorbate oxidation. Freshly prepared ascorbate (final concentration, 100 μM) was added to the cultures again after 16 h. Ascorbate loading was terminated 19 h after the initial exposure to the vitamin by washing the cells with Dulbecco's phosphate-buffered saline (catalog number 14040–133, Gibco BRL). Control cells were incubated with growth medium containing homocysteine but no ascorbate. All cells were incubated for an additional 1 h in ascorbate-free medium. Subsequently, some of the cultures were harvested for assay of intracellular ascorbate concentration by the HPLC-based assay with electrochemical detection (44). The intracellular ascorbate concentration was calculated on the basis of a cell water content of 4 μl/mg protein (44). After the ascorbate-loading incubation, the cells were incubated for 1 h with or without 100 μM hydrogen peroxide in culture medium containing the fluorescent probe ethidium bromide (1.25 μg/ml; Molecular Probes, Eugene, OR). Cells were examined by bright-field and fluorescent microscopy, and injury was assessed on the basis of nuclear permeability to ethidium bromide. Additionally, ultrastructural damage was observed by electron microscopy. The cell monolayers were fixed with 2% glutaraldehyde, postfixed in buffered 1% OsO_4, dehydrated in alcohol, and embedded in epoxy resin. Ultrathin sections (70–90 Å), contrasted with uranyl acetate and lead citrate, were examined with a Philips electron microscope 410 and photographed.

Statistical analysis. Data are presented as means ± SE for n independent experiments. The effects of a single level of treatment were evaluated by Student's pooled t-test or paired t-test. The effects of multiple levels of treatment on biochemical and cardiovascular parameters were assessed by ANOVA and the Tukey-Kramer test. The χ^2 test was used to evaluate the effects of treatments on mortality rates. $P <$ 0.05 was considered significant.

RESULTS

Biochemical measurements. Blood hemoglobin content, bicarbonate concentration, pH, P_{O_2}, and P_{CO_2} were not affected by CLP or by infusion of the low dose of ascorbate (Table 1). For technical reasons, these parameters were not measured in the rats that received the high dose of ascorbate. Blood lactate concentration was not changed significantly by CLP or by the

Table 1. *Systemic blood biochemical parameters*

	Control-Saline	CLP-Saline	Control-Ascorbate	CLP-Ascorbate
pH	7.43 ± 0.02	7.41 ± 0.02	7.44 ± 0.03	7.44 ± 0.02
Hb, g/l	140 ± 10	120 ± 10	130 ± 30	140 ± 10
P_{O_2}, Torr	113 ± 4	101 ± 7	83 ± 11	102 ± 13
P_{CO_2}, Torr	29 ± 3	37 ± 1	38 ± 4	32 ± 3
HCO_3^-, mM	26 ± 1	28 ± 1	25 ± 1	27 ± 1
Lactate, mM	0.74 ± 0.10	0.97 ± 0.13	1.19 ± 0.30	0.90 ± 0.14

Values are mean ± SE for n = 4–9 rats/group. A bolus infusion of the low dose of ascorbate (7.6 mg/100 g body wt) or saline vehicle was administered 30 min after catheterization (control-saline and control-ascorbate rats) and cecal ligation and perforation (CLP) surgery (CLP-saline and CLP-ascorbate rats). Systemic blood parameters were measured 24 h after surgery. Based on ANOVA, there were no statistically significant differences among the 4 groups.

Table 2. *Plasma urate and hemoglobin concentrations in control and CLP rats*

	Urate, μM		Hemoglobin, mg/dl	
Animal Group	1 h	24 h	1 h	24 h
Saline-infused				
Control	15 ± 4	10 ± 1	13 ± 8	5 ± 5
CLP	12 ± 3	10 ± 1	12 ± 8	10 ± 3
Ascorbate-infused (7.6 mg/100 g)				
Control	18 ± 9	21 ± 6	9 ± 6	7 ± 2
CLP	11 ± 1	14 ± 4	9 ± 2	9 ± 8

Values are mean ± SE for n = 3–7 rats/group. Rats were treated as described in Table 1. Based on ANOVA, there were no statistically significant effects of time or CLP.

low dose (Table 1) or high dose (data not shown) of ascorbate. Furthermore, there were no significant effects of CLP or ascorbate infusion on plasma urate or hemoglobin levels (Table 2).

The plasma concentration of ascorbate decreased by 50% between 1 and 24 h after CLP in the volume-resuscitated rats (Fig. 1). In contrast, plasma ascorbate levels did not change in the nonseptic control rats during the same period.

Infusion of the low dose of ascorbate (7.6 mg/100 g) elevated plasma concentrations of this antioxidant. In both CLP and nonseptic control rats, plasma ascorbate levels 1 h after the start of ascorbate infusion were
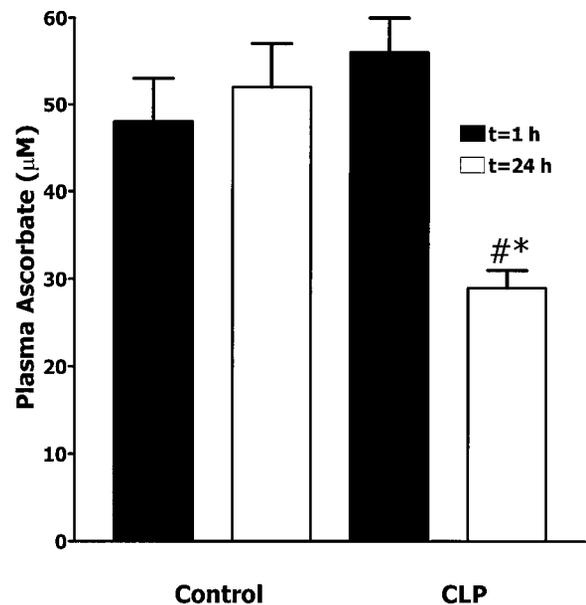

Fig. 1. Plasma ascorbate concentrations in saline-resuscitated cecal ligation and perforation (CLP) and control rats. Beginning 30 min after surgery, the animals were infused intravenously for 30 min with 3 ml of heparinized saline. They were infused subsequently with fentanyl in heparinized saline (10 ml·kg^{-1}·h^{-1}) for an additional 23 h. Values are means ± SE for the plasma ascorbate concentration 1 and 24 h after surgery (n = 6–11 rats) for control and CLP rats. t, Time. #$P <$ 0.05, ascorbate concentration at 24 h compared with that in the same group of animals 1 h after surgery. *$P <$ 0.05, plasma ascorbate concentration in the CLP group compared with that in the nonseptic control group at the corresponding time.
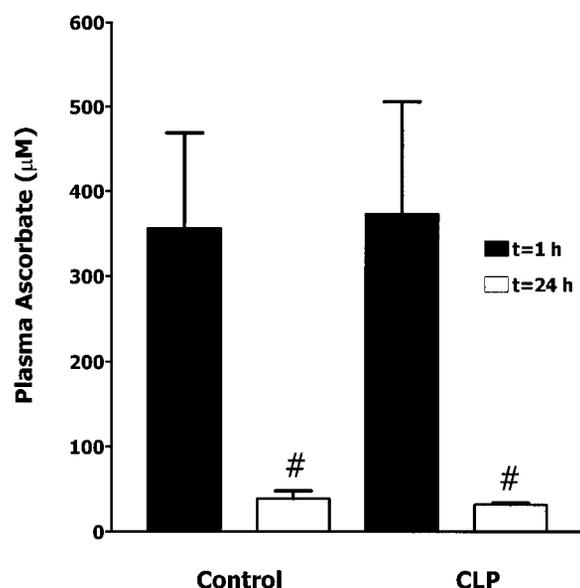
Fig. 2. Plasma ascorbate concentrations in CLP and control rats after both groups received the low dose of ascorbate. Procedure was the same as in Fig. 1 except that ascorbate (7.6 mg/100 g, dissolved in 3 ml of saline) was infused 30–60 min after surgery. Values are means ± SE for ascorbate concentration 1 and 24 h after surgery ($n = 3$–4 rats) in ascorbate-loaded control and CLP rats. $^{\#}P < 0.05$, value at 24 h compared with that for the same group of animals 1 h after surgery.
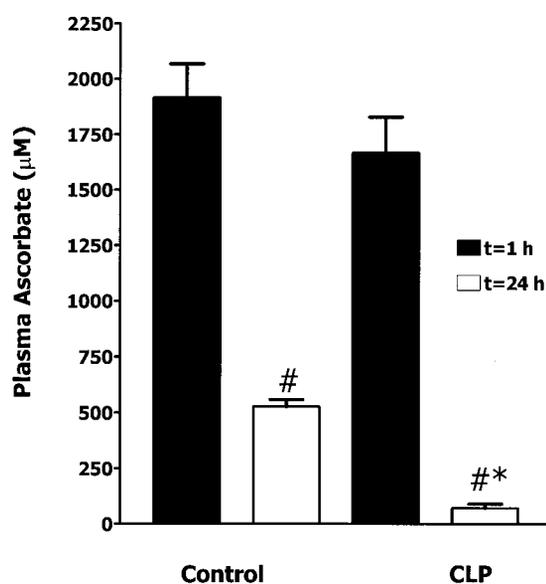

Fig. 3. Plasma ascorbate concentrations in CLP and control rats after both groups received the high dose of ascorbate. Procedure was the same as in Fig. 1 except that ascorbate (76 mg/100 g) was infused 30–60 min after surgery. Values are means ± SE for ascorbate concentration 1 and 24 h after surgery ($n = 3$–5 rats) in ascorbate-loaded control and CLP rats. $^{\#}P < 0.05$, value at 24 h compared with that for the same group of animals 1 h after surgery. $^{*}P < 0.05$, ascorbate concentration in CLP rats compared with that in the nonseptic control group at the corresponding time.

sevenfold higher (Fig. 2) than in the saline-infused groups (Fig. 1). The plasma ascorbate concentrations in the ascorbate-infused animals decreased subsequently and were not significantly different from those in the saline-infused groups at 24 h (saline-infused control, 56 ± 4 µM; saline-infused CLP, 29 ± 2 µM; ascorbate-infused control, 39 ± 9 µM; ascorbate-infused CLP, 32 ± 2 µM). Thus infusion of this dose of ascorbate abolished the difference between CLP and control rats at 24 h.

Infusion of a 10-fold higher dose of ascorbate led to larger and more persistent increases in plasma levels (Fig. 3). One hour after surgery, the effect of this dose was to raise plasma ascorbate concentrations ~40-fold higher (Fig. 3) than in the saline-infused rats (Fig. 1). Plasma ascorbate concentration after high-dose ascorbate infusion decreased within 24 h to significantly lower levels in CLP than in control rats (Fig. 3), but it remained higher than in saline-infused CLP rats (71 ± 11 vs. 29 ± 2 µM, $P < 0.05$).

CLP increased by 10-fold the urine ascorbate concentration in saline-infused rats (Fig. 4). Intravenous infusion of the low dose of ascorbate increased urine ascorbate concentration in nonseptic control rats but not in CLP rats (Fig. 4). Urine was not collected from those animals that received the high dose of ascorbate.

Cardiovascular measurements. Mean arterial blood pressure was not affected by infusion of saline or ascorbate in nonseptic control rats (Fig. 5). However, blood pressure fell by 20% 24 h after CLP. Both the low dose (Fig. 5) and the high dose of ascorbate prevented this small but significant decrease. The values obtained 24 h after surgery for rats that received the high dose

were 111 ± 6 mmHg for the CLP group and 116 ± 3 mmHg for the control group (not significantly different, $n = 4$).

Intravital videomicroscopy of the EDL muscle showed that neither CLP nor ascorbate altered red blood cell velocities in capillaries (Table 3). However,
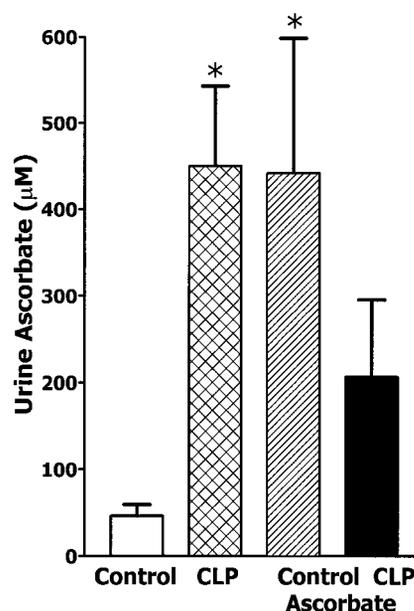

Fig. 4. Effects of CLP and ascorbate infusion on urine ascorbate concentration. Urine was collected from the bladder 25–26 h after surgery and assayed for ascorbate concentration. Values are means ± SE ($n = 4$–6 rats). $^{*}P < 0.05$ compared with saline-infused, nonseptic control group (open bar).
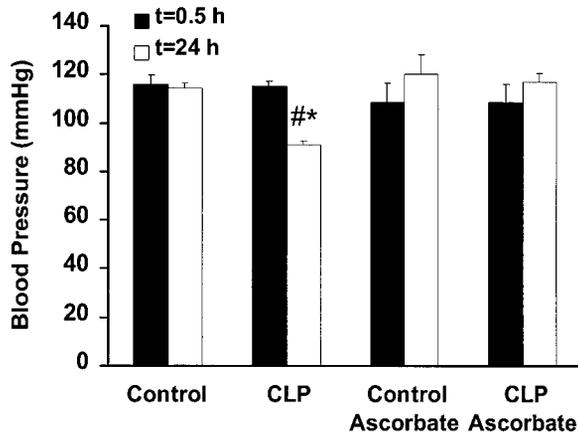
Fig. 5. Effect of CLP and ascorbate (7.6 mg/100 g) on blood pressure. Values are means ± SE for mean arterial blood pressure 0.5 and 24 h after surgery ($n = 4–10$ rats). #$P < 0.05$ compared with the value for the same group of animals 0.5 h after surgery. *$P < 0.05$ compared with the saline-infused, nonseptic control group at the corresponding time.

other parameters of microvascular function were affected. CLP tripled the number of nonperfused capillaries and doubled the number of intermittently perfused capillaries (Fig. 6). Conversely, CLP decreased by 30% the number of continuously perfused capillaries. Both the low and the high doses of ascorbate prevented these sepsis-induced changes in microvascular perfusion. Neither dose of ascorbate changed capillary blood flow in the nonseptic control rats. The densities of perfused capillaries in animals that received the low dose of ascorbate are shown in Fig. 6. For the rats that received the high dose, the average numbers of each type of capillary were 27 ± 2 continuously perfused, 2 ± 1 intermittent, and 2 ± 1 stopped flow in the CLP group ($n = 5$); and 27 ± 1 continuously perfused, 2 ± 1 intermittent, and 2 ± 1 stopped flow in the nonseptic control group ($n = 4$). The total number of capillaries per millimeter (perfused, intermittent, and stopped flow combined) did not differ among animal groups.

Table 3. *Velocity of red blood cells in capillaries*

Animal Group	Velocity, μm/s
Saline-infused	
Control	264 ± 36
CLP	283 ± 32
Ascorbate-infused (76 mg/100 g)	
Control	272 ± 85
CLP	167 ± 27

For the ascorbate-infused control and CLP groups and for the saline-infused control group, the mean ± SE values are based on $n = 4$, 5, and 16 rats, respectively. The value for the saline-infused CLP group is based on $n = 17$ rats from our laboratory's previous study (41). A bolus infusion of the high dose of ascorbate (76 mg/100 g body wt) or saline vehicle was administered 30 min after catheterization (control) and CLP surgery. Red blood cell velocity in capillaries of extensor digitorum longus muscle was measured using the video flying spot technique 24 h after surgery. There were no statistically significant differences among the 4 groups.

Postmortem and in vitro observations. There were no effects of CLP or ascorbate on mortality during the experimental period, as the number of animals that survived the 24-h period after surgery did not differ among treatment groups (Table 4). At autopsy, CLP rats were found to have an accumulation of purulent peritoneal fluid and inflamed intestine, marked by swelling of the intestinal wall. In contrast, a normal peritoneal cavity was found in control rats and those CLP rats that had been infused with either dose of ascorbate.

To investigate further the possibility that ascorbate has bacteriostatic activity, bacterial replication in dilute fecal samples was studied in vitro. Ascorbate was observed to inhibit bacterial replication significantly. The maximal effect was observed at 100 μM ascorbate, and no additional inhibition was seen at a 10-fold greater concentration (Fig. 7).

The possibility that ascorbate conferred antioxidant protection on skeletal muscle microvascular endothelial cells was also evaluated in vitro. The intracellular ascorbate concentration was nondetectable in control cultures that had been incubated with the homocysteine vehicle but was $1,490 \pm 220$ μM in cultures that had been incubated with 100 μM ascorbate. Subsequent incubation with hydrogen peroxide (100 μM in nominally ascorbate-free medium) caused 100% cell death in ascorbate-free cultures within 1 h but no detectable cell death in ascorbate-loaded cultures (based on nuclear staining by ethidium bromide; $n = 5$ experiments). Electron microscopy showed that hydrogen peroxide caused necrotic injury to the ascorbate-free cells, as indicated by damaged mitochondria, swollen endoplasmic reticulum, and clumping of nuclear material (Fig. 8). However, these ultrastructural
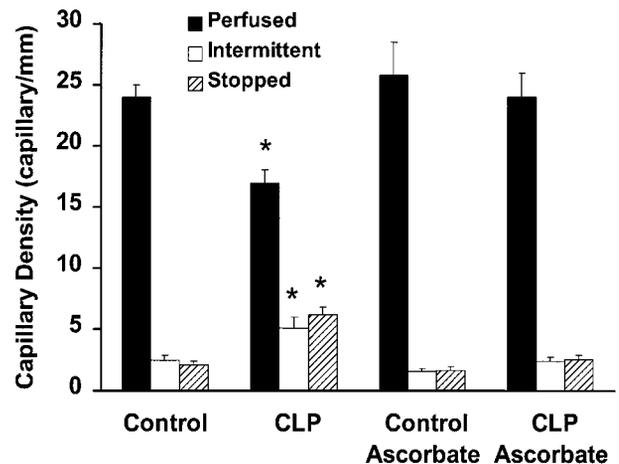

Fig. 6. Effect of CLP and ascorbate (7.6 mg/100 g) on microvascular perfusion in skeletal muscle. Microvascular blood flow in the extensor digitorum longus muscle was recorded by intravital videomicroscopy 25 h after surgery. Results are expressed as the no. of capillaries/mm of test line. Values are means ± SE for the no. of capillaries/mm that were perfused continuously with red blood cells (perfused), showed intermittent perfusion (intermittent), or were not perfused (stopped) ($n = 4–7$ rats). *$P < 0.05$ compared with the saline-infused, nonseptic control group.

Table 4. *Survival rates 24 h after surgery in control and CLP rats*

	Control-Saline	CLP-Saline	Control-Ascorbate (7.6 mg/100 g)	CLP-Ascorbate (7.6 mg/100 g)	Control-Ascorbate (76 mg/100 g)	CLP-Ascorbate (76 mg/100 g)
No. of survivors/total	7/8	12/16	4/5	5/5	7/9	6/7
Survival rate, %	88	75	80	100	78	86

Based on χ^2 test, there were no statistically significant differences among groups.

changes did not occur in ascorbate-loaded endothelial cells that were exposed to peroxide (Fig. 8).

DISCUSSION

Key findings of the present study were that CLP decreased plasma ascorbate concentration and microvascular perfusion in skeletal muscle. Administration of ascorbate prevented the ascorbate depletion and capillary blood flow impairment that otherwise occurred within 24 h after CLP. These findings are consistent with the hypothesis that oxidative stress causes microvascular dysfunction in sepsis. They also indicate that ascorbate therapy may be beneficial.

Oxidative stress in sepsis. There is considerable published evidence that implicates oxidative stress in the pathogenesis of septic shock and multiple organ dysfunction syndrome. For instance, contraction-related generation of reactive oxygen species is increased in skeletal muscle isolated from endotoxic rats (26). Generally, endotoxin triggers an inflammatory response in various tissues that is characterized by infiltration of neutrophils, lipid peroxidation, and microvascular leakage indicative of microvascular damage (37). As evidence for the important role of oxidative stress, a superoxide dismutase mimetic and peroxynitrite decomposition catalysts decrease the lipid peroxidation and microvascular leakage in the intestine of rats exposed to endotoxin (37). Furthermore, administration of an antioxidant steroid (lazaroid) protects against endotoxin-induced shock in rats (1).

There are many indications of oxidative stress in critically ill patients. In particular, circulating ascorbate concentrations are decreased in patients with sepsis, systemic inflammatory response syndrome, or acute respiratory distress syndrome (11, 24). Furthermore, ascorbate is effective in treating lactic acidosis in nonseptic patients in severe metabolic crisis (39).

Animal studies also have investigated the role of ascorbate in septic syndrome. Injection of bacterial endotoxin in animals decreases ascorbate content in the heart (35), lungs (3), and adrenal glands (13). Prior lowering of body ascorbate stores, by feeding an ascorbate-deficient diet, decreases survival after endotoxic shock in guinea pigs (3, 9). Conversely, ascorbate infusion lessens the impairment of cardiorespiratory function caused by endotoxin in sheep (7) and improves survival after endotoxin administration in mice (28). The protective effect of ascorbate likely is due, at least in part, to its antioxidant properties, because ascorbate inhibits the generation of oxidizing free radicals in endotoxin-exposed myocardium (35) and neutrophils (6), as well as decreases endotoxin-induced oxidative modification of proteins in liver (4).

The present rat model of sepsis. Sepsis was defined in our study as the outcome of CLP and laparotomy procedures to mimic the clinical situation of sepsis involving a surgical intervention. Because our CLP model involved a 24-h period of fluid resuscitation that could affect the availability of endogenous ascorbate, our control rats were subjected to the same resuscitation procedure. There were no differences between the CLP and control groups in body weight, blood gases, hemoglobin level, or urate and lactate concentrations (Tables 1 and 2). Whereas it has been reported that plasma lactate concentration increases before death in septic CLP animals (45), our saline-loading procedure for volume resuscitation was effective in preventing lactate acidosis during the period studied. These results indicate that severe metabolic problems do not occur during the first 24 h of the evolution of sepsis in our animal model. As further evidence that our study examines an early stage of sepsis, survival rate was not
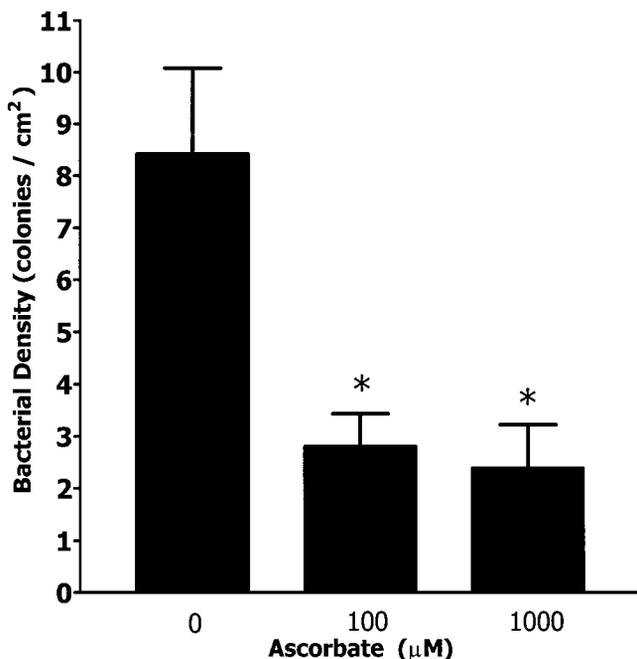

Fig. 7. Ascorbate inhibits fecal bacterial replication in vitro. Fecal samples obtained from the rat cecum were incubated with 0, 100, and 1,000 μM ascorbate in deionized water at 37°C for 30 min. Subsequently, 40-μl aliquots of these incubates were placed on agar plates that contained the same concentrations of ascorbate. Plates were incubated at 37°C for 8 h, and bacterial colonies were counted. Values are means ± SE for $n = 3$ experiments performed in triplicate. *$P < 0.05$ for the effect of ascorbate on bacterial growth.
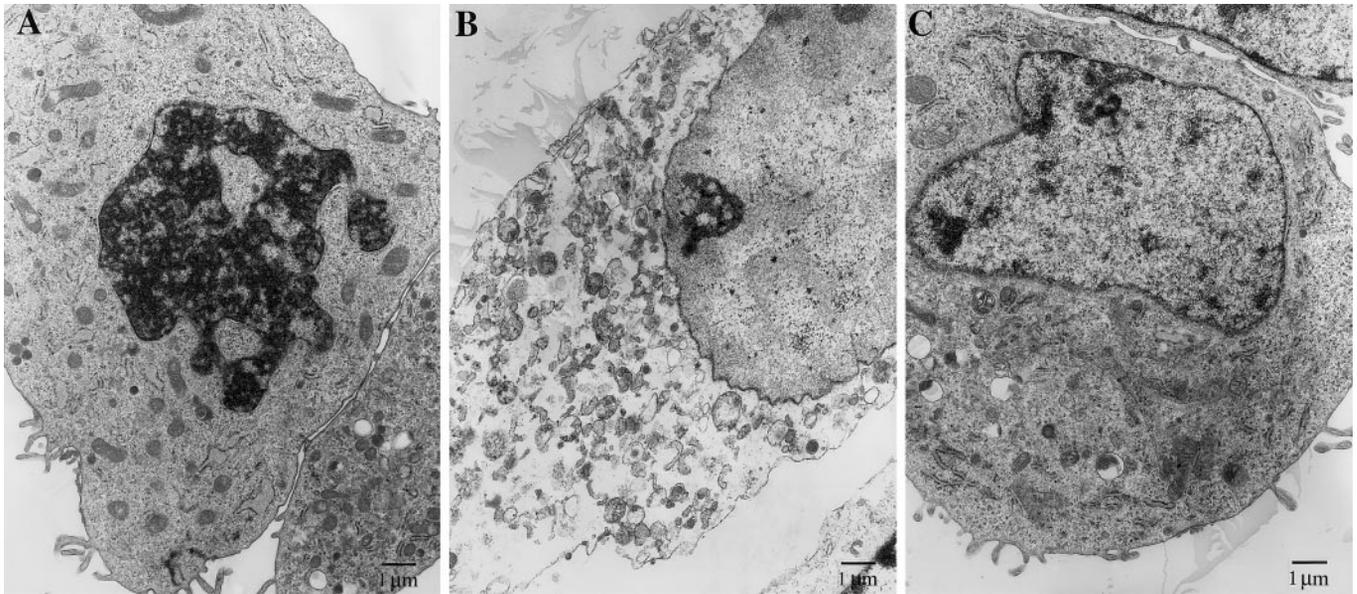
Fig. 8. Effects of hydrogen peroxide on endothelial cell ultrastructure. Rat skeletal muscle microvascular endothelial cells were first incubated with ascorbate (in homocysteine vehicle) or vehicle (homocysteine) for 19 h, were then incubated in ascorbate-free medium for a 1-h washout period, and were finally incubated with or without hydrogen peroxide (100 µM, 1 h). Representative electron micrographs are shown for the ascorbate-free and peroxide-free control (*A*), the ascorbate-free plus peroxide treatment (*B*), and the ascorbate-loaded plus peroxide treatment (*C*). Damaged mitochondria, swollen endoplasmic reticulum, and clumping of nuclear material were observed in the ascorbate-free cells after peroxide (*B*) but not in ascorbate-loaded cells (*C*). Bar, 1 µm; the magnification (×5,000) was the same for all 3 panels.

decreased by CLP at 24 h (Table 4). This is consistent with a previous study that reported that sepsis did not affect mortality at 24 h but decreased survival rate at 48 h (28). Administration of an ascorbate analog, 2-octadecylascorbic acid, improved survival rate 48 h after septic insult (28). Because microvascular dysfunction is a precursor of multiple-organ failure (21, 32), ascorbate's ability to maintain capillary perfusion may be beneficial to patients in preventing death.

Effect of sepsis on systemic ascorbate level. Plasma ascorbate concentration decreased within 24 h after CLP (Fig. 1). The underlying causes may be increased oxidation and excretion of the vitamin. Ascorbate may be oxidized by the reactive oxygen species produced both by the infected animal and by invading bacteria. The observation that the concentration of ascorbyl radical increases in patients with sepsis syndrome (11) is consistent with accelerated oxidation of endogenous ascorbate. The elevated ascorbyl radical level may be due to an increased generation of reactive oxygen species in these patients and the subsequent scavenging of these oxidants by ascorbate, leading to destruction of this antioxidant.

An alternative cause of the depressed plasma ascorbate concentration in sepsis may be slowed uptake of the antioxidant into cells and its increased excretion in urine. Renal retention of ascorbate becomes less effective under pathological conditions. For instance, water diuresis increases urinary excretion of vitamin C in human subjects (25). Urinary excretion of ascorbate also increases in diabetic patients with microvascular disease and may cause the lowering of plasma ascor-

bate concentration in these patients (38). The present experiments found that CLP greatly increased the ascorbate concentration in urine (Fig. 4), consistent with impaired reabsorption by the renal tubules of septic animals. Endotoxin and complement inhibit the active transport system responsible for concentrative uptake of ascorbate into cells (14, 29), and this may contribute to the increased concentration of ascorbate in the urine of septic animals.

Effect of sepsis on cardiovascular parameters. Mean arterial blood pressure fell after CLP, although it remained in the normotensive range (Fig. 5). Impaired microvascular responses to vasoconstrictors in mesentery and skeletal muscle (5), as well as decreased myocardial function (30), may have caused this fall in arterial pressure.

The mechanism that caused the decreased density of perfused capillaries in the septic EDL muscle (Fig. 6) is not known. The blood pressure reduction per se cannot be responsible because a much more severe hypotension (~40 mmHg) is required to decrease the density of perfused capillaries in the EDL muscle (40). Furthermore, neither accumulation of adhering leukocytes in the capillaries (31) nor tissue edema (32) has been observed in the EDL muscle 24 h after CLP, indicating that neither capillary plugging by leukocytes nor external compression of capillaries can account for the reduced capillary perfusion. Because sepsis reduces the deformability of red blood cell membrane (2), it is possible that red blood cells themselves became trapped in some capillaries. The significant increase in

the density of capillaries with stationary red blood cells after CLP (Fig. 6) is consistent with this explanation.

Beneficial effect of ascorbate infusion on the outcome of sepsis. Figures 5 and 6 demonstrate that a bolus infusion of ascorbate prevented the reduction in systemic blood pressure and the impairment of capillary perfusion observed 24 h after CLP. These beneficial effects in animal models support clinical observations that antioxidant administration may be a useful adjunct to conventional approaches in the management of sepsis and related pathologies. Intravenous injection of a combination of antioxidants (ascorbate, *N*-acetyl-L-cysteine, and α-tocopherol) in patients with septic shock increased heart rate and cardiac index while decreasing systemic vascular resistance (12). Administration of an antioxidant mixture (ascorbate, *N*-acetyl-L-cysteine, and glutathione) also decreased mortality in mice exposed to a burn injury combined with endotoxin (20). Chronic administration of a mixture of antioxidants (ascorbate, *N*-acetyl-L-cysteine, selenium, and vitamin E for 7 days) decreased the incidence of multiple organ dysfunction syndrome and infectious complications in severely injured trauma patients (33). Dietary supplementation with antioxidants also yielded beneficial effects on pulmonary gas exchange and reduction of new organ failures in patients with acute respiratory distress syndrome (10).

The mechanism of the protective effect of ascorbate bolus infusion after CLP has been investigated. The improvement in microvascular perfusion does not appear to be due to a nonspecific vasodilator effect, because ascorbate did not elevate red blood cell velocity. Our in vitro experiments evaluated two potential mechanisms responsible for the protection observed in vivo: one acting at the site of infection (i.e., peritoneal cavity) where ascorbate could have a bacteriostatic effect, and the other acting at the level of endothelial cells where it may protect against oxidative stress.

Our observations of an apparently normal peritoneal cavity in ascorbate-infused CLP rats and inhibition of bacterial replication by the vitamin in vitro (Fig. 7) are consistent with a protective effect at the site of infection. Previous studies have shown that ascorbate, even at concentrations too diluted to affect extracellular pH, inhibits the growth of numerous species of bacteria (36, 46). Gram-positive and gram-negative pathogenic bacteria are unable to take up either ascorbate or dehydroascorbic acid (42); therefore, the lethal effect of vitamin C must be initiated outside of these microorganisms. It is possible that free radicals, produced by the reaction of ascorbate with transition metals in the extracellular fluid, inhibit bacterial growth (18, 36).

The results of our experiments with endothelial cell cultures are consistent with another protective mechanism, because intracellular ascorbate prevented endothelial cell killing by peroxide (Fig. 8). Endothelial cell swelling is an early effect of sepsis in skeletal and cardiac muscle (17); therefore, it is possible that intracellular ascorbate protects volume-regulatory mechanisms from oxidative damage and thereby prevents this swelling. This explanation is plausible because

experiments with a different initiating insult, namely ischemia-reperfusion, have shown that antioxidants attenuate postischemic endothelial cell swelling and luminal membrane blebbing in capillaries (43). Prevention by ascorbate of endothelial swelling after sepsis-induced oxidative stress may have contributed to the improvement in capillary perfusion observed in the present study (Fig. 6).

In conclusion, ascorbate administration countered the depletion of this antioxidant in the CLP rat and improved arterial pressure and microvascular perfusion. Inhibition of bacterial replication and prevention of oxidative injury to endothelial cells may contribute to this beneficial outcome.

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