**Supplementary Digital Content**

**Reversal of the Pathophysiological Responses to Gram-negative Sepsis by Mega-dose Vitamin C**

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**METHODS**

*Surgical Preparation*

Two aseptic surgical procedures, separated by 3-4 weeks, were performed in 12 Merino ewes (35-45 kg) under general anesthesia. The sheep were healthy and had not been used previously for experimentation. Sheep had a carotid artery loop constructed and a transit-time flow probe (20 mm, Transonic Systems, Ithaca, NY) implanted on the pulmonary artery.1 The day before the second surgery, a carotid artery and jugular vein were cannulated. In a second surgery, a transit-time probe (4 mm) was placed on the left renal artery, the renal vein was cannulated and fiber-optic probes (Oxford Optronix, Abingdon, United Kingdom) were inserted into the renal cortex and medulla.2,3 A Foley catheter (size 12; Euromedical, Malaysia) was inserted into the bladder.

Arterial pressure, heart rate, cardiac output, renal blood flow (RBF), cortical and medullary tissue perfusion and oxygen tension (PO2) were continuously recorded at 100 Hz on a computer using a CED Micro 1401 interface with Spike 2 software (Cambridge Electronic Design, Cambridge, United Kingdom). Arterial and renal venous blood samples and urine samples were collected at baseline, at pre-defined time intervals during sepsis and during the recovery period following antibiotic therapy for measurement of blood gases and electrolytes (ABL Systems-625, Denmark) and biochemistry. Arterial blood and urine samples were simultaneously collected for assessment of plasma and urinary creatinine, sodium and potassium concentrations, osmolality and plasma liver enzymes. At 48-hours following recovery from sepsis, animals were euthanized with pentobarbital (100 mg/kg, i.v.) and the positions of the fiber-optic probes within the renal cortex and medulla were confirmed.

*Histological Methods*

At necropsy, the left kidney (n = 10) was cut transversely and one-half immersion fixed in 10% neutral buffered formalin. Segments (n = 3) containing papilla, medulla and overlying cortex were selected from each kidney and processed for paraffin embedding using standard histological protocols. Sections (5 µm) were prepared and stained with haematoxylin and eosin (acute tubular necrosis and interstitial mononuclear infiltrates), Masson trichrome (interstitial fibrosis and fibrin) and periodic acid Schiff (hyaline casts, glomerular and tubular basement membranes and loss of brush border in proximal tubules). Myeloperoxidase (Dako A0398) immunohistochemistry was used to label and localise polymorphonuclear cells. Haematoxylin and eosin stained sections were also examined with polarised light microscopy to detect birefringence (calcium oxalate crystals). Histological examination was performed blinded to experimental interventions and changes were compared with normal morphology and graded semi-quantitatively as 0 = no change, + = focal change (5 – 30% of the section), ++ = focal change (30 – 50% of the section) and +++ = diffuse change > 50% of the section.

*Statistical Analysis*

For the sheep study, variables are reported as mean ± standard error of mean for parametric data after passing the tests for normality (D’Agostino and Pearson Omnibus test, confirmed by Shapiro-Wilk test). Comparisons between baseline (Time 0) to 23-h of sepsis were performed using a Student’s paired t test. Variables during 23-31-h of sepsis, when animals were treated with either sodium ascorbate or vehicle, were analyzed using two-way repeated measures analysis of variance (Graphpad Software 6.0, La Jolla, CA). p values for within-subjects’ factors were conservatively adjusted using the Greenhouse-Geisser method. A Dunnett’s test was used to compare differences between the 16, 24, 40 and 48-h time points after antibiotic therapy against the baseline period. p value ≤0.05 was considered statistically significant.

**RESULTS**

**Infusion of Sodium Ascorbate or Vehicle (n=5/group)**

*Renal Oxygen Balance*

During sepsis there was an increase in renal oxygen delivery, consumption was unchanged and renal oxygen extraction decreased (Fig. S6). Sodium ascorbate reduced renal oxygen delivery (Fig. S6A), which decreased in parallel with the reduction in RBF (Fig. 2A)

*Plasma and Urine Osmolality*

Infusion of sodium ascorbate caused an expected progressive increase in plasma osmolality (Fig. S7A). Plasma osmolar gap (measured osmolality minus the calculated osmolality of all measured osmolytes) increased from 3.3 mOsmol/L during baseline to 15.0 mOsmol/L by 6-h of infusion (Fig. S7E), consistent with the predicted high levels of ascorbate in plasma.

*Liver Enzymes*

The sepsis-induced increases in plasma bilirubin and plasma aspartate aminotransferase were significantly reduced by infusion of sodium ascorbate (Fig. S8A and C). Plasma alanine transaminase was not significantly changed during sepsis or by treatment with sodium ascorbate (Fig. S8B).

*Renal Histology*

There were no overt signs of acute tubular necrosis and interstitial fibrosis within the renal cortex, corticomedullary junction nor medulla in either treatment group (Table S2). The renal cortex exhibited a focal distribution of interstitial mononuclear infiltrate in both groups (Fig. S9). Granular, cellular and hyaline casts associated with tubular epithelial cell breakdown were not observed. Polarised light microscopy of the haematoxylin and eosin stained sections did not detect birefringent crystal deposits in glomeruli, tubules, blood vessels nor the interstitium in either treatment group (Table S2). Myeloperoxidase (Dako A0398) immunohistochemistry applied for polymorphonuclear cell detection demonstrated that this was also not a feature in either treatment group.

**Infusion of NaHCO3**

In two septic sheep, infusion of NaHCO3 did not improve the clinical state, in fact early in the infusion the animals deteriorated, they started shivering and body temperature transiently increased 1-2 °C (Fig. S10C). This resolved after about 30 min, but their clinical state did not improve, as was seen with infusion of sodium ascorbate. Importantly, by the end of the infusion of sodium ascorbate body temperature had returned to normal, but with NaHCO3 although there was a slight decrease in body temperature, it remained substantially above pre-morbid baseline levels (Fig. S10C).

Sepsis reduced MAP, and during the period of treatment MAP was maintained at baseline levels by infusion of norepinephrine (Fig. S10B). In contrast to the withdrawal of norepinephrine that was possible during infusion of sodium ascorbate, during NaHCO3 infusion the dose of norepinephrine was not reduced (Fig. S10A). Unlike sodium ascorbate, which reduced heart rate, heart rate remained elevated with NaHCO3 (Fig. S10D).

In contrast to the beneficial effects of sodium ascorbate on renal blood flow and renal medullary perfusion and pO2 (Fig. 2), NaHCO3 caused no improvement in these variables (Fig. S11). Infusion of NaHCO3 caused a large increase in urine flow (Fig. S12A), but unlike sodium ascorbate it caused no reduction in plasma creatine and no increase in creatinine clearance (Fig. S12B, C).

Infusion of NaHCO3 caused large increases in blood pH (Fig. S13A), whilst there was no significant change with sodium ascorbate (Fig. 4B). NaHCO3 did not reduce blood lactate or increase arterial pO2 (Fig. S13B and C), in contrast to the significant improvements seen with sodium ascorbate (Fig. 4A and C). Since treatment with NaHCO3 did not decrease renal blood flow, it did not reduce renal oxygen delivery from the elevated level in sepsis (Fig. S14A).

NaHCO3 did not change plasma osmolality (Fig. S15A) and did not increase the plasma osmolar gap (Fig. S15E).

In contrast to the beneficial effect of sodium ascorbate to reduce the increase in plasma bilirubin, during NaHCO3 there were further increases in plasma bilirubin (Fig. S16).

**DISCUSSION**

The remarkable effect of sodium ascorbate to improve the clinical state of septic sheep, and the significant improvements in cardiovascular, renal, hepatic and pulmonary function, as well as the reductions in arterial lactate and body temperature, were not reproduced by treatment with Na HCO3. This indicates that these multiple beneficial effects of sodium ascorbate in sepsis on many organ systems is due to an action of ascorbate, not the sodium load.

*Strengths and limitations*

The sheep used in these experiments were not inbred or kept in a pathogen free environment, so they have a wild-type microbiome. We induced sepsis using *E. coli* isolated from an ICU patient with *E. coli* bacteremia and septic shock, instead of a laboratory bacterial strain or lipopolysaccharide. This is effectively a two-hit model, where an initial trauma, surgery 4 days before, is followed by induction of an infection, which resembles a frequent clinical scenario. We know time zero, i.e., the initiation of the septic insult, which clinically is usually unknown. Animals were treated at 23-h of sepsis when clinical triggers for intervention were present, at a clinically appropriate time at which patients are often diagnosed and treated. The ovine clinical phenotype resembles the human phenotype. Mortality was 2 out of 14 animals, similar to that seen in human sepsis. Studies were performed in conscious sheep to remove the confounding effects of general anesthesia (39), although unlike many septic patients, the animals were not sedated, mechanically ventilated or given prophylactic antibiotics until the end of the intervention period. Our studies were performed in young sheep without the comorbidities seen in some septic patients, but the clinical phenotype of septic AKI was comparable to that in humans.

**REFERENCES**

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**Table S1.** Systemic and renal variables, core temperature and blood lactate at baseline (pre-morbid) and after 23-h of gram-negative sepsis in conscious sheep

|  |  |  |
| --- | --- | --- |
| **Systemic and renal variables** | **Prior to intervention** | |
|  | **Baseline** | **23 h sepsis** |
| Mean arterial pressure (mmHg) | 85 ± 1 | 67 ± 2\*\*\* |
| Heart rate (beats/min) | 71 ± 2 | 141 ± 2 \*\*\* |
| Cardiac output (L/min) | 4.1 ± 0.2 | 6.1 ± 0.3 \*\*\* |
| Total peripheral conductance (mL/min/mmHg) | 49 ± 3 | 86 ± 5\*\*\* |
| Core temperature (ºC) | 39.2 ± 0.1 | 41.4 ± 0.2\*\*\* |
| Urine output (ml/kg/h) | 1.1 ± 0.3 | 0.4 ± 0.1\*\* |
| Plasma creatinine (µmol/L) | 71 ± 2 | 144 ± 15\*\*\* |
| Creatinine clearance (mL/min) | 137 ± 20 | 45 ± 10\*\* |
| Fractional sodium excretion (%) | 0.6 ± 0.1 | 0.4 ± 0.1 |
| Renal blood flow (mL/min) | 236 ± 10 | 384 ± 30\*\*\* |
| Renal vascular conductance (mL/min/mmHg) | 2.8 ± 0.1 | 5.7 ± 0.5\*\*\* |
| Renal oxygen delivery (mLO2/min) | 29 ± 2 | 46 ± 3\*\*\* |
| Renal oxygen consumption (mLO2/min) | 4.5 ± 0.5 | 4.4 ± 0.5 |
| Renal oxygen extraction (%) | 16 ± 2 | 9 ± 1\*\*\* |
| Renal medullary tissue perfusion (units) | 1077 ± 119 | 443 ± 64\*\*\* |
| Renal cortical tissue perfusion (units) | 1388 ± 160 | 2125 ± 177\*\*\* |
| Medullary tissue oxygen tension (mmHg) | 43 ± 4 | 20 ± 2\*\*\* |
| Cortical tissue oxygen tension(mmHg) | 40 ± 3 | 46 ± 3\* |
| Arterial blood lactate (mmol/L) | 0.6 ± 0.1 | 2.0 ± 0.2 |
| Arterial PO2 (mmHg) | 105 ± 2 | 80 ± 3\*\* |

Values are between-animal mean ± SEM. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 indicate significant differences between variables at baseline (pre-morbid) compared with variables at 23 h of gram-negative sepsis in all 10 sheep prior to treatment with sodium ascorbate or vehicle-crystalloid treatment. P values were derived from Student’s two-tailed paired t test.

**Table S2.** Variables for semiquantitative assessment of renal histopathology

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Acute tubular necrosis** | **Interstitial mononuclear infiltrates** | **Interstitial fibrosis** | **Tubular casts** | **Birefringence** |
| **Na ascorbate** | | | | | |
| Cortex | 0 | **+** | 0 | 0 | 0 |
| Corticomedullary junction | 0 | 0 | 0 | 0 | 0 |
| Medulla | 0 | 0 | 0 | 0 | 0 |
| **Vehicle-Crystalloid** | | | | | |
| Cortex | 0 | **+** | 0 | 0 | 0 |
| Corticomedullary junction | 0 | 0 | 0 | 0 | 0 |
| Medulla | 0 | 0 | 0 | 0 | 0 |

Sections of the renal cortex, corticomedullary junction and medulla were collected at autopsy following 48 h of recovery in sheep that received either Na ascorbate (N=5) or vehicle-crystalloids (N=4) during gram-negative sepsis. Formalin-fixed paraffin-embedded renal tissue were subjected to histochemical and immunohistochemical analysis for acute tubular necrosis (focal or diffuse denudation and/or flattening of tubular epithelial cells with tubular denudation, interstitial edema, loss of proximal tubular brush borders and tubular casts), interstitial mononuclear infiltrates (peritubular), fibrosis (interstitial), casts (hyaline, cellular or granular) and birefringence (oxalate) crystals. The grading system used include: Grade 1 (0) = no change compared with morphology on kidneys collected from healthy sheep; Grade 2 (+) = Focal change in 5-30% of total section; Grade 3 (+++) = Focal change in 30-50% of total section; Grade 4 (+++) = Diffuse change in > 50% of total section.



**Figure S6.** Renal oxygen handling in response to sodium ascorbate (*closed squares, n=5*) or vehicle (*open circles, n=5*) treatment during ovine sepsis and during recovery from gram-negative infection

Renal oxygen delivery (A), renal oxygen consumption (B) and renal oxygen extraction ratio (C) are presented during infusion of *Escherichia coli* from 0 to 31 hour of sepsis and then recovery over 48 hours following antibiotic therapy. All animals were resuscitated with a fluid bolus therapy (BT, 30 mL/kg balanced crystalloid over 30 minutes) from 23.5 to 24 hours of sepsis. Animals were randomised to receive sodium ascorbate (0.5g/kg) or vehicle-crystalloid from 24 to 24.5 hours of sepsis followed by an infusion of sodium ascorbate (0.5 g/kg/h) or vehicle-crystalloid from 24.5 to 31 hours of sepsis. Norepinephrine doses were titrated to return mean arterial pressure to 75-80 mmHg from 25 to 31 hours of sepsis. All animals received intravenous antibiotics at 31 hours of sepsis (1-gram ceftriaxone), with a repeated dose at 24 hours and their recovery from sepsis were monitored over 48 hours. Time 0 is the mean of the 24th hour of baseline and times 23-31 hours of sepsis and 48 hours of recovery are means of 1-hour periods. Data are presented as within-animal mean ± SEM. P values represent treatment-time interaction differences between sodium ascorbate and vehicle treatment from a two-way repeated measures analysis of variance from 23 to 31 hours of gram-negative sepsis



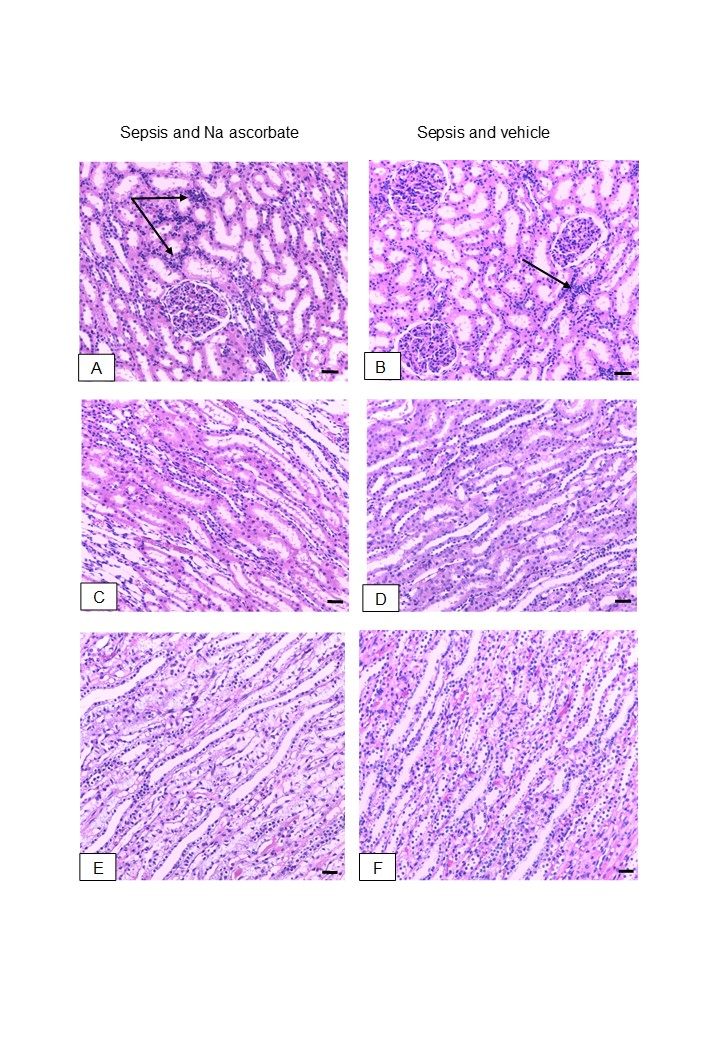
**Figure S7.** Plasma and urinary osmolality responses to sodium ascorbate (*closed squares, n=5*) or vehicle (*open circles, n=5*) treatment during ovine sepsis and during recovery from gram-negative infection

Measured plasma osmolality (A), measured urine osmolality (B), calculated plasma osmolality (C), calculated urine osmolality (D), plasma osmolar gap (E) and urine osmolar gap (F) are presented during infusion of *Escherichia coli* from 0 to 31 hour of sepsis and then recovery over 48 hours following antibiotic therapy. Fluid and drug infusions and statistical analyses are as detailed in Figure S6.



**Figure S8.** Plasma liver enzyme and electrolyte responses to sodium ascorbate (*closed squares, n=5*) or vehicle (*open circles, n=5*) treatment during ovine sepsis and during recovery from gram-negative infection

Plasma bilirubin (A), plasma magnesium (B), plasma alanine transaminase (C), plasma phosphate (D), plasma aspartate aminotransferase (E) and plasma chloride (F) are presented during infusion of *Escherichia coli* from 0 to 31 hour of sepsis and then recovery over 48 hours following antibiotic therapy. Fluid and drug infusions and statistical analyses are as detailed in Figure S6



**Figure S9.** Hematoxylin and eosin staining in renal cortical, corticomedullary and medullary tissue sections from sheep that received Na ascorbate or vehicle therapy during gram-negative sepsis. Representative images of sections (5 µm) of renal cortex (A & B), corticomedullary junction (C & D) and medulla (E & F) stained with haematoxylin and eosin. Kidneys were collected at post-mortem from sheep subjected to 31-h of sepsis, treatment with Na ascorbate (n=5) or vehicle (n=5) treatment, and subsequently 48-h recovery after antibiotic therapy. Black arrows depict focal presence of interstitial mononuclear infiltrates. Scale bar= 100 µm.



**Figure S10.** Changes in norepinephrine dose (A), mean arterial pressure (B), body temperature (C) and heart rate (D) in response to treatment with NaHCO3 during ovine sepsis. Sepsis was induced by infusion of *Escherichia coli* from 0 to 31-h of sepsis followed by recovery over 48-h following antibiotic therapy. Both sheep were initially resuscitated with fluid bolus therapy (Fluid BT, 30 mL/kg balanced crystalloid over 30 minutes) from 23.5 to 24-h of sepsis. This was followed by NaHCO­3 (0.21 g/kg) from 24 to 24.5-h of sepsis followed by an infusion of NaHCO3 (0.21 g/kg/h) from 24.5 to 31-h of sepsis. Norepinephrine doses were titrated to maintain mean arterial pressure at baseline levels (75-80 mmHg) from 25 to 31-h of sepsis. All animals received intravenous antibiotics at 31-h of sepsis (1-gram ceftriaxone), with a repeated dose at 24 hours and their recovery from infection was monitored over 48-h. Time 0 is the mean of the 24th hour of baseline and times 23-31-h of sepsis and 48-h of recovery are means of 0.5-h periods.



**Figure S11.** Changes in renal blood flow (A), renal vascular conductance (B), medullary perfusion (C), cortical perfusion (D), medullary oxygen tension (pO2) (E) and cortical pO2 (F) in response to treatment with NaHCO3 during ovine sepsis. Fluid and drug infusions are as detailed in Figure S10.



**Figure S12**. Changes in urine output (A), plasma creatinine (B), creatinine clearance (C), fractional sodium excretion (D), plasma osmolar gap (E) and fractional potassium excretion (F) in response to treatment with NaHCO3 during ovine sepsis. Fluid and drug infusions are as detailed in Figure S10.



**Figure S13.** Changes in arterial blood pH (A), arterial blood lactate (B), arterial blood partial pressure of oxygen (pO2) (C), arterial blood sodium (D), arterial partial pressure of carbon dioxide (pCO2) (E) and arterial blood potassium (F) in response to treatment with NaHCO3 during ovine sepsis. Fluid and drug infusions are as detailed in Figure S10.



**Figure S14.** Changes in renal oxygen delivery (A), renal oxygen consumption (B) and renal oxygen extraction ratio (C) in response to treatment with NaHCO3 during ovine sepsis. Fluid and drug infusions are as detailed in Figure S10.



**Figure S15.** Changes in plasma osmolality (A), measured urine osmolality (B), calculated plasma osmolality (C), calculated urine osmolality (D), plasma osmolar gap (E) and urine osmolar gap (F) in response to treatment with NaHCO3 during ovine sepsis. Fluid and drug infusions are as detailed in Figure S10.



**Figure S16.** Changes in plasma bilirubin (A), plasma alanine transaminase (B) and plasma aspartate aminotransferase (C) in response to treatment with NaHCO3 during ovine sepsis. Fluid and drug infusions are as detailed in Figure S10.