

Local Anesthetics Reduce Mortality and Protect against Renal and Hepatic Dysfunction in Murine Septic Peritonitis

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Background: **Mortality from sepsis frequently results from multiple organ injury and dysfunction. Cecal ligation and puncture is an established murine model of septic peritonitis that produces septic shock characterized by an initial hyperinflammatory response. In addition to their anesthetic properties, local anesthetics have been shown to attenuate inflammatory responses both *in vivo* and *in vitro*. In the current study, the ability of local anesthetic infusions to protect against sepsis-induced mortality, as well as renal and hepatic dysfunction after cecal ligation and puncture, was investigated.**

Methods: **C57BL/6 mice received mini-osmotic pumps containing saline (vehicle), 10% lidocaine, or 1% bupivacaine and were subjected to cecal ligation and puncture. Twenty-four hours after cecal ligation and puncture, renal and hepatic functions were assessed as well as markers of inflammation (proinflammatory cytokine protein and mRNA concentrations and myeloperoxidase activity). Renal apoptosis and 7-day survival was also assessed.**

Results: **Mice treated with lidocaine or bupivacaine infusion showed improved survival and had significantly lower plasma creatinine, aspartate aminotransferase, and alanine aminotransferase concentrations compared with mice receiving vehicle alone. Significant reduction in plasma tumor necrosis factor-α and keratinocyte-derived chemokine, as well as reductions in myeloperoxidase activity, intracellular adhesion molecule-1 protein expression, mRNA concentrations of proinflammatory markers, and apoptosis were observed in renal cortices from both local anesthetic groups.**

Conclusions: **The current data demonstrate that local anesthetic infusions confer a protective effect in mice from septic peritonitis by attenuating the hyperacute inflammatory response. This suppression resulted in improved mortality and less progression to acute kidney and liver injury and dysfunction.**

THE incidence of sepsis and the associated mortality are increasing. On an annual basis, sepsis afflicts approximately 750,000 patients in the United States and accounts for nearly $16.7 billion in health care costs.[1] When sepsis is associated with the development of acute multiorgan dysfunction, morbidity, mortality, and treatment expenditures greatly increase. For example, renal or hepatic dysfunction occurs in approximately 23% of all septic patients and is a significant clinical feature (38.2% and 54.3%, respectively) seen in patients who die from sepsis. Currently, no prophylactic therapy for patients in early sepsis has proven to avert progression to multiorgan dysfunction. Furthermore, no definitive treatment exists for those patients who progress to multiorgan failure.

Although the exact pathogenesis leading to multiorgan dysfunction remains to be elucidated, a complex inflammatory process seems to play a central role in initiating its development. An uncontrolled, hyperinflammatory response and associated uncontrolled, hyperexuberant cytokine production are known to be detrimental to organ function and are proposed to be the main cause precipitating multiorgan dysfunction in early sepsis. The murine cecal ligation and puncture (CLP) model resembles the human sepsis syndrome with respect to cytokine/chemokine generation and the development of fulminant multiorgan failure.[2–4] Although various attempts at targeting particular mediators of inflammation have not proved universally successful in human sepsis trials, some studies have suggested a beneficial role from more global antiinflammatory modulation.[5]

Local anesthetics have been shown to modulate inflammatory cascades and provide protection from ischemic reperfusion injury in the heart,[6,7] lung,[8,9] and liver.[10] They are capable of exerting antiinflammatory effects on various cell types including monocytes, macrophages, and neutrophils.[11] Therefore, we questioned whether infusion of local anesthetics after CLP would attenuate the expression of hyperinflammatory mediators, reduce organ injury and dysfunction, and provide a significant survival effect in this *in vivo* murine model of septic peritonitis.

Materials and Methods

Mice

We used male C57BL/6 mice (approximately 20–25g) purchased from Harlan Laboratories (Indianapolis, IN). All animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University (New York, NY).

Delivery of Saline Vehicle or Local Anesthetics

Mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg or to effect). A mini-osmotic pump (Model #2001; Durect Corporation, Cupertino, CA) was implanted into the subcutaneous space on the back of

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Table 1. Local Anesthetic Plasma Levels at 24 hours after Cecal Ligation and Puncture

Drug	Plasma Concentration (μM)
Lidocaine 10% (n = 5)	2.25 ± 0.44
Lidocaine 5% (n = 3)	0.89 ± 0.17
Bupivacaine 2% (n = 3)	0.16 ± 0.02
Bupivacaine 1% (n = 3)	0.09 ± 0.01

Values expressed as average ± SEM.

each animal. All pumps continuously delivered 1 μl/h of 10% lidocaine ($\sim$5.0 mg·kg^{-1}·h^{-1}), 5% lidocaine ($\sim$2.5 mg·kg^{-1}·h^{-1}), 2% bupivacaine ($\sim$1.0 mg·kg^{-1}·h^{-1}), 1% bupivacaine ($\sim$0.5 mg·kg^{-1}·h^{-1}), or saline vehicle for 7-day survival studies. Subsequent functional and molecular studies were then performed with 10% lidocaine and 1% bupivacaine at 24 h after CLP. These doses of local anesthetics were chosen to mimic clinically achieved plasma concentrations in patients receiving epidural local anesthetics for postoperative pain control. To confirm that clinically relevant local anesthetic plasma concentrations were achieved at 24 h with this delivery system, local anesthetic plasma concentrations from mice were confirmed by high performance liquid chromatography measurements at the Nathan Kline Institute (Orangeburg, NY) as described previously (table 1).[12]

Induction of Sepsis

Six hours after pump implantation (the time interval is required for pump priming), the mice were reanesthetized with intraperitoneal pentobarbital and were allowed to spontaneously breathe room air on an electric heating pad under a warming light. CLP was performed as described by others.[13] Briefly, the cecum was isolated through a 1-cm midline incision and the distal (0.5 cm) portion of the cecum below the ileocecal valve (to avoid bowel obstruction) was ligated with a 4–0 silk suture. The cecum was then punctured through and along its antimesenteric border (double puncture) with a 20-gauge needle and a small amount of stool was extruded through the puncture site. After instillation of 0.5 ml normal saline into the peritoneal cavity, the abdomen was closed in two layers. The mice remained on the heating pad until they recovered from the anesthetic. Twenty-four hours after CLP, mice were killed with an overdose of intraperitoneal pentobarbital, and specimens (plasma and kidneys) were collected. The left kidney was used for histologic evaluation and the right kidney was snap frozen in liquid nitrogen for all *in vitro* studies described (*e.g.*, DNA laddering, semiquantitative reverse transcriptase polymerase chain reaction, and immunoblotting).

Survival Studies

To determine the effect that local anesthetic infusions would have on mortality from CLP-induced septic peritonitis, continuous subcutaneous infusion of lidocaine and bupivacaine was administered to mice for 7 days until the pump reservoir was exhausted. Survival studies were performed with infusions of varying concentrations of lidocaine (5% and 10%) and bupivacaine (1% and 2%) to determine if dose dependency existed. All mice had free access to water and food and were frequently observed by dedicated research personnel to determine 7-day survival statistics. All severely moribund animals were euthanized with an overdose injection of anesthetic in adherence with our animal protocol. To examine if the manner of administration played an important role in survival, a separate small cohort of mice (n = 8) were bolused with lidocaine every 8 h totaling the dose they would have received with the continuous osmotic pump infusion. Specifically, a 0.8 mg subcutaneous lidocaine bolus every 8 h for 24 h was equivalent to an infusion of 10% lidocaine at 1 μl/h over 24 h.

Assessment of Renal and Hepatic Function after Sepsis

Renal function was assessed by measuring plasma creatinine 24 h after CLP by a colorimetric method based on the Jaffe reaction.[14] Hepatic function 24 h after CLP was assessed by measuring plasma, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) concentrations using a commercially available colorimetric method (Sigma, St. Louis, MO).

Measurement of Systemic Cytokines by ELISA

Murine plasma tumor necrosis factor (TNF)-α and keratinocyte-derived chemokine (KC) concentrations taken 24 h after CLP were measured using commercially available ELISA kits according to manufacturer instructions (ALPCO, Windham, NH and R&D Systems, Minneapolis, MN, respectively).

Assessment of Renal Inflammation

Renal inflammation 24 h after CLP was determined by measurement of renal cortical myeloperoxidase activity (a marker of leukocyte infiltration) and immunoblotting for intracellular adhesion molecule (ICAM)-1 as described previously.[12] In addition, measurements of mRNA encoding for the markers of inflammation (KC, macrophage inflammatory protein [MIP]-2, ICAM-1, monocyte chemoattractant protein [MCP]-1), TNF-α, regulated on activation normal T-cell expressed and secreted, interferon-induced protein 10, and glyceraldehyde 3-phosphate dehydrogenase (control for RNA quantity) in renal cortices with semiquantitative reverse transcriptase polymerase chain reaction were also performed as described previously.[12] For each experiment, we also performed semiquantitative reverse transcriptase polymerase chain reaction under conditions yielding linear results for glyceraldehyde 3-phosphate dehydrogenase to confirm equal RNA input. Primers were

Table 2. Proinflammatory Sense and Anti-sense Primers Based on Published GenBank Sequences for Mice

Primer	Accession No. Mouse	Sequence (Sense, Antisense)	Product Size, bp	Annealing Temperature (°C)
KC	J04596	5′-CAATGAGCTGCGCTGTCAGTG-3′ 5′-CTTGGGGACACCTTTTAGCATC-3′	203	60
MIP-2	X53798	5′-CCAAGGGTTGACTTCAAGAAC-3′ 5′-AGCGAGGCACATCAGGTACG-3′	282	60
ICAM-1	X52264	5′-TGTTTCCTGCCTCTGAAGC-3′ 5′-CTTCGTTTGTGATCCTCCG-3′	409	60
MCP-1	NM_011333	5′-ACCTGCTGCTACTCATTCAC-3′ 5′-TTGAGGTGGTTGTGGAAAAG-3′	312	60
TNF-α	X02611	5′-CCTCAGCCTCTTCTCCTTCCT-3′ 5′-GGTGTGGGTGAGGAGCA-3′	290	65
IP-10	M33266	5′-CCTATCCTGCCCACGTGTTGAG-3′ 5′-CGCACCTCCACATAGCTTACAG-3′	430	60
RANTES	M77747	5′-CCTCACCATCATCCTCACTGCA-3′ 5′-TCTTCTCTGGGTTGGCACACAC-3′	214	65
GAPDH	M32599	5′-ACCACAGTCCATGCCATCAC-3′ 5′-CACCACCCTGTTGCTGTAGCC-3′	450	65

Respective anticipated reverse transcriptase polymerase chain reaction product size and annealing temperatures used for each primer are provided.

bp = basepair; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; ICAM-1 = intracellular adhesion molecule-1; IP-10 = interferon-induced protein 10; KC = keratinocyte derived chemokine; MCP-1 = monocyte chemoattractant protein 1; MIP-2 = macrophage inflammatory protein 2; RANTES = regulated upon activation normal T-cell expressed and secreted; TNFα = tumor necrosis factor-alpha.

designed based on published GenBank sequences (National Institutes of Health database, Bethesda, MD) for mice (table 2).

Assessment of Renal Necrosis and Apoptosis

Both necrosis and apoptosis was assessed by histologic evaluation. Renal apoptosis was also evaluated by DNA laddering as well as by *in situ* Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling assay Assay (TUNEL).

Histologic Examinations

Explanted kidneys were bisected along the long axis and fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Morphological assessment was performed by an experienced renal pathologist (SHN) who was blinded to the treatments that each animal had received. An established grading scale (0–4) of necrotic injury to the proximal tubules was used for the histopathological assessment of damage as outlined by Jablonski *et al.*[15] For apoptotic assessment the pathologist examined hematoxylin and eosin stained mouse kidney sections under light microscopy for apoptotic bodies. As previously described, the degree of renal tubular apoptosis was quantified by counting the number of apoptotic bodies in proximal tubules in the corticomedullary area of the kidney.[16] Twenty-five to fifty tubules were counted per field from each treatment group and at least six fields were examined per section. Results are representative of at least four separate experiments.

Detection of DNA Fragmentation by DNA Laddering

To assess potential differences in DNA laddering (indicative of apoptosis), extracted DNA (Wizard; Promega, Madison, WI) from dissected renal cortices taken at 24 h after CLP were subjected to electrophoresis for 6 h at 70 V in a 2.0% agarose gel in Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide and photographed under ultraviolet illumination. DNA ladder markers (100 bp) were used as a reference for the analysis of internucleosomal DNA fragmentation.

Assessment of Renal Apoptosis by TUNEL Assay

To further evaluate potential differences in renal apoptosis, TUNEL assay was performed on formalin fixed mice kidney sections obtained at 24 h after CLP. Briefly, sections were deparaffinized and rehydrated. *In situ* labeling of fragmented DNA was performed with TUNEL (green fluorescence) using a commercial *in situ* cell death detection kit (Roche, Indianapolis, IN) according to manufacturer instructions. To visualize the total number of cells in the field, sections were also concomitantly stained with a red fluorescent monomeric cyanine nucleic acid stain (Molecular Probes, Eugene, OR).

Statistical Analysis

One-way analysis of variance was used to compare mean values across multiple treatment groups with a Dunnett *post hoc* multiple comparison test. The ordinal values of the Jablonski scale were analyzed by the Kruskal-Wallis nonparametric test with Dunn posttest comparison between groups. Survival statistics were compared with a Kaplan-Meier curve and log-rank test. In all cases, a probability value less than 0.05 was taken to indicate significance.

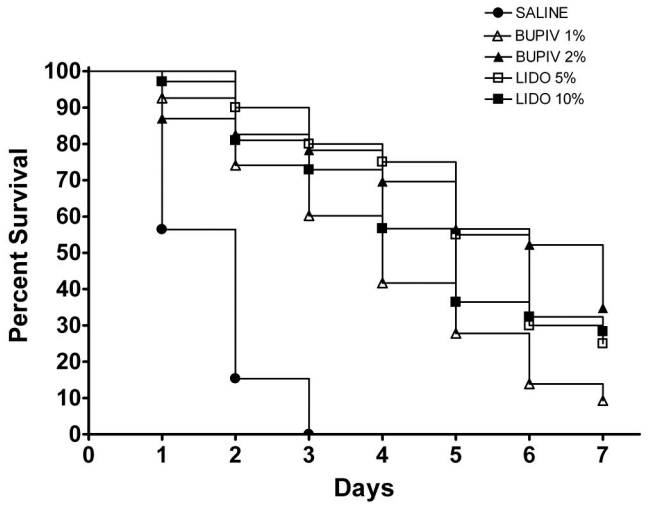

Fig. 1. Kaplan-Meier survival curves were generated for C57 mice subjected to cecal ligation and double puncture with saline infusion (n = 35), C57 mice subjected to cecal ligation and double puncture while receiving infusion of 5% (n = 20) or 10% (n = 35) lidocaine, and C57 mice subjected to cecal ligation and double puncture treated with infusion of 1% (n = 27) or 2% (n = 21) bupivacaine. Log-rank analysis demonstrated a significant improvement in survival for all local anesthetic infusion groups *versus* the saline vehicle group ($P < 0.05$). Log-rank analysis also demonstrated a significant improvement in survival in the 2% bupivacaine infusion group *versus* the 1% bupivacaine infusion group ($P < 0.05$). No statistical differences in mortality were observed between the lidocaine infusion groups. *BUPIV* = bupivacaine; *LIDO* = lidocaine.

Reagents

Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, MO). Protein content was determined with the Pierce Chemical (Rockford, IL) bicinchoninic acid protein assay reagent using bovine serum albumin as a standard.

Results

Local Anesthetic Infusion Protects against CLP-induced Mortality

We initially measured the effects of chronic local anesthetic infusion on mortality from CLP-induced septic peritonitis. As demonstrated in figure 1, infusion of lidocaine and bupivacaine significantly protected mice against CLP-induced mortality. Both lidocaine infusion groups (5% and 10%) significantly reduced mortality to 0% (n = 20) and 3% (n = 25), respectively, at 24 h compared with the saline vehicle group (24 h mortality = 49%, n = 31; $P < 0.05$). Log-rank analysis demonstrated that a significant level of protection was conferred by lidocaine throughout the study. Similar results were observed in the mice that received continuous subcutaneous infusions of bupivacaine. CLP-induced mortality with 1% and 2% bupivacaine infusion was 7% (n = 22) and 14% (n = 21, respectively) at 24 h, and a significant degree of protection compared with saline vehicle persisted throughout the study ($P < 0.05$). The 2% bupivacaine infusion significantly improved survival *versus* the 1% bupivacaine infusion ($P < 0.05$). However, we chose to use the 10% lidocaine and 1% bupivacaine treatments in the remaining studies because these more accurately reflect clinically relevant doses. We also found the manner of administration had an impact on survival benefit. We observed no benefit in mortality with bolus administration (every 8 h for 24 h) of 10% lidocaine (data not shown).

Local Anesthetic Infusion Significantly Reduced CLP-induced Organ Dysfunction/Injury

The development of organ dysfunction and injury as a complication of sepsis is associated with increased mortality. We examined the degree of renal dysfunction by measuring plasma creatinine and the degree of hepatic dysfunction by measuring ALT and AST 24 h after CLP. In this model of sepsis, plasma creatinine was significantly increased in saline vehicle treated mice (1.2 ± 0.10 mg/dl, n = 13) compared with sham surgery controls (0.50 ± 0.10 mg/dl, n = 8) (table 3). Mice treated with 10% lidocaine or 1% bupivacaine infusion had significantly lower plasma creatinine at 24 h (0.50 ± 0.10 mg/dl, n = 10 and 0.50 ± 0.10 mg/dl, n = 5; $P < 0.001$ respectively) compared with mice treated with saline vehicle alone Therefore, local anesthetic infusion alleviates CLP-induced renal injury. Similarly, 24 h after CLP operation AST and ALT concentrations were significantly increased in saline vehicle treated mice (AST, 349 ± 15 U/ml, n = 15 and ALT, 142 ± 10 U/ml, n = 12) compared with sham surgery controls (AST, 91 ± 11 U/ml, n = 8 and ALT, 20 ± 2 U/ml, n = 8; table 3). At 24 h after CLP, the lidocaine and bupivacaine treated groups also demonstrated lower AST (134 ± 13 U/ml, n = 7 and 169 ± 28 U/ml, n = 4 respectively; $P < 0.001$) and ALT (78 ± 7 U/ml, n = 7 and 95 ± 23 U/ml, n = 4 respectively; $P < 0.001$) compared with mice receiving vehicle alone. This data also demonstrates that local anesthetic infusion ameliorates CLP-induced hepatic injury in mice.

Local Anesthetic Infusion Significantly Attenuated Proinflammatory KC and TNF-α Plasma Concentrations after CLP

TNF-α concentrations were undetectable in most sham surgery control mice (n = 5), indicating that baseline concentrations were less than 18.2 pg/ml (the sensitivity of our ELISA). In contrast, plasma TNF-α concentrations in mice undergoing CLP with saline vehicle was significantly increased (1626 ± 553 pg/ml, n = 6) in comparison with the sham and the local anesthetic groups. Infusion of lidocaine or bupivacaine resulted in undetectable plasma TNF-α concentrations, indicating that local anesthetics reduced TNF-α concentrations after CLP-induced sepsis to concentrations indistinguishable from sham controls (table 3). KC plasma concentrations

Table 3. Markers of Organ Dysfunction, Markers of Systemic Inflammation, and Markers of Renal Inflammation 24 hours after the induction of sepsis

Test	Sham	Vehicle	Lidocaine 10%	Bupivacaine 1%
Markers of Organ Injury				
Creatinine (mg/dl)	0.5 ± 0.1 (8)	1.2 ± 0.1 (13)*	0.48 ± 0.06 (10)†	0.5 ± 0.1 (5)†
AST (SF U/ml)	91 ± 11 (8)	349 ± 15 (15)*	134 ± 13 (7)†	169 ± 28 (4)†
ALT (SF U/ml)	20 ± 2 (8)	142 ± 10 (12)*	78 ± 7 (7)*†	95 ± 23 (4)*†
Markers of Systemic Inflammation				
TNF-α ELISA (pg/ml)	<18.2 (5)	1626 ± 553 (6)*	<18.2 (6)†	<18.2 (4)†
KC ELISA (pg/ml)	173 ± 19 (4)	21375 ± 5353 (5)*	1346 ± 150 (7)*†	3292 ± 770 (4)*†
Markers of Renal Inflammation				
MPO assay (ΔOD/min/mg protein)	0.02 ± 0.02 (7)	2.1 ± 0.24 (9)*	0.57 ± 0.11 (3)†	0.31 ± 0.21 (5)†
ICAM-1 protein expression (% of sham)	1 ± 0.08 (6)	2.64 ± 0.49 (6)*	0.91 ± 0.16 (6)†	0.98 ± 0.6 (4)†

Values expressed as average ± SEM (n).

ALT = alanine aminotransferase levels; AST = aspartate aminotransferase; ICAM-1 = intracellular adhesion molecule 1; KC = keratinocyte derived chemokine; MPO = myeloperoxidase.

* $P < 0.05$ *versus* sham; † $P < 0.05$ *versus* saline vehicle.

at 24 h after operation were significantly increased in mice undergoing CLP with saline vehicle (21375 ± 5353 pg/ml, n = 5) compared with concentrations observed in the sham group (173 ± 19 pg/ml, n = 4). Lidocaine and bupivacaine significantly reduced KC plasma concentrations to 1346 ± 150 pg/ml, n = 7 and 3292 ± 770 pg/ml, n = 4, respectively (table 3).

Renal Myeloperoxidase Activity 24 h after CLP Is Reduced by Local Anesthetic Infusion

Myeloperoxidase is an enzyme present in leukocytes and is an index of tissue leukocyte infiltration.[17] CLP increased myeloperoxidase activity (2.10 ± 0.24 ΔOD/min/mg protein, n = 9) in the renal cortex of mice compared with sham controls (0.02 ± 0.02 ΔOD/min/mg protein, n = 7). However, mice receiving 10% lidocaine or 1% bupivacaine infusion demonstrated significantly reduced myeloperoxidase concentrations (0.57 ± 0.11 ΔOD/min/mg protein, n = 3 and 0.31 ± 0.21 ΔOD/min/mg protein, n = 5, respectively) as compared with mice treated with vehicle alone (table 3).

Renal Protein and mRNA Expression of Proinflammatory Markers after CLP Is Reduced by Local Anesthetic Infusion

Immunoblotting for ICAM-1 protein from dissected renal cortices taken 24 h after CLP revealed that ICAM-1 protein expression was significantly higher in the CLP group receiving saline vehicle (263 ± 49% of sham, n = 6) *versus* the lidocaine and bupivacaine treated groups (91 ± 16% of sham, n = 6 and 98 ± 55% of sham, n = 5; respectively) (table 3). In a separate set of experiments, saline-treated mice subjected to CLP demonstrated increased gene expression of KC, MIP-2, ICAM-1, MCP-1, TNF-α, regulated upon activation normal T-cell expressed and secreted, and interferon-induced protein 10 compared with sham controls. However, infusion of 10% lidocaine or 1% bupivacaine resulted in reduced mRNA quantities of KC, MIP-2, ICAM-1, and MCP-1 (fig. 2). mRNA quantity of TNF-α, regulated upon activation normal T-cell expressed and secreted, and interferon-induced protein 10 were not significantly different between treatment groups (data not shown). This occurred despite a decrease in systemic plasma concentrations of TNF-α with local anesthetic infusion as described above. This is consistent with other published findings that suggest suppression of TNF-α can occur at a posttranscriptional level.[18]

Local Anesthetic Treatment Decreases CLP-induced Renal Apoptosis

Although no appreciable renal necrosis was observed 24 h after CLP-induced sepsis (Jablonski scores were 0 for all groups), CLP-induced sepsis in mice receiving saline vehicle resulted in a significant increase in renal cortical apoptosis. However, local anesthetic infusion decreased the magnitude of renal apoptosis after CLP, as indicated by quantification of apoptotic bodies, the appearance of DNA laddering, and TUNEL positive cells.

There was a minimal occurrence of renal apoptotic bodies seen in sham operated mice (0.023 ± 0.022 apoptotic bodies/tubule). CLP resulted in a significantly higher number of renal apoptotic bodies (0.320 ± 0.117 apoptotic bodies/tubule; $P < 0.05$). In contrast, infusion of 10% lidocaine (0.018 ± 0.008 apoptotic bodies/tubule, n = 6) or 1% bupivacaine (0.006 ± 0.004 apoptotic bodies/tubule, n = 4) resulted in decreased renal apoptosis at 24 h after CLP ($P < 0.05$).

Similar results were observed when renal cortices were assessed for DNA laddering (fig. 3). Sham-operated mice displayed little to no DNA laddering. Samples from mice subjected to CLP and saline infusion showed obvious DNA laddering composed of DNA fragments in multiples of 180 basepairs, whereas samples from mice re-

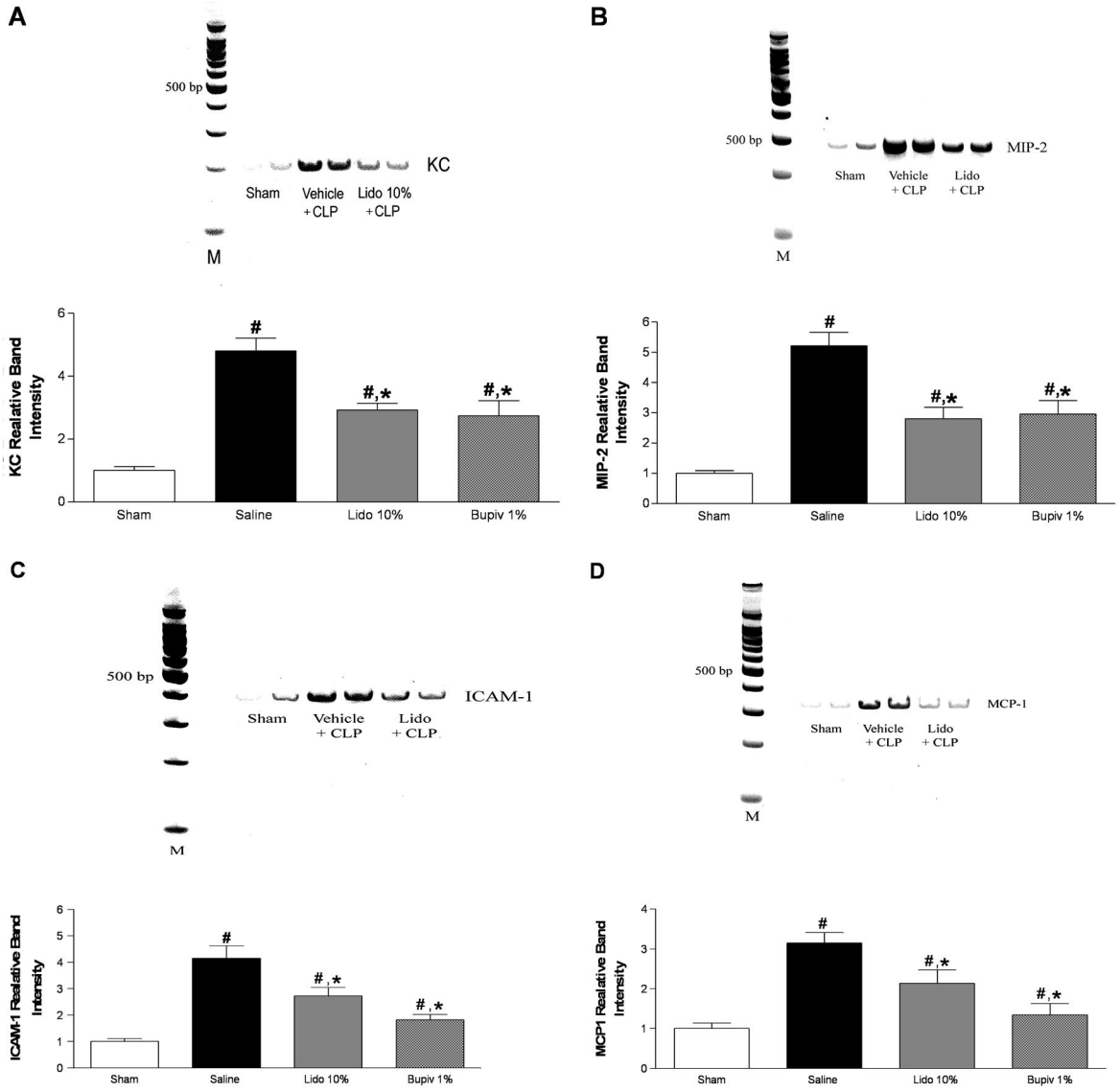

Fig. 2. Semiquantitative reverse transcriptase polymerase chain reaction of proinflammatory markers keratinocyte derived chemokine (KC) (*A*), macrophage inflammatory protein 2 (MIP-2) (*B*), intracellular adhesion molecule-1 (ICAM-1) (*C*), and monocyte chemoattractant protein 1 (MCP-1) (*D*) from renal cortices of sham operated C57 control mice, C57 mice subjected to cecal ligation and double puncture (CLP) with saline infusion, C57 mice subjected to CLP while receiving infusion of 10% lidocaine, and C57 mice treated with infusion of 1% bupivacaine. Bar graphs represent densitometric quantifications of relative band intensities from reverse transcriptase polymerase chain reactions for each indicated mRNA, and are compilations of at least three separate experiments. A representative image of the semiquantitative reverse transcriptase polymerase chain reaction gels for each gene with sham, saline CLP, and 10% lidocaine with CLP is also shown. *bp* = basepair; *M* = 100 basepair DNA marker; *Bupiv* = bupivacaine; *Lido* = lidocaine. # $P < 0.05$ *versus* sham; *$P < 0.05$ *versus* saline vehicle.

ceiving local anesthetic infusion demonstrated minimal to no evidence of DNA laddering. Figure 3 is representative of three separate experiments.

Furthermore, we failed to detect TUNEL positive corticomedullary kidney cells in sections from sham-operated mice (fig. 4). Consistent with our above findings, mice treated with saline infusion undergoing CLP showed increased TUNEL positive staining in the corticomedullary junction. However, mice receiving local anesthetic infusion and subjected to CLP showed reduced TUNEL-positive cells in the corticomedullary junction after 24 h. Figure 4 is representative of three separate experiments.

Discussion

The major findings of the current study are that chronic infusions of lidocaine or bupivacaine improve mortality, provide significant protection from acute renal and hepatic dysfunction, and attenuate the hyperinflammatory response and renal apoptosis associated with murine septic peritonitis induced by CLP.

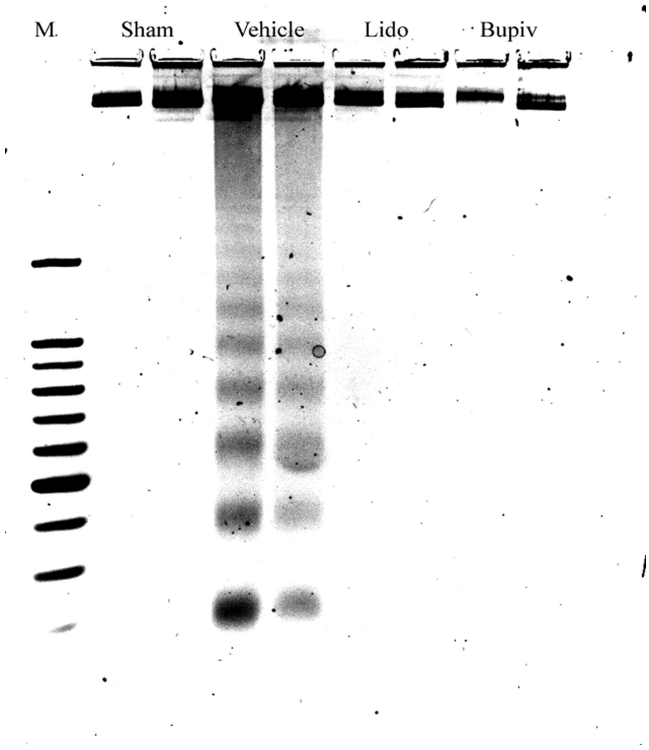

Fig. 3. Representative image of DNA laddering in renal cortices of sham operated C57 control mice, C57 mice subjected to cecal ligation and double puncture with saline infusion, C57 mice subjected to cecal ligation and double puncture while receiving infusion of 10% lidocaine, and C57 mice treated with infusion of 1% bupivacaine. Image is representative of three separate experiments. *M* = 100 basepair DNA marker; *Bupiv* = bupivacaine; *Lido* = lidocaine

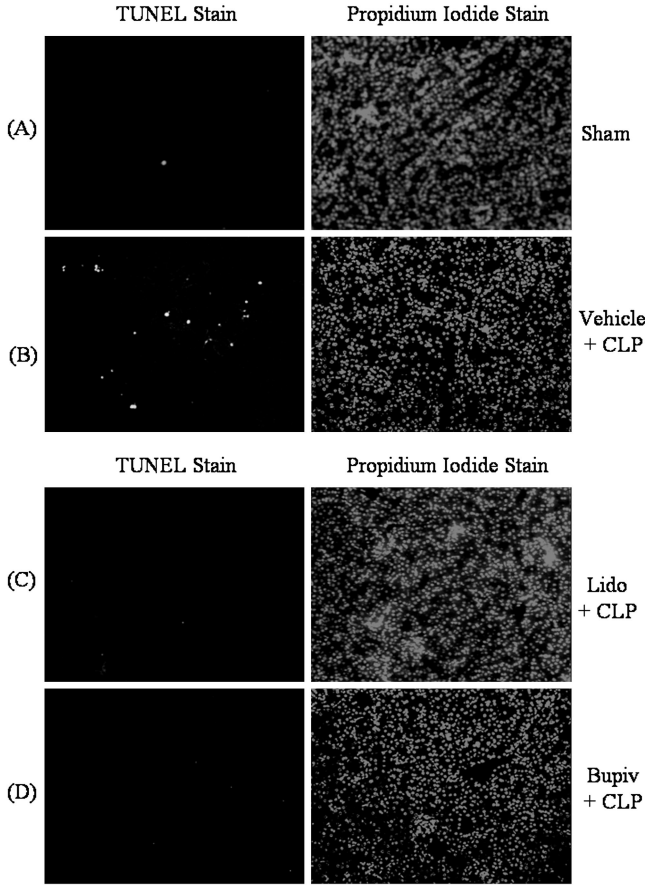

Fig. 4. Representative image of TUNEL staining of renal cortices of sham operated C57 control mice (*A*), C57 mice subjected to cecal ligation and double puncture with saline infusion (*B*), C57 mice subjected to cecal ligation and double puncture while receiving infusion of 10% lidocaine (*C*), and C57 mice treated with infusion of 1% bupivacaine (*D*). Image is representative of three separate experiments. *Bupiv* = bupivacaine; *Lido* = lidocaine.

Sepsis remains a major health challenge facing contemporary medicine. Despite advances in antibiotic, hemodynamic, and ventilatory support, sepsis remains responsible for approximately 215,000 deaths in the United States each year.[1] Given the high mortality rate associated with sepsis (which ranges between 30% and 70%), therapeutic interventions are primarily judged by their impact on survival.[19]

To more accurately recapitulate the complex immunology of human sepsis, we adopted the cecal ligation and double puncture model that produces a clinical picture that more accurately reflects human sepsis than animal models that utilize endotoxin or bacteria.[3,20] This model induces septic peritonitis and has been shown to resemble human sepsis with respect to cytokine generation and progression to multiorgan failure and response to certain therapeutic interventions.[21] Another advantage of this model is the ability to manipulate the magnitude of sepsis. In preliminary studies, we compared survival responses to graded injuries from 18-gauge, 20-gauge, and 22-gauge CLP. We chose to employ a 20-gauge needle to induce a lethal degree of sepsis with a high predilection for fulminant multiple organ failure in the absence of any interventions. Our mortality rates correspond well with other published survival studies using this genetic strain in CLP.[22] We deliberately chose this severe model of sepsis to elucidate whether local anesthetic infusion could improve survival, even in the face of a known lethal injury. By 48 h after CLP, we observed a profound level of mortality (78%) and morbidity in the majority of mice receiving saline vehicle alone. They uniformly exhibited piloerection and conjunctival secretions, were disheveled in appearance, remained sedentary, and abstained from food and drink. No mouse from this treatment group survived beyond 3 days. In contrast, mice receiving local anesthetic infusion were more robust, mobile, and resumed drinking and eating by 4 days, and a significant number survived past 7 days.

It is well known that mortality in sepsis is strongly influenced by the development of organ injury and dysfunction.[1] In concordance, our study showed chronic local anesthetic infusion not only decreased CLP-induced mortality, it significantly reduced the magnitude of CLP-induced renal and hepatic injury. Local anesthetics were

able to decrease both creatinine and ALT/AST, suggesting that the protection conferred by local anesthetics was global and not restricted to a particular organ. Creatinine concentrations were not statistically different between sham and local anesthetic groups. Taken together, chronic local anesthetic infusion provided global protection from organ injury and dysfunction resulting from severe sepsis.

The fact that global antiinflammatory regimens have shown some degree of success in human sepsis lends credence to interventions predicated upon antiinflammatory modulation.[5] These beneficial human sepsis studies utilizing insulin, low dose steroids, and activated protein C all share antiinflammatory characteristics.[23–25] Similarly, there exists ample evidence of the potent antiinflammatory effects of local anesthetics. In a rabbit model of lung injury, Takao *et al.* demonstrated that lidocaine infusion decreased various chemotactic factors and resulted in less neutrophil infiltration.[26] In a separate study lidocaine and bupivacaine inhibited the release of inflammatory mediators leukotriene B4 and interleukin-1 from human neutrophils and monocytes.[27] Several studies have also demonstrated that local anesthetics, in addition to possessing antithrombotic effects, can inhibit inflammatory cell migration and proinflammatory cytokine generation.[28–30] On the basis of these studies, we hypothesized that modulation of initial inflammatory responses after septic insult with local anesthetics would lead to improved mortality rates as well as hepatic and renal protection, as inflammation is a major component of cell death associated with renal and hepatic injury in sepsis. In accordance with other published findings, significant inflammatory processes are elicited during CLP-induced sepsis in our saline vehicle group (shown by considerable elevations in proinflammatory plasma cytokines, renal mRNA expression, myeloperoxidase activity, and ICAM-1 protein expression). Although no mechanistic data are provided in this study, we show that local anesthetic treatment reduced inflammatory responses after CLP in mice. We speculate that the protective effects of local anesthetics occur by limiting inflammatory cell (macrophage, neutrophil, lymphocyte) activation or by attenuating inflammation on renal and hepatic parenchymal cells.

We first examined plasma concentrations of proinflammatory cytokines (TNF-α and KC) to assess the general state of inflammation in our mice. TNF-α is a prototypic example of a proinflammatory cytokine that plays an important role in propagating a host of secondary inflammatory cascades. As such, it has been shown in a variety of studies to mediate many of the immunopathologic features seen in sepsis. It also has been implicated to directly cause acute renal failure in sepsis by acting on the TNF-1 receptor[31] and TNF-α has also been shown to cause multiorgan failure and lethality in sepsis.[32] We demonstrated that local anesthetic infusion resulted in a dramatic decrease in circulating concentrations of TNF-α 24 h after CLP. Local anesthetics mitigated TNF-α plasma concentrations to undetectable concentrations (comparable to sham controls), and this may in part account for the decreased renal injury we observed in local anesthetic treated mice. Murine KC is a proinflammatory alpha chemokine that putatively represents the functional homologue of human interleukin-8. As such, KC not only serves as a potent leukocyte attractant and activator but its overexpression in experimental peritonitis has also been associated with liver enzyme abnormalities and increased early mortality.[33] We also demonstrated a reduction in plasma KC concentrations with local anesthetic infusion, which may have served to prevent overamplification of proinflammatory responses mediated by KC. These results illustrate that local anesthetic infusion attenuates the overexuberant production of proinflammatory cytokines induced during severe sepsis.

To further examine our hypothesis that local anesthetics decrease inflammation, we then focused on gene expression in the kidney to examine if this phenomenon also occurred at the organ level. Previous studies have demonstrated that up-regulation of chemokines within the kidney accompanies the acute renal failure resulting from polymicrobial sepsis.[34] We found reduced gene transcription of KC, MIP-2, ICAM-1, and MCP-1 in mice receiving local anesthetic infusion. In addition to the systemic plasma reductions in KC described above, we also observed renal KC gene expression was attenuated with local anesthetic infusions. Similar to KC, MIP-2 plays a pivotal role in leukocyte recruitment. Previous studies have shown a positive correlation with blunting MIP-2 overexpression and increased survival in sepsis models.[35] Similarly, we observed substantial elevations in KC and MIP-2 mRNA concentrations with CLP, and a significant attenuation of this overexpression in KC and MIP-2 mRNA concentrations was seen in those mice treated with chronic infusion of lidocaine and bupivacaine. ICAM-1 is a proinflammatory adhesion molecule that participates in leukocyte recruitment and is upregulated during acute inflammatory responses. MCP-1 is a chemokine also involved in the pathogenesis of sepsis that has been shown by other authors to indirectly contribute to renal injury.[36] It plays an important role in the elicitation of both macrophages and neutrophils during sepsis. We also show that chronic local anesthetic infusion results in a decrease in MCP-1 gene expression in lidocaine and bupivacaine treated mice. We did not observe significant differences in gene expression of TNF-α, regulated on activation normal T-cell expressed and secreted, or interferon-induced protein 10. We did see a decrease in systemic plasma concentrations of TNF-α with local anesthetic infusion, suggesting that inhibition of TNF-α expression may occur at a posttranscriptional level.

Renal apoptosis is often an important component in the development of acute renal failure. In renal ischemia and reperfusion injury models, it has been demonstrated that increases in apoptosis were directly associated with increased renal inflammation.[37] Daemen *et al.* also demonstrated that blocking apoptosis prevented renal inflammation.[38] Evidence is emerging that renal apoptosis also plays a significant role in the development of acute renal failure in sepsis.[39,40] In the current study, we observed increased levels of apoptosis in renal tubule cells 24 h after CLP and a concomitant increase in renal dysfunction. In contrast, mice treated with local anesthetic infusion demonstrated decreased renal apoptosis and significantly improved renal function. Whether the decrease in renal apoptosis was mediated by local anesthetic suppressing proinflammatory mediators or by a direct effect on apoptotic pathways remains to be elucidated. We conclude from our data that in this sepsis model local anesthetic infusion is associated with decreased apoptosis and improved renal function after CLP.

A great deal of valuable work and attention has recently been dedicated to the immunosuppressive and hypoinflammatory phase that follows the initial hyperinflammatory phase of sepsis.[20] There is a growing body of evidence to suggest that uncontrolled, hyperexuberant cytokine regulation, as occurs in sepsis, can lead to apoptosis in a variety of cell types and subsequently manifest as immunosuppression. The fact that immunosuppression may arise secondary to the uncontrolled hyperinflammatory phase leads to the question of whether attenuation of the hyperinflammatory phase would aid in preventing subsequent immunosuppression. We agree that once a generalized state of immunosuppression is achieved, it logically follows that further immunosuppressive or antiinflammatory modulation would be deleterious. However, as already mentioned, there are three clinical studies that have demonstrated a benefit from antiinflammatory modulation in sepsis. We believe the benefits we have demonstrated in this study likely follow similar antiinflammatory mechanisms.

In conclusion, we demonstrate that chronic infusion of local anesthetics provides protection from CLP-induced mortality and acute organ dysfunction. As the pathogenesis of organ dysfunction in sepsis is largely mediated by an imbalanced, uncontrolled inflammatory response, we believe that local anesthetics improve organ function after septic insult by attenuating this hyperinflammatory process. This attenuation is global in nature, exerting its effect on a variety of proinflammatory molecules, and culminates in a reduction in organ injury and thereby protects from progression to multiple organ dysfunction. Given the protective benefit on survival and organ dysfunction observed with chronic local anesthetic infusion, our findings could have important therapeutic implications for patients in early sepsis. Although it is premature to advocate intravenous anesthetics in septic patients, this intervention may someday prove beneficial for patients in early sepsis. Intense work is now being performed to elucidate the cell types involved in mediating the protective effects of local anesthetics and the cellular signaling mechanisms involved in protection.

References

1. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. Crit Care Med 2001; 29:1303–10
2. Freise H, Bruckner UB, Spiegel HU: Animal models of sepsis. J Invest Surg 2001; 14:195–212
3. Ebong S, Call D, Nemzek J, Bolgos G, Newcomb D, Remick D: Immunopathologic alterations in murine models of sepsis of increasing severity. Infect Immun 1999; 67:6603–10
4. Miyaji T, Hu X, Yuen PS, Muramatsu Y, Iyer S, Hewitt SM, Star RA: Ethyl pyruvate decreases sepsis-induced acute renal failure and multiple organ damage in aged mice. Kidney Int 2003; 64:1620–31
5. Cohen J: The immunopathogenesis of sepsis. Nature 2002; 420:885–91
6. Strohm C, Barancik T, Bruhl ML, Kilian SA, Schaper W: Inhibition of the ER-kinase cascade by PD98059 and UO126 counteracts ischemic preconditioning in pig myocardium. J Cardiovasc Pharmacol 2000; 36:218–29
7. Lee R, Nitta T, Schmid RA, Schuessler RB, Harris KM, Gay WA Jr: Retrograde infusion of lidocaine or L-arginine before reperfusion reduces myocardial infarct size. Ann Thorac Surg 1998; 65:1353–9
8. Das KC, Misra HP: Prevention of reperfusion lung injury by lidocaine in isolated rat lung ventilated with higher oxygen levels. J Postgrad Med 2003; 49:17–20
9. Schmid RA, Yamashita M, Ando K, Tanaka Y, Cooper JD, Patterson GA: Lidocaine reduces reperfusion injury and neutrophil migration in canine lung allografts. Ann Thorac Surg 1996; 61:949–55
10. Tomori H, Shiraishi M, Koga H, Toure M, Taira K, Higa T, Okuhama Y, Hiroyasu S, Muto Y: Protective effects of lidocaine in hepatic ischemia/reperfusion injury in vitro. Transplant Proc 1998; 30:3740–2
11. Hollmann MW, Durieux ME: Local anesthetics and the inflammatory response: A new therapeutic indication? ANESTHESIOLOGY 2000; 93:858–75
12. Lee HT, Krichevsky IE, Xu H, Ota-Setlik A, D'Agati VD, Emala CW: Local anesthetics worsen renal function after ischemia-reperfusion injury in rats. Am J Physiol Renal Physiol 2004; 286:F111–9
13. Walley KR, Lukacs NW, Standiford TJ, Strieter RM, Kunkel SL: Balance of inflammatory cytokines related to severity and mortality of murine sepsis. Infect Immun 1996; 64:4733–8
14. Heinegard D, Tiderstrom G: Determination of serum creatinine by a direct colorimetric method. Clin Chim Acta 1973; 43:305–10
15. Jablonski P, Howden BO, Rae DA, Birrel CS, Marshall VC, Tange J: An experimental model for assessment of renal recovery from warm ischemia. Transplantation 1983; 35:198–204
16. Lee HT, Gallos G, Nasr SH, Emala CW: A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. J Am Soc Nephrol 2004; 15:102–11
17. Ysebaert DK, De Greef KE, Vercauteren SR, Ghielli M, Verpooten GA, Eyskens EJ, De Broe ME: Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. Nephrol Dial Transplant 2000; 15:1562–74
18. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN: TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 2000; 103:1071–83
19. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ: Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 1992; 101:1644–55
20. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. N Engl J Med 2003; 348:138–50
21. Remick D, Manohar P, Bolgos G, Rodriguez J, Moldawer L, Wollenberg G: Blockade of tumor necrosis factor reduces lipopolysaccharide lethality, but not the lethality of cecal ligation and puncture. Shock 1995; 4:89–95
22. Gold JA, Parsey M, Hoshino Y, Hoshino S, Nolan A, Yee H, Tse DB, Weiden MD: CD40 contributes to lethality in acute sepsis: In vivo role for CD40 in innate immunity. Infect Immun 2003; 71:3521–8
23. Van den BG, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P, Bouillon R: Intensive insulin therapy in the critically ill patients. N Engl J Med 2001; 345:1359–67
24. Annane D, Sebille V, Charpentier C, Bollaert PE, Francois B, Korach JM, Capellier G, Cohen Y, Azoulay E, Troche G, Chaumet-Riffaut P, Bellissant E: Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. JAMA 2002; 288:862–71
25. Vincent JL, Angus DC, Artigas A, Kalil A, Basson BR, Jamal HH, Johnson G

III, Bernard GR: Effects of drotrecogin alfa (activated) on organ dysfunction in the PROWESS trial. Crit Care Med 2003; 31:834–40

26. Takao Y, Mikawa K, Nishina K, Maekawa N, Obara H: Lidocaine attenuates hyperoxic lung injury in rabbits. Acta Anaesthesiol Scand 1996; 40:318–25

27. Sinclair R, Eriksson AS, Gretzer C, Cassuto J, Thomsen P: Inhibitory effects of amide local anaesthetics on stimulus-induced human leukocyte metabolic activation, LTB4 release and IL-1 secretion in vitro. Acta Anaesthesiol Scand 1993; 37:159–65

28. Li CY, Tsai CS, Hsu PC, Chueh SH, Wong CS, Ho ST: Lidocaine attenuates monocyte chemoattractant protein-1 production and chemotaxis in human monocytes: Possible mechanisms for its effect on inflammation. Anesth Analg 2003; 97:1312–6

29. Hammer R, Dahlgren C, Stendahl O: Inhibition of human leukocyte metabolism and random mobility by local anaesthesia. Acta Anaesthesiol Scand 1985; 29:520–3

30. Tuman KJ, McCarthy RJ, March RJ, DeLaria GA, Patel RV, Ivankovich AD: Effects of epidural anesthesia and analgesia on coagulation and outcome after major vascular surgery. Anesth Analg 1991; 73:696–704

31. Cunningham PN, Dyanov HM, Park P, Wang J, Newell KA, Quigg RJ: Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney. J Immunol 2002; 168:5817–23

32. Leon LR, White AA, Kluger MJ: Role of IL-6 and TNF in thermoregulation and survival during sepsis in mice. Am J Physiol 1998; 275:R269–R277

33. Mercer-Jones MA, Shrotri MS, Peyton JC, Remick DG, Cheadle WG: Neutrophil sequestration in liver and lung is differentially regulated by C-X-C chemokines during experimental peritonitis. Inflammation 1999; 23:305–19

34. Maier S, Emmanuilidis K, Entleutner M, Zantl N, Werner M, Pfeffer K, Heidecke CD: Massive chemokine transcription in acute renal failure due to polymicrobial sepsis. Shock 2000; 14:187–92

35. Remick DG, Call DR, Ebong SJ, Newcomb DE, Nybom P, Nemzek JA, Bolgos GE: Combination immunotherapy with soluble tumor necrosis factor receptors plus interleukin 1 receptor antagonist decreases sepsis mortality. Crit Care Med 2001; 29:473–81

36. Viedt C, Dechend R, Fei J, Hansch GM, Kreuzer J, Orth SR: MCP-1 induces inflammatory activation of human tubular epithelial cells: Involvement of the transcription factors, nuclear factor-kappaB and activating protein-1. J Am Soc Nephrol 2002; 13:1534–47

37. Daemen MA, de Vries B, Buurman WA: Apoptosis and inflammation in renal reperfusion injury. Transplantation 2002; 73:1693–700

38. Daemen MA, van 't Veer C, Denecker G, Heemskerk VH, Wolfs TG, Clauss M, Vandenabeele P, Buurman WA: Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. J Clin Invest 1999; 104:541–9

39. Jo SK, Cha DR, Cho WY, Kim HK, Chang KH, Yun SY, Won NH: Inflammatory cytokines and lipopolysaccharide induce Fas-mediated apoptosis in renal tubular cells. Nephron 2002; 91:406–15

40. Wan L, Bellomo R, Di Giantomasso D, Ronco C: The pathogenesis of septic acute renal failure. Curr Opin Crit Care 2003; 9:496–502