Preclinical trial of L-arginine monotherapy alone or with *N*-acetylcysteine in septic shock*

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Objective: **L-arginine supplementation in sepsis is controversial. Septic shock has been alternatively viewed as an L-arginine-deficient state or as a syndrome caused by excess nitric oxide, an end-product of L-arginine metabolism.**

Design: **Randomized, placebo-controlled, and double-blinded (investigators, veterinarians, and pharmacists).**

Setting: **Laboratory.**

Subjects: **Purpose-bred, 1- to 2-yr-old, 10- to 12-kg beagles.**

Interventions: **The effects of parenteral L-arginine alone or in combination with *N*-acetylcysteine were compared with vehicle alone in a well-characterized canine model of *Escherichia coli* peritonitis. Two doses were studied that delivered approximately 1.5-fold (10 mg·kg^{-1}·hr^{-1}) and 15-fold (100 mg·kg^{-1}·hr^{-1}) the L-arginine dose typically administered with standard total parenteral nutrition. Animals in the low- and high-dose L-arginine arms were further randomized to receive vehicle alone or *N*-acetylcysteine (20 mg·kg^{-1}·hr^{-1}) as an antioxidant to prevent peroxynitrite formation.**

Measurements and Main Results: **The main measurements were hemodynamics, plasma arginine and ornithine, serum nitrate/nitrite, laboratory studies for organ injury, and survival. Both doses of L-arginine similarly increased mortality ($p = .02$), and worsened shock ($p = .001$ for reduced mean arterial pressure). These effects were associated with significant increases in plasma arginine ($p = .0013$) and ornithine ($p = .0021$). In addition, serum nitrate/nitrite ($p = .02$), liver enzymes ($p = .08$), and blood urea nitrogen/creatinine ratios ($p = .001$) rose, whereas arterial pH ($p = .001$) and bicarbonate levels ($p = .001$) fell. *N*-acetylcysteine did not significantly decrease any of the harmful effects of L-arginine. Thus, parenteral L-arginine monotherapy was markedly harmful in animals with septic shock.**

Conclusions: **These findings suggest that supplemental parenteral L-arginine, at doses above standard dietary practices, should be avoided in critically ill patients with septic shock. (Crit Care Med 2006; 34:2719–2728)**

KEY WORDS: L-arginine; N-acetylcysteine; sepsis; nitric oxide; immunonutrition

The administration of pharmacologic doses of L-arginine in sepsis is controversial. Two authoritative groups, writing on nutritional support in critical illness based on the best available evidence, have given conflicting advice on supplemental L-arginine (1–4). Recent commentaries have suggested that sepsis may be an L-arginine-deficient state that would respond favorably to L-arginine therapy (5, 6). Conversely, others have cautioned that exogenous L-arginine may worsen mortality rate (1, 7) or possibly trigger cardiovascular collapse in sepsis (8). L-arginine, the focus on these disparate views, has numerous physiologic and pharmacologic effects (9) that invite such speculations about its potential to affect the survival of septic patients.

L-arginine is the substrate for and sole source of nitric oxide ($NO^{\cdot}$), the endogenous mediator most closely linked to sepsis-induced shock (10). This suggests that enhancing $NO^{\cdot}$ production by providing supplemental L-arginine might be harmful. However, $NO^{\cdot}$ also has effects that are potentially beneficial in sepsis such as maintaining microvascular perfusion, blocking platelet aggregation and adhesion, scavenging superoxide, and otherwise protecting the endothelium (11). Furthermore, nonselective nitric oxide synthase (NOS) inhibitors were found to be markedly harmful in patients with septic shock (12), thereby suggesting that the opposite approach of increasing $NO^{\cdot}$ production might be beneficial. In addition to the $NO^{\cdot}$ pathway, which accounts for <2% of its utilization, L-arginine is also metabolized by arginases into urea and ornithine (6, 9). The latter can be

*See also p. 2844.

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converted to proline, the backbone of collagen, and polyamines, essential regulators of cell proliferation. Therefore, L-arginine is important to wound healing and tissue repair (13). Another metabolite of L-arginine, agmatine, regulates a number of ligand-gated calcium channels and thereby affects the brain, heart, and vasculature (14–16). Finally, pharmacologic doses of L-arginine have a myriad of neuroendocrine activities including modulating both sympathetic (17) and parasympathetic tone (18) and stimulating the release of hormones such as prolactin, growth hormone, insulin, insulin-like growth factor, and glucagon (6, 9). Overall, the potential impact of these diverse activities of L-arginine on the outcome of sepsis is largely unknown.

In healthy adults, L-arginine is a nonessential amino acid (6, 9). Dietary intake is typically 5–6 g/day, whereas daily endogenous production is ≥15 g. However, in immature animals and pathologic states such as severe sepsis, L-arginine demand may exceed supply. Plasma levels of L-arginine often fall precipitously in sepsis (19, 20). Furthermore, sepsis-induced catabolism and organ failure result in elevated levels of dimethylarginine (ADMA), a by-product of protein breakdown that antagonizes L-arginine utilization (21). ADMA competes for L-arginine transport and is a potent endogenous inhibitor of NOS (22, 23). Importantly, plasma levels of L-arginine (19) and ADMA (24) correlate directly and inversely with survival, respectively, in sepsis and critical illness. Collectively, these observations have led to the notion that L-arginine becomes conditionally essential in severe sepsis and septic shock (5, 6).

Although some of the preceding arguments are compelling, experimental evidence indicating that L-arginine might improve survival in septic shock is very limited. Only a few small animal studies have been conducted that have included survival as an end point, and these have produced inconsistent results (25–30). Clinical trials in critically ill patients have been limited to mostly unblinded studies of immune-enhanced enteral nutrition that contains a mixture of experimental components in addition to L-arginine (31). Only one of these trials demonstrated that high-L-arginine enteral nutrition significantly benefited survival in sepsis (32), whereas three studies suggested no benefit, or possibly harm (33–35). To date, human trials of L-arginine alone have employed bolus infusions (200–500 mg/kg) and have only examined short-term physiologic effects (36–39). These studies have shown that parenteral L-arginine can significantly decrease blood pressure and increase $NO^{\cdot}$ production in patients with sepsis or vascular disease.

Here, we used a well-characterized canine model of septic shock to test the therapeutic efficacy of parenteral L-arginine monotherapy. This canine model has been employed for 2 decades to study various therapies for sepsis and has yielded results concordant with subsequent clinical trials (40, 41). L-arginine was studied at two doses, 1.5- and 15-fold higher than standard formulations of total parenteral nutrition (TPN). Both L-arginine doses were studied alone and with an infusion of *N*-acetylcysteine, which was given as an antioxidant and to increase $NO^{\cdot}$ bioavailability (42–44). *N*-acetylcysteine might further augment beneficial effects of L-arginine by decreasing the production of peroxynitrite, a harmful metabolite formed from $NO^{\cdot}$ and superoxide.

METHODS

Animal Care. This experimental protocol was approved by the Animal Care and Use Committee of the Clinical Center of the National Institutes of Health. Throughout the studies, every effort was made to minimize animal pain and suffering. The research protocol required the veterinarian staff to kill any animal that became moribund or experienced unexpected pain or distress during the induction of sepsis. During the study, all animals that were killed were preterminal in the judgment of an experienced senior veterinarian.

Animal Model. Purpose-bred, 1- to 2-yr-old, 10- to 12-kg beagles were studied. Intraperitoneal placement of the fibrin-thrombin clot on day 0 required general anesthesia. Intravascular catheters were placed in awake animals at baseline and on days 0 and 28 with subcutaneous infiltration of local anesthesia (1% lidocaine); the catheters were removed after completion of laboratory and hemodynamic evaluations at baseline and on study days 2 and 28.

On the day of clot placement (day 0), animals were sedated with xylazine hydrochloride (2 $mg \cdot kg^{-1}$) and atropine sulfate (0.03 $mg \cdot kg^{-1}$) intramuscularly. The trachea was intubated after mask induction of anesthesia by inhalation of isoflurane. Intraoperative fluid (50 $mL \cdot kg^{-1}$ of 0.9% saline) was given for 30 mins. Animals breathed isoflurane (1–3% in 100% oxygen) spontaneously during intraperitoneal placement of 1 or 1.5×10^{10} colony-forming units of *Escherichia coli* 0111:B4 per kilogram of body weight. Bacterial dose was balanced across the study groups. The *E. coli* were prepared and impregnated in the fibrin-thrombin clot as previously described (45). After closure of the incision, 6–10 mL of 0.25% bupivicaine hydrochloride was injected subcutaneously into the surgical area, the isoflurane was discontinued, and animals were extubated awake.

Study Drugs. L-arginine (10% arginine hydrochloride, USP, at a final concentration of 0.05 $g \cdot mL^{-1}$, Pharmacia, Clayton, NC) was given as a continuous infusion at a rate of either 10 or 100 $mg \cdot kg^{-1} \cdot hr^{-1}$ for 36 hrs. These doses are respectively equivalent to 1.5 and 15 times the dose routinely used in patient TPN on a per-kilogram basis. However, canines have a substantially higher metabolic rates and protein requirements than humans per kilogram of body weight. A 10-kg beagle consumes 600–900 kcal per day (60–90 kcal/kg/day). Typical chow (such as Premium Edge, 1.55% L-arginine; Meta, MO) contains 0.42 g per 100 kcal of L-arginine.

N-acetylcysteine (Zambon Corporation, East Rutherford, NJ), in a concentration of 50 $mg \cdot mL^{-1}$, was infused as an intravenous bolus of 100 $mg \cdot kg^{-1}$ for 30 mins, followed by continuous intravenous infusion of 20 $mg \cdot kg^{-1} \cdot hr^{-1}$ for 36 hrs. This is similar to the dose used clinically to treat acetaminophen toxicity. Both L-arginine and *N*-acetylcysteine were formulated for infusion by the NIH Pharmacy, Pharmaceutical Development Section. NaOH (0.1 N) was used as needed to adjust the final pH of these solutions (pH 5 to 6.5 for both).

Study Design. Animals were randomly assigned to a treatment or placebo arm. Fifty-six animals were used in this study: Nine received low-dose (10 $mg \cdot kg^{-1} \cdot hr^{-1}$) and seven high-dose L-arginine (100 $mg \cdot kg^{-1} \cdot hr^{-1}$). These doses are respectively equivalent to 1.5 and 15 times the dose routinely used in TPN. Another 12 received low- and 12 high-dose L-arginine in combination with *N*-acetylcysteine. As controls, 16 animals received vehicle alone. Three to four animals were studied each week for 16 wks. Each week, one control and two to three animals from each of the four treatment groups were studied to produce a connected, balanced design. All animals had a thrombin-fibrin clot infected with *E. coli* placed intraperitoneally and were followed for 28 days. Animals were continuously observed for the first 48 hrs after clot implantation. Any unobserved death was considered to have occurred when the animal was found.

Therapeutic Interventions. Starting 4 hrs after clot implantation, animals were randomized to intravenous low- or high-dose L-arginine alone, *N*-acetylcysteine in combination with L-arginine, or vehicle for 36 hrs. Ceftriaxone sodium (100 $mg \cdot kg^{-1}$; Hoffman-LaRoche Laboratories, Nutley, NJ) was given intravenously 6 hrs after clot implantation and then daily for 4 days. A fluid infusion (10 $mL \cdot kg^{-1} \cdot hr^{-1}$) of D5 Ringer's lactate solution was given from 4 to 36 hrs. From 6 to 36 hrs,

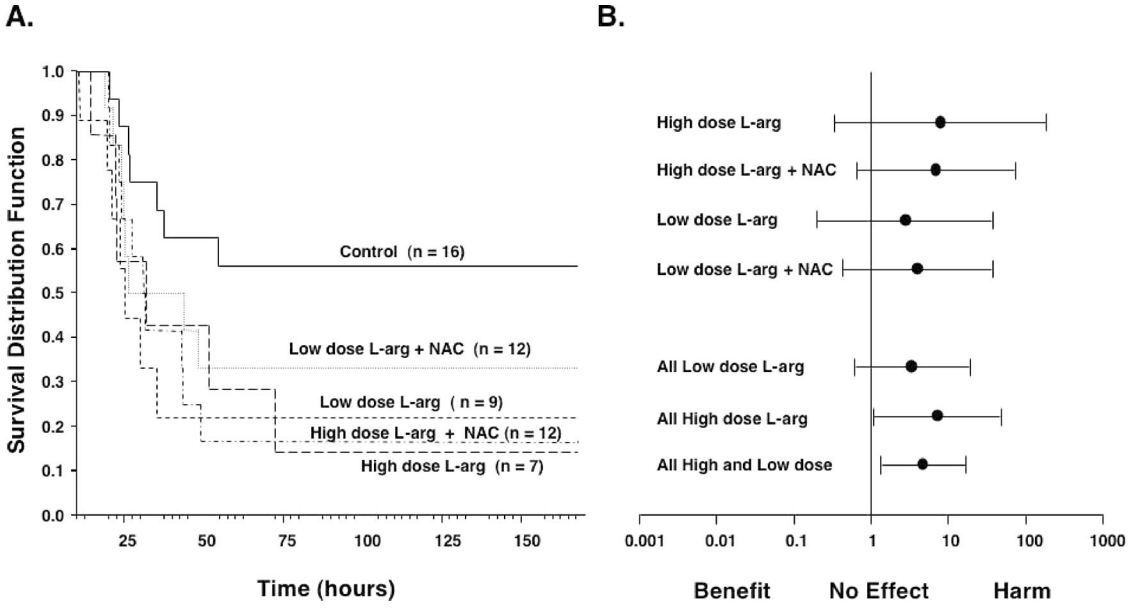

Figure 1. Effect of low- and high-dose L-arginine (*arg*) alone or combined with *N*-acetylcysteine (*NAC*) on survival. *A*, proportion surviving vs. time for different treatment groups. *B*, odds ratios for survival for different treatment groups and 95% confidence intervals are shown. Odds ratios were calculated for each treatment group using concurrently run controls.

animals were kept in Kirschner cages (PlasLabs, Lansing, MI) at 27°C with infused oxygen to maintain an F_{IO_2} of 40%. Animals were removed for 30 mins from cages at 8, 24, and 36 hrs after clot implantation to allow equilibrium with room air for hemodynamic and blood evaluations. Animals had unrestricted access to food and water throughout the study except for 8–10 hrs before surgery.

Hemodynamics and Blood Chemistries. For baseline evaluations, we measured hemodynamic and laboratory values ≥7 days before clot implantation. Data were obtained from femoral arterial and balloon flotation thermodilution pulmonary arterial catheters. Afterward, a fluid challenge of Ringer's lactate solution (20 $mg \cdot kg^{-1}$ of body weight for 30 mins) was given and the hemodynamic studies were repeated. To determine the serial effects of sepsis for 28 days, we repeated the same hemodynamic and blood chemistry evaluations 8, 24, and 48 hrs and 28 days after bacterial clot. Measurements included heart rate (beats/min), mean arterial pressure (MAP, mm Hg), central venous pressure (mm Hg), mean pulmonary artery pressure (mm Hg), pulmonary artery occlusion pressure (mm Hg), and cardiac output ($mL \cdot min^{-1}$). To determine left ventricular ejection fraction, we performed radionuclide-gated blood pool scans using previously described techniques (45). Hemodynamic data were indexed to body weight in kilograms. Cardiac index ($mL \cdot min^{-1} \cdot kg^{-1}$), stroke volume index ($mL \cdot kg^{-1}$), left ventricular stroke work index ($g \cdot mL \cdot^{-1} \cdot kg^{-1}$), systemic vascular resistance index ($dynes \cdot cm^{-1} \cdot kg^{-1}$), oxygen delivery index ($mL \cdot min^{-1} \cdot kg^{-1}$), left ventricular end-diastolic volume index ($mL \cdot kg^{-1}$), left ventricular end-systolic volume index ($mL \cdot kg^{-1}$), and alveolar-arterial oxygen gradient (torr) were calculated using standard formulas.

Arterial and mixed venous blood gases were measured at 37°C with a blood gas analyzer (288; CBI-Corning, Midfield, MA). Blood lactate levels were measured using a glucose-lactate analyzer (2300 STAT, Yellow Springs Instrument, Yellow Springs, OH). Complete blood counts were performed using an automatic analyzer (STK-S; Counter Electronics, Hialeah, FL). Measurements of electrolyte levels and chemical analyses of the blood (e.g., calcium, phosphorus, glucose, urea nitrogen, creatinine, uric acid, alanine aminotransferase, albumin, aspartate aminotransferase, γ-glutamyl-transpeptidase, alkaline phosphatase, lactate dehydrogenase, total bilirubin, triglyceride, and cholesterol levels) were done using an automated chemistry analyzer (AU 500; Olympus, Irving, TX). Each study day, blood was obtained for tumor necrosis factor (TNF) and nitrite/nitrate concentrations and quantitative blood cultures. Blood collected into pyrogen-free tubes containing heparin was used to measure endotoxin. This plasma was diluted, heat-treated, and assayed using a kinetic modification of the chromogenic Limulus amebocyte lysate assay (Whitaker Bioproducts, Walkersville, MD), as previously described (45). TNF concentrations were determined with a quantitative TNF cytotoxicity assay using WEHI 164 cells in 96-well plates; results were calculated based on the values obtained from a recombinant human TNF standard as previously described (46). Serum nitrate/nitrite (NO_x) levels ($\mu mol \cdot L^{-1}$) were measured by converting nitrate to nitrite using a colorimetric assay based on the Griess reaction (47). Plasma for arginine and ornithine determinations was collected at 24 hrs after the start of L-arginine or vehicle alone. Levels were analyzed at the Department of Laboratory Medicine, Children's Hospital, Washington, DC. At this laboratory, the normal ranges in children for arginine and ornithine are 20–179 μmol/L and 23–155 μmol/L, respectively.

Statistical Methods. Survival was analyzed using a Cox proportional hazards model and included effects for dose of L-arginine, the addition of *N*-acetylcysteine therapy, and the interactions between these effects. Animals killed by the veterinary staff were considered deaths at the time they were killed. All animals that were killed were in septic shock and/or multiple organ failure and judged to be preterminal by an experienced veterinarian. No significant differences in survival were observed between the low- vs. high-dose L-arginine groups nor between L-arginine alone vs. L-arginine with *N*-acetylcysteine. Therefore, data were pooled across L-arginine dose without and with *N*-acetylcysteine to increase power for the primary analysis comparing L-arginine with control. Kaplan-Meier survival plots are shown in Figure 1*A*. Arginine and ornithine levels were analyzed using the Cochran-Armitage test of linear trend.

To determine the effects of L-arginine on nonsurvival variables, analyses of variance comparing the control group and the other four groups (low- or high-dose L-arginine alone or with *N*-acetylcysteine) were performed. If no significant differences were observed among the four groups, variables were pooled to increase power. All variables were analyzed as changes from baseline at 8 hrs and 24 hrs. After 24 hrs, there were not enough

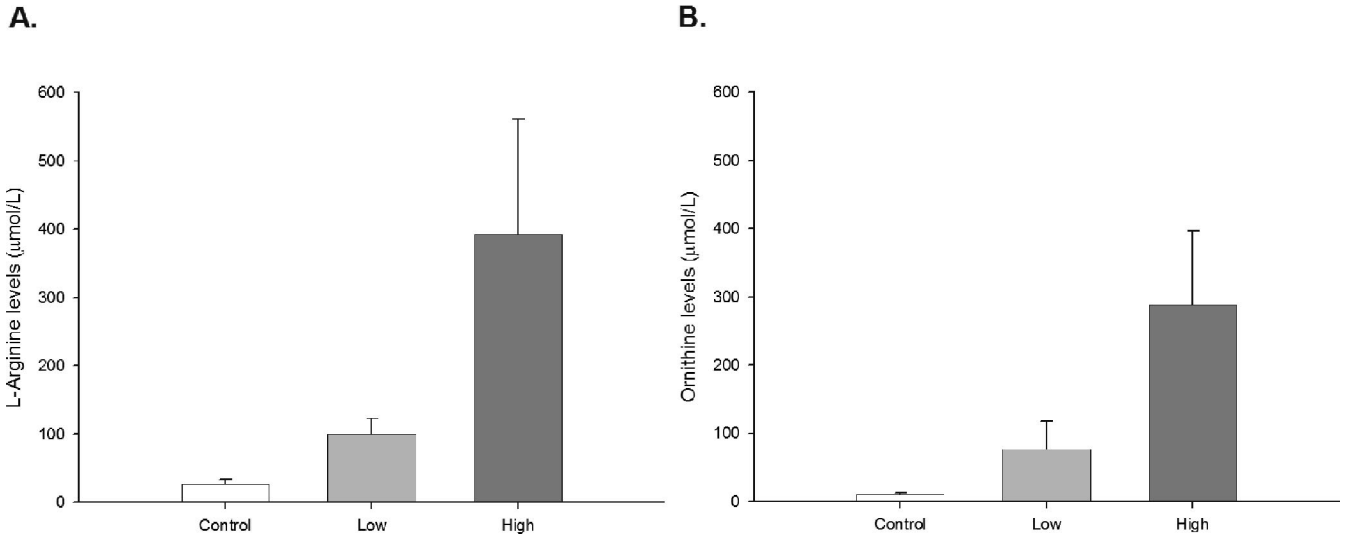

Figure 2. Plasma levels of (*A*) arginine and (*B*) ornithine. Four animals from each of the control, low-dose, and high-dose groups were randomly selected. Levels were measured at 24 hrs after the start of vehicle alone or L-arginine infusion. Values for each group, indicated by differently shaded bars, are shown as mean ± SE.

surviving animals in each treatment group studied to analyze. To increase power, data at 8 and 24 hrs were pooled for analysis if there were no significant differences between the time points. A liver injury score was constructed by normalizing and summing the four variables measured. A modified Bonferroni procedure was used to correct for the number of variables examined at baseline. Harmful effects over time were not adjusted for multiple comparisons. Two-sided p values $<.05$ were considered statistically significant. All results are presented as mean ± SE of the mean.

RESULTS

Clinical Manifestations and Survival. Following *E. coli*-infected clot implantation, all animals had typical signs of sepsis (i.e., weakness, lethargy, and anorexia). Compared with controls, there was a significant increase in mortality rates for all animals given L-arginine therapy ($p = .02$; Fig. 1), irrespective of the dose or combination with *N*-acetylcysteine. Raising L-arginine dose increased mortality rates, but this effect was not statistically significant ($p = .46$). The addition of *N*-acetylcysteine resulted in mortality rates that were similar to L-arginine alone ($p = .84$).

Arginine and Ornithine Levels. Arginine and ornithine plasma concentrations were measured in four animals randomly selected from each of the control, low-dose, and high-dose treatment groups. Levels for both arginine ($p = .0013$) and ornithine ($p = .0021$) were significantly dose ordered (Fig. 2).

Temperature, Nitrate/Nitrite Levels, and Intravascular Pressures. At baseline, the temperature, NO_x levels, and all intravascular pressure measurements were similar (Bonferroni adjusted; all p values nonsignificant) in groups of animals treated with L-arginine, alone and in combination with *N*-acetylcysteine, and controls (Fig. 3). At 8 hrs, temperatures similarly decreased from baseline in animals treated with L-arginine, alone and in combination with *N*-acetylcysteine, and controls. However, from 8 to 24 hrs, temperature progressively increased less in animals treated with L-arginine alone and in combination with *N*-acetylcysteine, compared with controls ($p = .03$). This loss of an increase in temperature was greater in animals treated with higher vs. lower doses of L-arginine ($p = .06$; Fig. 3*A*). At 8 hrs, the increase in serum NO_x levels from baseline in animals treated with L-arginine alone and in combination with *N*-acetylcysteine was not significantly greater compared with controls (all p values nonsignificant). However, from 8 to 24 hrs there was a significant increase in mean serum NO_x levels in animals treated with L-arginine alone and in combination with *N*-acetylcysteine, compared with controls ($p = .02$; Fig. 3*B*). At 8 but not 24 hrs, there was significantly less of an increase from baseline in mean pulmonary artery pressure in animals treated with low-dose L-arginine alone and in combination with *N*-acetylcysteine, compared with controls ($p = .02$). At 8 but not 24 hrs, there was also less of an increase in mean pulmonary artery pressure in animals treated with higher doses of L-arginine compared with controls, but this was not statistically significant (p nonsignificant; Fig. 3*C*). At 8 and 24 hrs after clot implantation, MAP decreased further from baseline in animals treated with L-arginine alone and in combination with *N*-acetylcysteine, compared with controls ($p = .001$). This further decrease in MAP was greater in animals treated with lower vs. higher doses of L-arginine ($p = .06$; Fig. 3*D*).

Organ Injury and Effects on pH. At baseline, serum liver enzymes, serum markers of renal function, arterial pH, arterial lactate levels, and serum electrolytes were similar (Bonferroni adjusted; all p values nonsignificant) in animals treated with L-arginine alone and in combination with *N*-acetylcysteine, and controls (Figs. 4–6). At 8 and 24 hrs after clot implantation, there was a dose-ordered (control < low-dose < high-dose L-arginine) increase from baseline in serum levels of all four liver enzymes measured in animals that received L-arginine alone and in combination with *N*-acetylcysteine ($p = .08$; Fig. 4) (see Statistical Methods). At 8 and 24 hrs compared with baseline, there were significant dose-ordered increases in blood urea nitrogen and serum blood urea nitrogen/creatinine ratios in animals treated with L-arginine, alone and in combination with *N*-acetylcysteine, compared with controls (both $p = .001$; Fig. 5). From 8 to 24 hrs

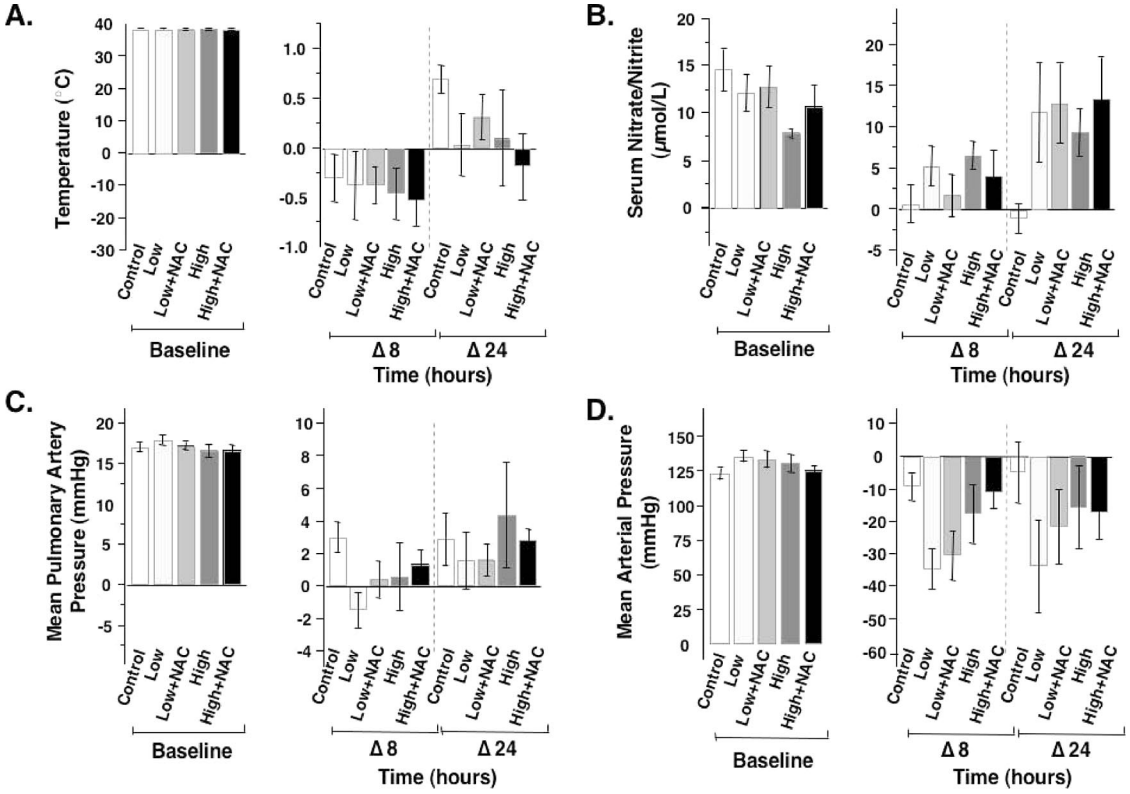

Figure 3. Effect of low- and high-dose L-arginine alone or combined with *N*-acetylcysteine (*NAC*) on (*A*) temperature, (*B*) serum nitrate/nitrite, (*C*) mean pulmonary artery pressure, and (*D*) mean arterial pressure. Absolute values at baseline (mean ± SE) are shown on the left and changes over time (mean ± SE) on the right of each panel. The various treatment groups are labeled and indicated by differently shaded bars.

compared with baseline, animals treated with L-arginine alone and in combination with *N*-acetylcysteine had a greater decrease in mean arterial pH ($p = .001$) and bicarbonate levels ($p = .001$) and increase in potassium ($p = .025$) and chloride ($p = .0002$) compared with controls. These effects on acid/base status and electrolytes were greater in animals treated with higher vs. lower doses of L-arginine (all p values = .02; Fig. 6). At 8 and 24 hrs there was no significant difference (p nonsignificant) in mean creatinine, arterial lactate levels, and serum sodium among the four treatment groups and controls (data not shown).

At baseline, all measurements of cardiopulmonary function were similar (Bonferroni adjusted; p nonsignificant) in groups of animals treated with L-arginine alone and in combination with *N*-acetylcysteine and in controls (data not shown). At 8 and 24 hrs, with L-arginine alone and in combination with *N*-acetylcysteine, there was no significant difference (p nonsignificant) in the change from baseline in mean cardiac index, left ventricular ejection fraction, Pa_{O_2}, alveolar-arterial oxygen gradient, and pulmonary artery occlusion pressure compared with controls (all p values nonsignificant; data not shown).

Other Mediators of Sepsis. At baseline, quantitative blood culture, endotoxin levels, and TNF levels were similar (Bonferroni adjusted; all p values nonsignificant) in groups of animals treated with L-arginine alone and in combination with *N*-acetylcysteine and in controls (data not shown). From 8 to 24 hrs there were significant elevations in blood bacterial counts and in plasma levels of endotoxin and TNF that were similar (p nonsignificant) in animals treated with L-arginine alone and in combination with *N*-acetylcysteine, and controls (data not shown).

DISCUSSION

L-arginine, a dibasic amino acid with a diverse repertoire of physiologic and pharmacologic activities (9), has an important but incompletely defined role in the pathophysiology of sepsis (6). Treatment with supradietary doses of this nutrient in critical illness remains controversial (1–4). We investigated the effects of intravenous L-arginine alone or in combination with *N*-acetylcysteine. The L-arginine doses studied, 1.5 and 15 times that typically delivered in TPN, lowered MAP, worsened organ injury as evidenced by laboratory findings, and significantly increased mortality rate. Importantly, L-arginine administration was associated with increases in plasma levels of arginine and ornithine; L-arginine is converted to ornithine and urea by arginase. Likewise, serum NO_x, a marker of $NO^{\cdot}$ production, significantly rose in association with L-arginine infusion. *N*-acetylcysteine is an antioxidant that regenerates intracellular glutathione and thereby may reduce production of peroxynitrite (42–44), a toxic free radical formed from $NO^{\cdot}$ and O_2^-. However, despite the addition of *N*-acetylcysteine, the harmful effects of L-arginine were not reduced.

Although the precise mechanisms of L-arginine toxicity in this study cannot be determined, these results do not support proceeding to clinical trials of parenteral L-arginine monotherapy in septic shock. Notably, this study indicates that L-arginine doses only modestly above those of standard dietary practices for TPN might negatively affect survival in septic shock. Typically, full-support TPN in patients delivers about 5–7 $mg \cdot kg^{-1} \cdot hr^{-1}$ of L-arginine. In the low-dose group (10

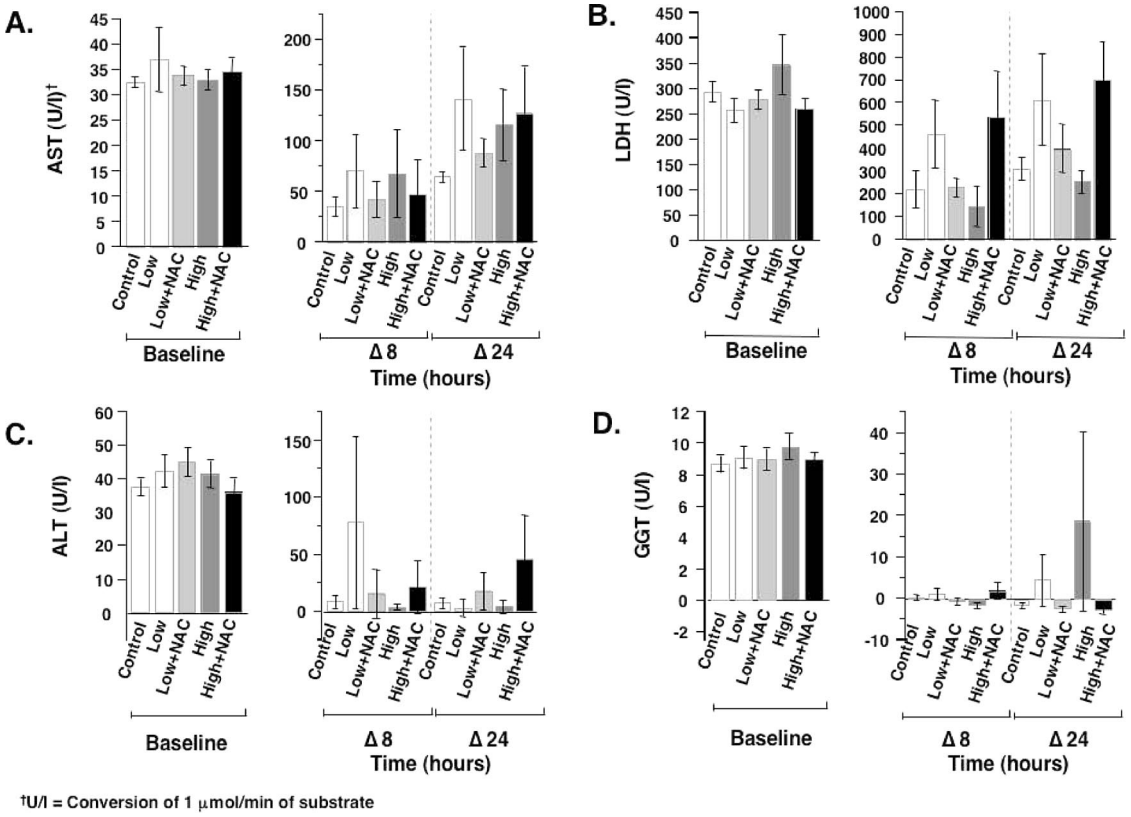

Figure 4. Effect of low- and high-dose L-arginine alone or combined with *N*-acetylcysteine (*NAC*) on liver function tests: (*A*) aspartate aminotransferase (*AST*), (*B*) lactate dehydrogenase (*LDH*), (*C*) alanine aminotransferase (*ALT*), and (*D*) γ-glutamyl transpeptidase (*GGT*). Absolute values at baseline (mean ± SE) are shown on the left and changes over time (mean ± SE) on the right of each panel. The various treatment groups are labeled and indicated by differently shaded bars.

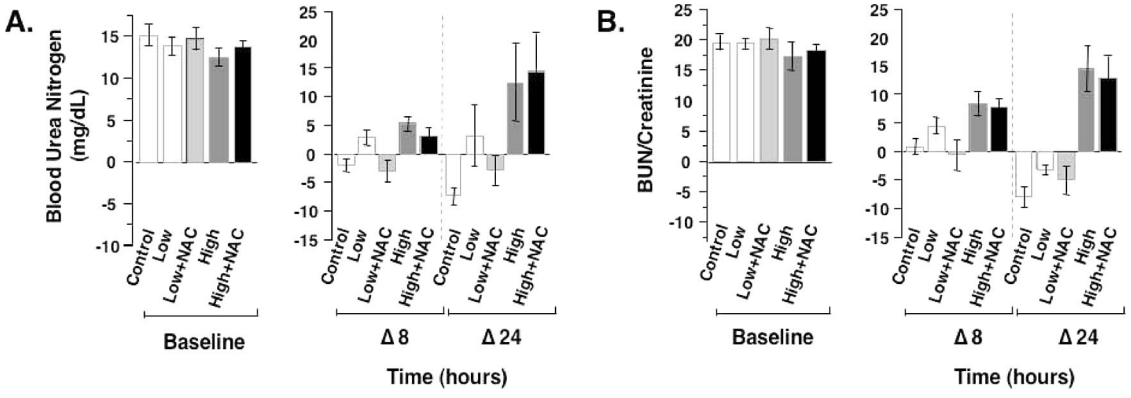

Figure 5. Effect of low- and high-dose L-arginine alone or combined with *N*-acetylcysteine (*NAC*) on renal function tests: (*A*) blood urea nitrogen (*BUN*), and (*B*) BUN/creatinine ratio. Absolute values at baseline (mean ± SE) are shown on the left and changes over time (mean ± SE) on the right of each panel. The various treatment groups are labeled and indicated by differently shaded bars.

$mg \cdot kg^{-1} \cdot hr^{-1}$) examined here, a 10-kg animal received 2.4 g of L-arginine per day. Given the higher caloric and protein demands of canines compared with humans, this represents a standard amount of dietary L-arginine. Chow for the same animal before the study (Premium Edge, 1.55% L-arginine; Meta, MO) provides 2.52–3.78 g of daily L-arginine (0.42 g/100 kcal). At 1.25 g/100 kcal, some immune-enhancing enteral nutrition formulas used clinically deliver almost three-fold more L-arginine on a per-kcal basis (32). However, due to first-pass metabolism of enterally delivered L-arginine and the presence of other additives in these formulations, the current findings cannot be directly extrapolated to the use of immune-enhancing nutrition in patients with septic shock.

The effect of L-arginine on survival in sepsis has been investigated previously in mice, rats, guinea pigs, and rabbits. Mouse models included cecal ligation and puncture or *E. coli* gavage in burn injured animals (25). Both challenges were given 5 days after transfusion with allogeneic blood from endotoxin-sensitive C3H/HeJ mice. Prefeeding for 15 days with a 2% L-arginine-enriched diet compared with two control feeds, containing either 0.7% or 0.5% L-arginine, improved survival (survival for cecal ligation and puncture, 47% vs. 27%; for burn injury 100% vs. 50%). Importantly, the benefit of L-arginine in burn injury was lost

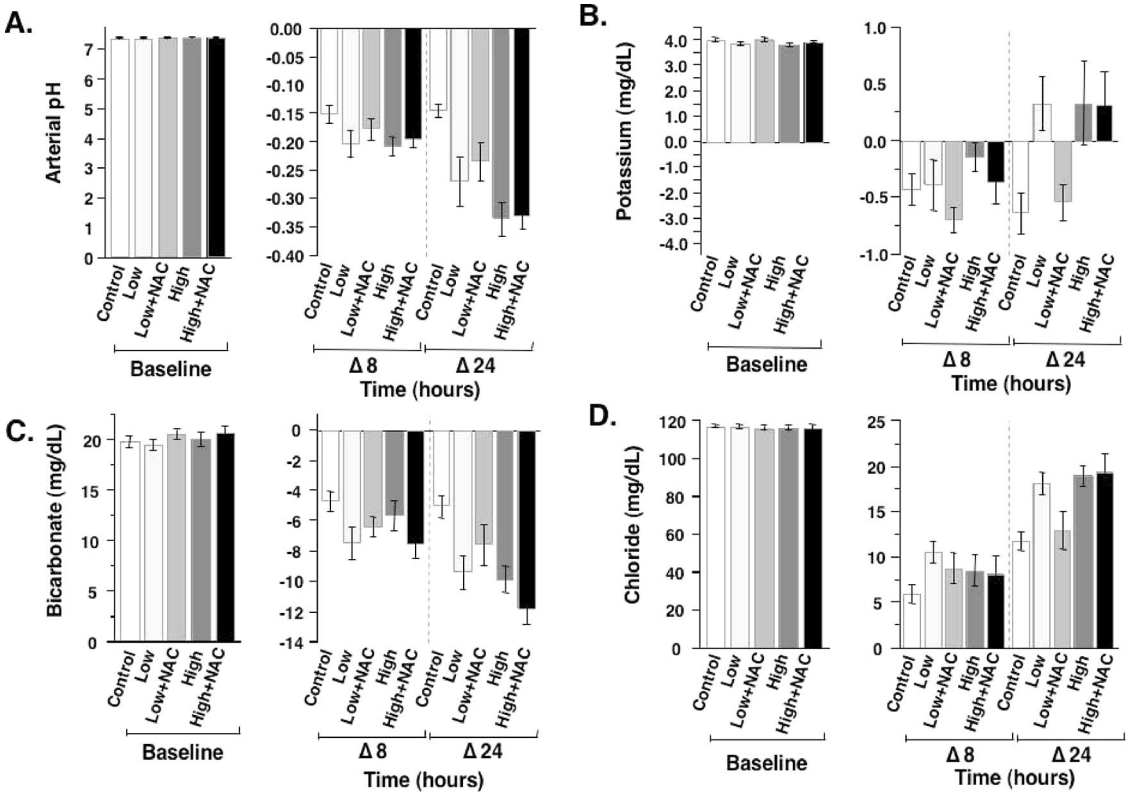

Figure 6. Effect of low- and high-dose L-arginine alone or combined with *N*-acetylcysteine (*NAC*) on serum chemistries: (*A*) arterial pH, (*B*) potassium, (*C*) bicarbonate, and (*D*) chloride. Absolute values at baseline (mean ± SE) are shown on the left and changes over time (mean ± SE) on the right of each panel. The various treatment groups are labeled and indicated by differently shaded bars.

when N-nitro-L-arginine, a NOS inhibitor, was coadministered. A rat study of cecal ligation and puncture also demonstrated a survival benefit comparing intravenous L-arginine monotherapy (1.2 $g \cdot kg^{-1} \cdot day^{-1}$) with a vehicle control (26). However, another report of cecal ligation and puncture in rats comparing two total parenteral nutrition groups that received 1.65 or 0.79 $g \cdot kg^{-1} \cdot day^{-1}$ of L-arginine found no effect on survival (27).

Two studies in guinea pigs, one using enteral feeding in burn injury and the other using parenteral nutrition in a peritonitis model, produced conflicting results. Saito et al. (28) investigated burn-injured animals treated with continuous isonitrogenous, isocaloric, and isovolemic tube feedings. Supplementation with 0%, 1%, 2%, and 4% L-arginine resulted in mortality rates of 56%, 29%, 22%, and 56%, respectively, suggesting that intermediary doses might be beneficial. In contrast, Gonce et al. (29), studying combined *E. coli* and *Staphylococcus* peritonitis in guinea pigs, failed to find a beneficial dose of L-arginine. Animals were randomized to isocaloric and isovolumetric parenteral nutrition containing 0%, 2%, 4%, or 6% L-arginine with mortality rates of 46%, 59%, 59%, and 91%, respectively ($p = .014$). Finally, in a rabbit model, L-arginine supplementation (1.5 $g \cdot kg^{-1} \cdot day^{-1}$) added to drinking water 3 days before and for 5 days after challenge with intravenous endotoxin did not alter mortality compared with D-arginine or N-nitro-L-arginine, a NOS inhibitor (30).

Clinical trials of L-arginine monotherapy in critical illness have not been performed. Although unblinded studies of immune-enhancing enteral nutrition have been conducted, these formulas are complex mixtures. Therefore, outcomes cannot be directly associated with L-arginine supplementation alone. Nonetheless, one of the larger trials of immune-enhancing formula included 181 randomized patients with Acute Physiology and Chronic Health Evaluation II scores ≥10 and laboratory or clinical signs of infection and demonstrated a survival benefit (32). Overall mortality was reduced from 32% to 19% with the immunonutrition formula. However, length of stay in the intensive care unit was not decreased by the experimental therapy, and a subgroup analysis revealed that most of the survival benefit was observed in patients with low disease severity (baseline Acute Physiology and Chronic Health Evaluation II score 10–15) or in patients with bacteremia (1, 32). Interestingly, other immune modulators studied in sepsis have shown the opposite effect; that is, benefit was greatest in the sickest patients (48, 49). Three other clinical trials that used immune-enhancing enteral nutrition containing supplemental L-arginine have suggested potential harmful effects, at least in some subgroups, with respect to survival (33–35). The most recent of these studies was a large, multiple-center trial in critically ill patients comparing immune-enhancing enteral nutrition with standard TPN. A planned interim analysis revealed an excess in intensive care unit mortality among participants with severe sepsis in the experimental arm (44.4% for enteral nutrition vs. 14.3% for TPN; $p = .04$) (35). This was particularly surprising since standard enteral nutrition compared with TPN has generally been shown to be beneficial due to reduced infectious complications (50). The trial, which had recruited 237 patients with a planned enrollment of 1,500 participants, was continued but with an amendment that ex-

cluded patients with severe sepsis. In a meta-analysis of immune-enhancing nutrition in 2001 (31) and a subsequent commentary (1), Heyland and colleagues suggested that these formulas might actually increase mortality rates in septic patients. Collectively, these findings led to a recommendation against supplemental L-arginine in the Canadian Clinical Practice Guidelines for nutritional support in critically ill adults (2). Although our results might be interpreted as supporting these guidelines, intravenous L-arginine given alone in severe sepsis may have physiologic and clinical consequences that are different from those associated enteral L-arginine as part of a complex formula.

The sustained effects we observed on MAP and mean pulmonary artery pressure in our canine model have been reported to occur acutely in human studies that administered relatively large bolus doses of L-arginine to patients with sepsis or other forms of endothelial dysfunction. Lorente and colleagues (36) challenged seven patients with vasopressor-dependent septic shock with 200 $mg \cdot kg^{-1}$ L-arginine, which produced immediate but transient hemodynamic changes consistent with systemic and pulmonary vasodilation. Likewise, 500 $mg \cdot kg^{-1}$ bolus doses of L-arginine have transiently (<60 mins) decreased blood pressure in healthy men with somewhat larger effects in obese hypertensive men (37). Furthermore, this same bolus dose of L-arginine caused short-term pulmonary vasodilation in patients with pulmonary hypertension (38). These bolus doses were shown to increase $NO^{\cdot}$ production (38, 39), as was documented for the continuous infusions of L-arginine in our canine study.

The mechanisms by which L-arginine infusion worsened outcome in this study are uncertain. *N*-acetylcysteine, despite its well-documented antioxidant activity and potential to decrease the formation of peroxynitrite (42–44), had no effect on L-arginine-associated vasodilation or organ injury. L-arginine did increase plasma levels of arginine and serum levels of NO_x. The L-arginine-induced rise in NO_x levels suggests that supplying substrate to inducible NOS may have increased $NO^{\cdot}$ production, thereby worsening shock and mortality. Recent studies have also suggested a role for endothelial NOS-derived $NO^{\cdot}$ in early septic shock, which appears to optimize the later expression of inducible NOS (51). However, simply attributing our findings to feeding the $NO^{\cdot}$ pathway ignores the complexity of L-arginine biology and the difficulty of identifying a single mechanism. The remarkable efficiency of the y+ transport system that maintains intracellular levels of L-arginine (52, 53) and the ability of endothelium to synthesize its own L-arginine from citrulline (54) suggest that exogenous L-arginine may not affect $NO^{\cdot}$ production directly. Likewise, L-arginine boluses have failed to decrease MAP in healthy adults when the secretion of insulin and/or growth hormone was blocked by an infusion of octreotide or somatistatin (55, 56). Furthermore, incubation of isolated, atherosclerotic rabbit aortas with high concentrations of L-arginine did not affect vascular tone or modulate endothelium-dependent relaxation (57).

L-arginine effects in normal volunteers and isolated vessels are unlikely to simulate its *in vivo* actions during septic shock, when inducible NOS is highly expressed in the vasculature. Furthermore, continuous L-arginine infusions at the doses used in our study are not known to have the prominent neuroendocrine effects that have been associated with large boluses (9). In sepsis, low L-arginine and high ADMA levels may substantially alter L-arginine transport and utilization. ADMA not only acts as a nonselective inhibitor of NOS but also competes for L-arginine transport into cells (21–24).

In addition to increasing mortality rate and worsening hemodynamics, L-arginine also appeared to worsen measures of liver injury and renal function, and it decreased pH. $NO^{\cdot}$ has been shown to either protect or harm the liver in models of sepsis or reperfusion injury, depending on the level of redox stress (58). L-arginine-mediated liver toxicity in our model may also be attributed to worsened shock rather than $NO^{\cdot}$ *per se*. Notably, the ability of *N*-acetylcysteine to regenerate glutathione and thereby reduce oxidant stress had no effect on liver injury. Unlike elevations in liver enzymes, increases in blood urea nitrogen may not reflect $NO^{\cdot}$-mediated organ injury or actual changes in glomerular filtration rates. Arginase metabolizes L-arginine into ornithine and urea (6, 9). Therefore, supplying substrate to this pathway might have increased blood urea nitrogen independent of altered renal function. The metabolic acidosis seen in L-arginine-treated animals was hyperchloremic; lactate levels and anion gap were not increased. Diarrhea appeared to occur more frequently with L-arginine treatment than in controls, suggesting that it may have contributed to bicarbonate loss and the low pH. Although NOS is prominently expressed in the bowel, its possible role in the L-arginine-associated diarrhea we observed has not been described.

CONCLUSIONS

L-arginine monotherapy in this canine model of septic shock augmented $NO^{\cdot}$ production, lowered blood pressure, increased evidence of organ injury, and worsened survival. These harmful effects were seen at doses below that supplied in standard chow and only 1.5-fold higher than that delivered clinically (on a $g \cdot kg^{-1} \cdot day^{-1}$ basis) in standard formulations of TPN. These findings provide some preclinical evidence for the recommendation of the Canadian Clinical Practice Guidelines for nutrition support in critical illness that "diets supplemented with L-arginine. . .not be used for critically ill patients" (2). Our investigation cautions against recent calls for clinical trials of L-arginine monotherapy in patients with severe sepsis or septic shock.

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