# Failure of nitroglycerin (glyceryl trinitrate) to improve villi hypoperfusion in endotoxaemic shock in sheep

Vanina S Kanoore Edul, Gonzalo Ferrara, Mario O Pozo, Gastón Murias, Enrique Martins, Carlos Canullán, Héctor S Canales, Elisa Estenssoro, Can Ince and Arnaldo Dubin

**ABSTRACT**

**Objective:** To evaluate the effects of nitroglycerin (glyceryl trinitrate) on intestinal microcirculation during endotoxaemic shock.

**Design:** Controlled experimental study.

**Setting:** Research laboratory.

**Subjects:** 20 anaesthetised, mechanically ventilated sheep.

**Interventions:** Septic shock was induced by endotoxin infusion. After 60 minutes without resuscitation, sheep received fluid resuscitation and were randomised to control or nitroglycerin groups. Nitroglycerin was infused at a rate of 0.2 μg/kg/min for 90 minutes.

**Main outcome measure:** Improved villi microcirculation.

**Results:** Endotoxin lowered arterial blood pressure, cardiac output and intestinal blood flow, which were improved by fluid resuscitation. Mean (SD) ileal intramucosal–arterial $P_{CO_2}$ gradient increased during shock and remained elevated after resuscitation in control and nitroglycerin groups (8 [8], 15 [9] and 17 [9], and 6 [6], 13 [11] and 14 [9] mmHg, respectively; $P < 0.05$, baseline v shock and resuscitation for both groups). Villi microvascular flow index was reduced during shock and remained lower than baseline after the resuscitation in both groups (3.0 [0.0], 2.5 [0.2] and 2.7 [0.2], and 3.0 [0.0], 2.3 [0.3] and 2.6 [0.3], respectively; $P < 0.05$, baseline v shock and resuscitation for both groups). The red blood cell velocity behaved similarly (859 [443], 553 [236] and 670 [276], and 886 [440], 447 [124] and 606 [235] μm/s, respectively; $P < 0.05$, baseline v shock and resuscitation for both groups).

**Conclusions:** In endotoxaemic sheep, low doses of nitroglycerin failed to improve the subtle but persistent villi hypoperfusion that remains present after fluid resuscitation.

Crit Care Resusc 2011; 13: 252–261

Septic patients frequently show evidence of tissue hypoperfusion, such as lactic acidosis and tissue hypercapnia, despite normal or increased blood flow and oxygen transport. These findings might be explained by the presence of microvascular alterations.[1] Moreover, microcirculatory alterations have been described in septic patients. De Backer and colleagues showed that septic patients exhibited abnormalities in sublingual microcirculation that were more severe in the non-survivors,[2] and that the persistence of these alterations was related to multiorgan failure and subsequent death.[3]

In a sheep model of endotoxaemic shock, we have previously shown that the gut intramucosal–arterial $P_{CO_2}$ gradient ($\Delta P_{CO_2}$) increases during reductions in intestinal blood flow.[4] The normalisation of systemic and intestinal haemodynamics by fluid resuscitation, however, failed to improve $\Delta P_{CO_2}$. The underlying explanation was a blood-flow redistribution within the intestinal wall that rendered the villi hypoperfused.[4] These alterations in the perfusion of the gut mucosa are relevant to patient outcome because mucosal ischaemia can produce an intestinal barrier dysfunction and a subsequent bacterial translocation, leading to multiorgan failure.[5]

Therefore, improvement of microcirculation could be a valuable therapeutic goal. One approach to recruit microcirculation is the use of vasodilators in general, and the use of nitrovasodilators in particular.[6] Sepsis is characterised not only by the overproduction of nitric oxide (NO), but also by regional deficits in its production.[7] Consequently, the use of nitrovasodilators is an appealing therapeutic alternative. Nevertheless, the clinical and experimental evidence are contradictory, and the effects of nitroglycerin (glyceryl trinitrate) on intestinal microcirculation in septic shock have not been studied.

Our goal in these experiments was, therefore, to evaluate the effects of nitroglycerin on sublingual and gut microcirculation during the resuscitation of sheep from endotoxaemic shock. We hypothesised that nitroglycerin would correct the villi hypoperfusion that persists after fluid resuscitation of endotoxaemic shock.

## Methods

This study was approved by the local animal research committee (approval number 0800-009634/11-000). Care of animals was in accordance with National Institutes of Health (United States) guidelines.

### Surgical preparation

Twenty sheep (mean weight, 25 kg [SD, 8 kg]) were anaesthetised with 30 mg/kg of sodium pentobarbital, intubated and mechanically ventilated with a Dual Phase Control

Respirator Pump Ventilator (Harvard Apparatus, South Natick, Mass, USA) with a tidal volume of 15 mL/kg, an $Fio_2$ of 0.21 and a positive end-expiratory pressure of 8 cm $H_2O$. The initial respiratory rate was set to keep the arterial $Pco_2$ between 35 and 40 mmHg. This respiratory setting was maintained during the rest of the experiments. Neuromuscular blockade was performed with intravenous pancuronium bromide (0.06 mg/kg). Additional pentobarbital boluses (1 mg/kg) were administered hourly and when clinical signs of inadequate depth of anaesthesia were seen. Analgesia was provided by fentanyl as a bolus of 2 μg/kg followed by 1 μg/kg/h. These medicines were intravenously administered.

Catheters were inserted through the left femoral vein to administer fluids and drugs, and through the left femoral artery to measure blood pressure and to obtain blood samples. A 7.5 French Swan-Ganz Standard Thermodilution Pulmonary Artery Catheter was inserted through the right external jugular vein (Edwards Life Sciences, Irvine, Calif, USA).

A midline laparotomy was performed followed by a gastrostomy to allow drainage of gastric contents. A splenectomy was then carried out to avoid spleen contraction during shock. An electromagnetic flow probe was placed around the superior mesenteric artery to measure intestinal blood flow. A catheter was situated in the superior mesenteric vein through a small vein proximal to the gut to measure pressure and draw blood samples. A tonometer was inserted through a small ileotomy to measure intramucosal $Pco_2$. A catheter was left in the abdomen to measure intra-abdominal pressure. A 10–15 cm segment of the ileum was mobilised, placed outside the abdomen, and opened 2 cm on its antimesenteric border to allow an examination of the mucosa. The exteriorised intestinal segment was covered, and moisture and temperature preserved by a device. Finally, after complete haemostasis, the abdominal wall incision was closed, excepting a short segment for externalisation of the ileal loop.

### Measurements and derived calculations

Arterial, systemic, pulmonary and central venous pressures were measured with corresponding transducers (Statham P23 AA, Statham, Hato Rey, Puerto Rico). Cardiac output was measured by thermodilution with 5 mL of 0°C saline solution (HP OmniCare Model 24 A 10, Hewlett Packard, Andover, Mass, USA). The mean value from three measurements taken randomly during the respiratory cycle was subsequently expressed per kilogram of body weight. Intestinal blood flow was measured by the electromagnetic method (Spectramed Blood Flowmeter model SP 2202 B, Spectramed Inc, Oxnard, Calif, USA) with in-vitro calibrated transducers with diameters of 5 and 6 mm (Blood Flowmeter Transducer, Spectramed Inc). Occlusive zero was controlled before and after each experiment. Non-occlusive zero was corrected before each measurement. Superior mesenteric blood flow was normalised to gut weight.

Arterial, mixed venous and mesenteric venous $Po_2$, $Pco_2$ and pH were measured with an ABL5 blood gas analyser (Radiometer, Copenhagen, Denmark), and haemoglobin and oxygen saturation were measured with an OSM3 co-oximeter calibrated for sheep blood (Radiometer). Systemic and intestinal oxygen transports and consumptions ($Do_2$ and $Vo_2$) were calculated by standard equations. Superior mesenteric artery blood flow and intestinal $Vo_2$ and $Do_2$ are expressed as indices of intestinal weight.

Intramucosal $Pco_2$ was measured with a tonometer (Tonometrics Catheter, Datex-Ohmeda, Helsinki, Finland) through the use of an automated air tonometry system (Tonocap, Datex-Ohmeda). Its value was used to calculate $\Delta Pco_2$.

Arterial lactate was measured with an amperometric electrode containing lactate oxidase (Rapidlab 865, Chiron Diagnostics Corp, East Walpole, Mass, USA).

### Microcirculatory measurements and analysis

We evaluated the microcirculatory network in the sublingual mucosa and the intestinal mucosa and serosa using a MicroScan sidestream dark field (SDF) imaging device (MicroVision Medical, Amsterdam, Netherlands).[8]

Different precautions and specific steps were taken both to obtain images of adequate quality and to ensure good reproducibility. Experienced researchers performed the video acquisitions and image analyses. After gentle removal of saliva or faeces with isotonic-saline-drenched gauze, steady images of at least 20 seconds were obtained, so as to avoid pressure artefacts, by means of a portable computer and an ADVC110 analogue-to-digital video converser (Canopus Co, San Jose, Calif, USA). Clips were stored as AVI files on the hard disk to allow computerised frame-by-frame image analysis. SDF images were acquired from five different regions within the site of interest. Adequate focus and contrast adjustment were verified. Images of poor quality were discarded. The whole sequence was used to characterise the semi-quantitative characteristics of microvascular blood flow, particularly the presence of stopped or intermittent flow.

Video clips were analysed blindly and randomly using different approaches. First, we used a modification of a semi-quantitative score.[9] It distinguishes no flow (0), intermittent flow (1), sluggish flow (2), and continuous flow (3). A value was assigned to each individual vessel. The overall score, called the microvascular flow index (MFI), is the mean of the individual values. For each animal, values obtained from three fields were averaged.

**Table 1. Haemodynamic and oxygen transport parameters, during baseline, shock and resuscitation, in control and nitroglycerin groups**

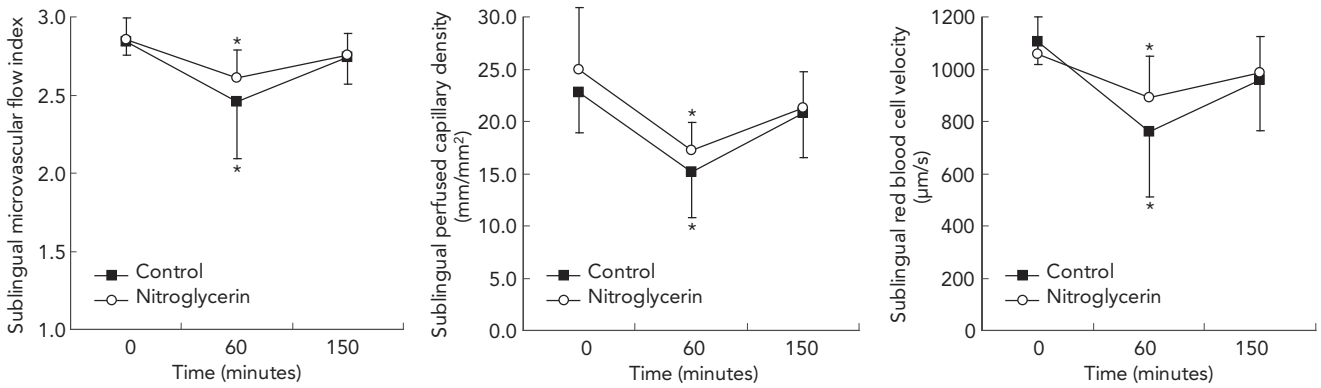
| | | Baseline | Shock | | Resuscitation | | |
|---|---|---|---|---|---|---|---|
| | Group | 0 min | 30 min | 60 min | 90 min | 120 min | 150 min |
| Heart rate (beats/min) | Control | 162 (33) | 143 (32) | 152 (43) | 184 (45) | 188 (27)* | 189 (40) |
| | Nitroglycerin | 150 (38) | 150 (36) | 166 (51) | 199 (23)* | 203 (127)* | 188 (35) |
| Mean arterial pressure (mmHg) | Control | 97 (13) | 65 (28)* | 56 (24)* | 86 (22)* | 87 (20)* | 91 (25) |
| | Nitroglycerin | 92 (15) | 66 (26)* | 51 (17)* | 75 (15)* | 81 (16)* | 75 (22)* |
| Mean pulmonary pressure (mmHg) | Control | 12 (5) | 26 (8)* | 21 (6)* | 25 (8)* | 26 (5)* | 25 (5)* |
| | Nitroglycerin | 13 (3) | 28 (7)* | 20 (4)* | 25 (6)* | 23 (6)* | 23 (6)* |
| Pulmonary artery occlusion pressure (mmHg) | Control | 4 (3) | 8 (7) | 7 (5) | 6 (5) | 8 (5) | 8 (6) |
| | Nitroglycerin | 4 (3) | 6 (4) | 4 (3) | 7 (4) | 6 (3) | 6 (3) |
| Central venous pressure (mmHg) | Control | 2 (3) | 1 (4) | 2 (3) | 4 (3) | 3 (2) | 3 (3) |
| | Nitroglycerin | 4 (4) | 2 (3) | 3 (4) | 4 (3) | 3 (4) | 3 (3) |
| Mesenteric venous pressure (mmHg) | Control | 6 (2) | 7 (4) | 7 (3) | 8 (2)* | 8 (2)* | 9 (3)* |
| | Nitroglycerin | 7 (3) | 8 (4) | 7 (4) | 9 (4)* | 9 (5)* | 10 (3)* |
| Cardiac output/body weight (mL/kg/min) | Control | 111 (38) | 80 (38)* | 95 (38) | 163 (55)* | 146 (54)* | 134 (45)* |
| | Nitroglycerin | 110 (32) | 88 (27)* | 104 (39) | 164 (48)* | 155 (53)* | 134 (48)* |
| Superior mesenteric artery blood flow (mL/min/kg) | Control | 640 (434) | 416 (238)* | 532 (351)* | 863 (364)* | 744 (345) | 670 (344) |
| | Nitroglycerin | 643 (327) | 445 (219)* | 500 (297)* | 702 (327) | 650 (306) | 578 (299) |
| Superior mesenteric blood flow/cardiac output (%) | Control | 15 (7) | 15 (7) | 14 (5) | 14 (5) | 14 (7) | 14 (6) |
| | Nitroglycerin | 16 (6) | 15 (8) | 14 (7)* | 12 (6)* | 12 (6)* | 12 (6)* |
| Systemic vascular resistance (dyn.s/cm$^5$) | Control | 2908 (717) | 2686 (683) | 1891 (654)* | 1669 (394)* | 1920 (357)* | 2184 (529)* |
| | Nitroglycerin | 3077 (1115) | 2719 (954)* | 1802 (875)* | 1705 (731)* | 1968 (848)* | 2038 (622)* |
| Pulmonary vascular resistance (dyn.s/cm$^5$) | Control | 241 (134) | 777 (402)* | 505 (240)* | 374 (224)* | 395 (146)* | 424 (150)* |
| | Nitroglycerin | 321 (176) | 992 (436)* | 647 (469)* | 456 (309)* | 458 (289)* | 572 (512)* |
| Mesenteric vascular resistance (dyn.s/cm$^5$) | Control | 22 (10) | 20 (13)* | 14 (10)* | 13 (7)* | 15 (8)* | 17 (8)* |
| | Nitroglycerin | 23 (14) | 22 (16)* | 16 (13)* | 16 (12)* | 18 (12)* | 18 (12)* |
| Systemic oxygen transport (mL/min/kg) | Control | 14.3 (4.4) | 9.3 (4.6)* | 11.6 (4.8)* | 16.6 (6.3)* | 15.2 (6.2) | 14.2 (4.8) |
| | Nitroglycerin | 14.1 (4.8) | 8.6 (4.1)* | 11.3 (5.8)* | 16.1 (5.9) | 15.1 (6.3) | 12.9 (5.4) |
| Systemic oxygen consumption (mL/min/kg) | Control | 5.5 (1.8) | 5.0 (2.7) | 5.2 (2.0) | 5.8 (2.7) | 5.6 (2.6) | 5.6 (2.2) |
| | Nitroglycerin | 5.3 (1.4) | 4.7 (1.4) | 5.2 (1.4) | 5.8 (1.4) | 5.7 (1.4) | 5.5 (1.6) |
| Intestinal oxygen transport (mL/min/kg) | Control | 85.3 (62.7) | 48.0 (28.4)* | 65.8 (44.2) | 88.8 (40.3) | 77.8 (37.8) | 72.2 (38.9) |
| | Nitroglycerin | 80.6 (39.3) | 45.8 (30.2)* | 52.8 (32.9)* | 67.8 (33.8) | 63.1 (33.5) | 56.3 (35.0) |
| Intestinal oxygen consumption (mL/min/kg) | Control | 30.0 (24.1) | 27.0 (16.9) | 30.4 (17.7) | 32.5 (13.7) | 32.7 (19.1) | 30.8 (17.5) |
| | Nitroglycerin | 27.6 (11.6) | 22.9 (12.6) | 23.4 (12.3) | 25.7 (14.2) | 22.0 (15.7) | 24.1 (13.2) |
| Intra-abdominal pressure (mmHg) | Control | 2 (2) | 2 (2) | 2 (2) | 2 (2) | 2 (2) | 2 (2) |
| | Nitroglycerin | 4 (3) | 3 (3) | 4 (3) | 4 (3) | 4 (3) | 4 (3) |

Data are shown as mean (SD). * $P < 0.05$ v baseline within each group.

Second, we used AVA (Automated Vascular Analysis) 3.0, an image analysis software package (Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands) developed for SDF video images, to determine vascular density and generate "space–time diagrams" of single vessels for quantitative measurement of red blood cell velocity (RBCV).[10] RBCV was not measured in vessels with intermittent or absent flow. Finally, the percentage of perfused vessels and the total and perfused vascular densities were calculated.[4,11] The former was calculated from the number of vessels with flows of 2 and 3 after division by the total number of vessels and multiplication by 100. The latter was calculated from the total density after multiplication by the fraction of perfused vessels.

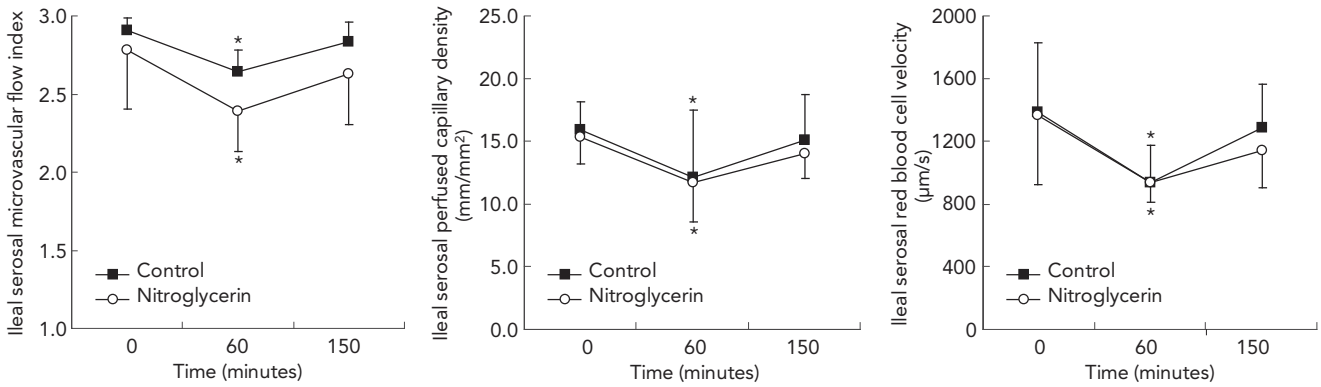
We also determined the heterogeneity of perfusion within each territory, referred to as the heterogeneity flow

**Figure 1. Sublingual microvascular flow index, perfused capillary density, and red blood cell velocity at baseline and during shock and resuscitation in the control and nitroglycerin groups**




Data are shown as mean (SD). * $P < 0.05$ v baseline within each group.

**Figure 2. Ileal serosal microvascular flow index, perfused capillary density, and red blood cell velocity at baseline and during shock and resuscitation in the control and nitroglycerin groups**




Data are shown as mean (SD). * $P < 0.05$ v baseline within each group.

index, from the difference between the highest and lowest MFI divided by the mean MFI.[12]

In sheep, most of sublingual and ileal serosal vascular density (97% [SD, 1%] of total vessel length),[13] and all villi vessels consist of capillaries (diameter below 20 μm),[11] so the analysis was focused on these vessels.

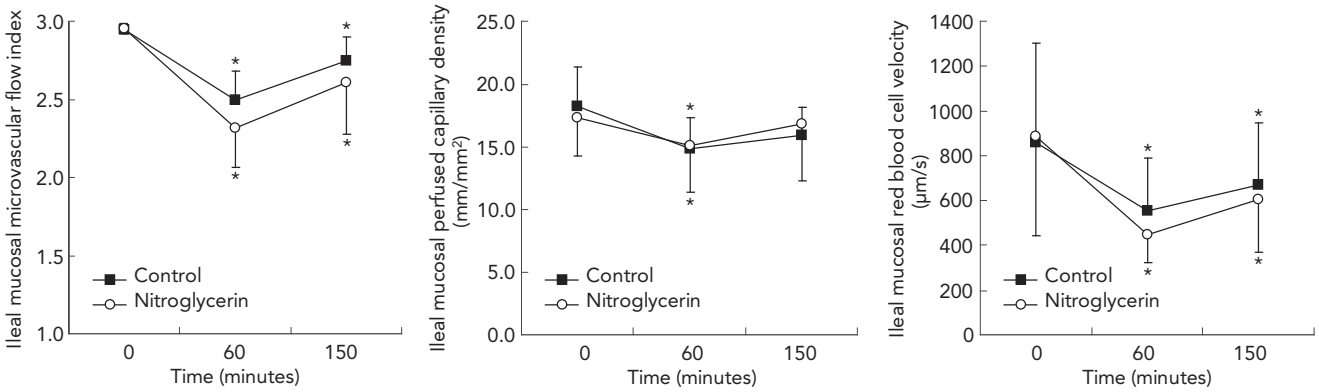
## Experimental procedure

Basal measurements were taken after a stabilisation period of no less than 30 minutes. After the basal measurements, a bolus of 5 μg/kg of *Escherichia coli* lipopolysaccharide was given over 1 minute followed by a continuous infusion of 4 μg/kg/h during the rest of the experiment. After 1 hour without resuscitation (endotoxaemic shock), the sheep received 6% hydroxyethylstarch 130/0.4 (Voluven, Fresenius Kabi, Bad Homburg, Germany) to rapidly reach basal blood pressure and intestinal blood flow. According to previous data,[4] the time to achieve these goals by means of a fluid challenge is less than 5 minutes. After this step, the sheep were randomised to control ($n = 10$) or nitroglycerin ($n = 10$) groups. Nitroglycerin was infused at a rate of 0.2 μg/kg/min for the rest of the experiments. The same volume of fluid used during the initial resuscitation was infused in both groups from the time when the aims of blood pressure and intestinal blood flow were accomplished until the end of the experiments.

Except for lactate levels and microcirculation — measured only at baseline, after 60 minutes of shock and after 90 minutes of resuscitation — all measurements were recorded every 30 minutes.

At the end of the experiment, the animals were euthanised with an additional dose of pentobarbital and a potassium chloride bolus. A catheter was inserted in the

**Figure 3. Ileal mucosal microvascular flow index, perfused capillary density, and red blood cell velocity at baseline and during shock and resuscitation in the control and nitroglycerin groups**




Data are shown as mean (SD). * $P < 0.05$ v baseline within each group.

superior mesenteric artery for the instillation of India ink. The dyed intestinal segments were dissected, washed, and weighed to calculate the gut indices.

### Statistical analysis

Using the mucosal MFI as the primary measure of outcome and taking into account previous data,[4] we calculated that a study of 20 sheep would have an 80% power of detecting an increase in MFI of 0.5 in nitroglycerin group with a certainty of 95%.

Data were assessed for normality using the Shapiro–Wilk test and expressed as mean (SD). Two-way repeated measures analysis of variance (ANOVA) was used to compare both groups. $P < 0.05$ was considered to be significant. Spearman rank correlation coefficients between MFIs and RBCVs were calculated.

## Results

### Effects on haemodynamics and oxygen transport

The infusion of endotoxin induced arterial hypotension, low cardiac output, and diminished intestinal blood flow; and systemic and intestinal $Do_2$ values likewise decreased. The systemic vascular resistance fell, and the pulmonary vascular resistance increased. Both groups received a similar volume of fluid throughout the resuscitation period (550 mL [SD, 346 mL] v 538 mL [SD, 335 mL]; $P$ = not significant). After the initial fluid challenge, similar values of mean arterial blood pressure (92 mmHg [SD, 21 mmHg] v 89 mmHg [SD, 22 mmHg]; $P$ = not significant v baseline for both) and superior mesenteric artery blood flow (918 mL/min/kg [SD, 367 mL/min/kg] v 914 mL/min/kg [SD, 356 mL/min/kg]; $P < 0.05$ v baseline for both) were reached in both groups. Fluid resuscitation normalised cardiac output and systemic $Do_2$ values, but the systemic and pulmonary vascular resistances remained altered.

Despite slight though significant increases in the mesenteric venous pressure, the mesenteric vascular resistance decreased throughout the experiment. The intra-abdominal pressure, however, remained normal. No statistically significant differences were found between the two groups in any parameter, although the nitroglycerin group showed a trend toward lower arterial blood pressures, intestinal blood flow and superior mesenteric artery blood flow and cardiac output ratio (Table 1).

### Effects on arterial blood gases and lactate

The two groups developed similar degrees of metabolic acidosis, hypoxaemia, and hyperlactataemia; moreover, in both groups these changes persisted after resuscitation (Table 2).

### Effects on carbon dioxide gradients

Systemic and intestinal venoarterial $Pco_2$ differences widened during shock and normalised with resuscitation. By contrast, the increase in $\Delta Pco_2$ that occurred during shock persisted after resuscitation. These alterations were similar in both groups (Table 2).

### Effects on microcirculation

Endotoxaemic shock decreased the MFI; the fraction of perfused capillaries; the perfused capillary density; and the RBCV in the sublingual mucosa, the intestinal mucosa, and the intestinal serosa. In addition, the heterogeneity flow index increased in those territories. Fluid resuscitation improved most of these microvascular variables. The MFI and the RBCV in the intestinal mucosa, however, remained significantly reduced compared with basal values. We recorded no differences between the two experimental groups (Figure 1, Figure 2, Figure 3 and Table 3).

**Table 2. Lactate, arterial, mixed venous and mesenteric venous blood gases, and carbon dioxide gradients, during baseline, shock and resuscitation, in control and nitroglycerin groups**

| | | Baseline | Shock | | Resuscitation | | |
|---|---|---|---|---|---|---|---|
| | Group | 0 min | 30 min | 60 min | 90 min | 120 min | 150 min |
| Arterial lactate (mmol/L) | Control | 2.1 (0.8) | | 4.3 (1.6)* | | | 5.6 (1.7)* |
| | Nitroglycerin | 1.7 (0.6) | | 3.6 (1.2)* | | | 5.1 (2.3)* |
| Arterial pH | Control | 7.42 (0.04) | 7.38 (0.06)* | 7.38 (0.06)* | 7.34 (0.05)* | 7.35 (0.05)* | 7.33 (0.07)* |
| | Nitroglycerin | 7.43 (0.05) | 7.37 (0.06)* | 7.35 (0.06)* | 7.34 (0.07)* | 7.34 (0.07)* | 7.33 (0.08)* |
| Arterial $PCO_2$ (mmHg) | Control | 38 (3) | 40 (8) | 40 (7) | 41 (6) | 39 (7) | 40 (7) |
| | Nitroglycerin | 38 (2) | 43 (6) | 43 (7) | 42 (7) | 41 (8) | 41 (8) |
| Arterial $PCO_2$ (mmHg) | Control | 79 (13) | 61 (18)* | 69 (14)* | 70 (20)* | 69 (21)* | 69 (19)* |
| | Nitroglycerin | 78 (6) | 50 (14)* | 59 (13)* | 62 (13)* | 64 (16)* | 59 (13)* |
| Arterial $HCO_3^-$ (mmol/L) | Control | 25 (2) | 23 (3) | 23 (3)* | 22 (3)* | 21 (2)* | 21 (3)* |
| | Nitroglycerin | 25 (2) | 25 (2) | 23 (3)* | 22 (3)* | 22 (3)* | 21 (2)* |
| Arterial base excess (mmol/L) | Control | 1 (2) | – 1 (3)* | – 1 (3)* | – 3 (3)* | – 4 (2)* | – 4 (3)* |
| | Nitroglycerin | 1 (3) | 0 (3) | – 2 (3)* | – 3 (3)* | – 3 (3)* | – 4 (3)* |
| Mixed venous pH | Control | 7.38 (0.04) | 7.33 (0.06)* | 7.32 (0.06)* | 7.31 (0.05)* | 7.32 (0.06)* | 7.30 (0.07)* |
| | Nitroglycerin | 7.39 (0.06) | 7.33 (0.06)* | 7.31 (0.07)* | 7.30 (0.07)* | 7.32 (0.06)* | 7.29 (0.08)* |
| Mixed venous $PCO_2$ (mmHg) | Control | 44 (3) | 48 (7) | 49 (6)* | 47 (7) | 46 (9) | 47 (8) |
| | Nitroglycerin | 45 (3) | 51 (5)* | 53 (7)* | 49 (8) | 46 (10) | 50 (9) |
| Mixed venous $PO_2$ (mmHg) | Control | 38 (5) | 29 (3)* | 35 (3)* | 40 (7) | 39 (8) | 38 (7) |
| | Nitroglycerin | 37 (4) | 26 (7)* | 30 (6)* | 37 (7) | 35 (6) | 32 (6)* |
| Mixed venous $HCO_3^-$ (mmol/L) | Control | 26 (2) | 24 (4) | 24 (4) | 23 (4)* | 22 (5)* | 22 (4)* |
| | Nitroglycerin | 27 (2) | 27 (2) | 27 (2) | 24 (2)* | 23 (4)* | 24 (2)* |
| Mixed venous base excess (mmol/L) | Control | 1 (2) | – 2 (4)* | – 2 (4)* | – 3 (5)* | – 4 (5)* | – 4 (5)* |
| | Nitroglycerin | 2 (3) | 0 (3)* | 0 (3)* | – 2 (3)* | – 2 (3)* | – 3 (3)* |
| Mesenteric venous pH | Control | 7.39 (0.04) | 7.34 (0.06)* | 7.32 (0.05)* | 7.31 (0.05)* | 7.31 (0.06)* | 7.30 (0.07)* |
| | Nitroglycerin | 7.39 (0.05) | 7.32 (0.06)* | 7.30 (0.06)* | 7.30 (0.08)* | 7.30 (0.08)* | 7.30 (0.08)* |
| Mesenteric venous $PCO_2$ (mmHg) | Control | 45 (4) | 51 (6)* | 50 (6)* | 48 (9) | 48 (8) | 49 (9) |
| | Nitroglycerin | 45 (4) | 52 (8 )* | 54 (6)* | 52 (10) | 51 (9) | 50 (9) |
| Mesenteric venous $PO_2$ (mmHg) | Control | 40 (5) | 29 (2)* | 34 (4)* | 40 (9) | 37 (8) | 36 (8) |
| | Nitroglycerin | 39 (7) | 27 (7)* | 32 (7)* | 38 (10) | 34 (8) | 32 (5)* |
| Mesenteric venous $HCO_3^-$ (mmol/L) | Control | 27 (2) | 26 (5) | 25 (4) | 24 (5)* | 23 (4)* | 23 (4)* |
| | Nitroglycerin | 28 (2) | 27 (3) | 27 (2) | 25 (2)* | 25 (2)* | 24 (2)* |
| Mesenteric venous base excess (mmol/L) | Control | 2 (2) | 0 (6)* | – 2 (5)* | – 4 (5)* | – 4 (5)* | – 4 (5)* |
| | Nitroglycerin | 3 (3) | 0 (3)* | 0 (3)* | – 2 (2)* | – 2 (3)* | – 3 (3)* |
| Arterial mixed–venous $\Delta PCO_2$ (mmHg) | Control | 7 (2) | 8 (3) | 10 (2)* | 6 (2) | 7 (3) | 7 (3) |
| | Nitroglycerin | 7 (2) | 8 (2) | 10 (4)* | 8 (5) | 5 (4) | 9 (5) |
| Arterial–mesenteric venous $\Delta PCO_2$ (mmHg) | Control | 7 (2) | 11 (4)* | 10 (5)* | 7 (4) | 8 (4) | 9 (5) |
| | Nitroglycerin | 8 (2) | 10 (3)* | 11 (5)* | 10 (8) | 10 (7) | 9 (5) |
| Ileal intramucosal–arterial $\Delta PCO_2$ (mmHg) | Control | 8 (8) | 12 (11) | 15 (9)* | 14 (10)* | 16 (11)* | 17 (9)* |
| | Nitroglycerin | 6 (6) | 9 (11) | 13 (11) | 12 (8)* | 13 (9)* | 14 (9)* |

Data are shown as mean (SD. )* $P < 0.05$ v baseline within each group.

The variations in MFIs and RBCVs were significantly correlated in sublingual mucosa (r=0.73; $P<0.001$), intestinal mucosa (r=0.70; $P<0.001$), and intestinal serosa (r=0.75; $P<0.001$).

## Discussion

In our study using a sheep experimental model, we found that nitroglycerin fails to improve the subtle but persistent villi hypoperfusion that remains present after the normalisa-

**Table 3. Microcirculatory parameters during baseline, shock and resuscitation, in control and nitroglycerin groups**

| | Group | Baseline | Shock | Resuscitation |
|---|---|---|---|---|
| Fraction of perfused capillaries | | | | |
| Sublingual mucosa | Control | 0.98 (0.03) | 0.89 (0.10)* | 0.97 (0.03) |
| | Nitroglycerin | 0.98 (0.03) | 0.93 (0.05)* | 0.98 (0.01) |
| Intestinal serosa | Control | 0.99 (0.01) | 0.96 (0.04)* | 0.98 (0.03) |
| | Nitroglycerin | 0.99 (0.03) | 0.90 (0.06)* | 0.94 (0.08) |
| Intestinal mucosa | Control | 0.99 (0.01) | 0.93 (0.04)* | 0.98 (0.02) |
| | Nitroglycerin | 0.99 (0.01) | 0.84 (0.13)* | 0.93 (0.11) |
| Heterogeneity flow index | | | | |
| Sublingual mucosa | Control | 0.88 (0.30) | 1.18 (0.32)* | 0.88 (0.27) |
| | Nitroglycerin | 0.68 (0.37) | 1.01 (0.21)* | 0.85 (0.27) |
| Intestinal serosa | Control | 0.66 (0.31) | 0.96 (0.30)* | 0.68 (0.37) |
| | Nitroglycerin | 0.66 (0.53) | 1.23 (0.21)* | 0.90 (0.35) |
| Intestinal mucosa | Control | 0.55 (0.24) | 1.12 (0.29)* | 0.65 (0.25) |
| | Nitroglycerin | 0.55 (0.29) | 1.39 (0.24)* | 0.84 (0.27) |

Data are shown as mean (SD). * $P < 0.05$ v baseline within each group.

tion of systemic and intestinal haemodynamics and oxygen transport by fluid resuscitation. Similarly, neither intramucosal acidosis nor lactic acidosis was ameliorated by nitroglycerin.

### The experimental model of septic shock

Despite the use of only a short-term infusion of endotoxin, this sheep experimental model mirrors several findings reported for non-resuscitated human septic shock. The endotoxin produced a shock state characterised by arterial hypotension, low cardiac output, decreased intestinal blood flow and diminished systemic vascular resistance, along with lactic acidosis, intramucosal acidosis and microvascular alterations. Fluid administration improved the arterial blood pressure, the systemic and gut blood flows, and some of the microvascular abnormalities. Nevertheless, hyperlactataemia, increased $\Delta P_{CO_2}$ values and villi hypoperfusion persisted. From a pathophysiological standpoint, our results exclude the role of intra-abdominal pressure as the cause of microvascular alterations, but support the notion that increases in portal and mesenteric venous pressures could contribute to the development of these abnormalities.[14]

Our findings are similar to a previous report,[4] but differ from a study in pigs with hyperdynamic septic shock secondary to cholangitis.[15] In this latter study, microcirculatory alterations were more severe, exhibiting reductions in the perfused capillary density and RBCV values to lower than 50% and 25% of the baseline, respectively. In addition, microvascular changes were not only restricted to the intestinal villi, but were also comparable in the sublingual and gut mucosae.[15] These differences could be related to the species studied and the type and magnitude of the septic insult. The presence of more subtle alterations in our study, however, might resemble human septic shock more closely. For example, in most clinical studies, the MFI was consistently higher than 2.[16-19] Consequently, resuscitated septic shock is more frequently associated with moderate — but not with severe — microcirculatory alterations.

As had been recently recommended,[11] we used a comprehensive approach for the assessment of the microcirculation. The analysis included measurements of perfusion, density and heterogeneity. The evaluation showed that each parameter could behave in a different manner from the other. Although all the components of the analysis were altered during shock, the perfusion indices alone continued to remain decreased after resuscitation. This finding emphatically indicates that a complete evaluation of recovery from septic shock requires the quantification of all variables. In addition, our results confirm the satisfactory correlation between the quantitative and semi-quantitative measurements of perfusion that had already been found in haemorrhage.[13]

### The role of nitric oxide in sepsis

NO is a key mediator in the pathophysiology of septic shock, mainly through the development of refractory vasodilation. Nevertheless, NO is necessary to produce vasodilation and to guarantee oxygen transport to tissues. An inhibition of NO synthesis increases arterial blood pressure and systemic vascular resistance but reduces car-

diac output.[7] Accordingly, a non-selective inhibition of NO synthase worsens the outcome of septic shock.[20] In addition, regional deficiencies in NO production may contribute to tissue hypoperfusion. Engelberger and colleagues recently showed that endotoxin inhibits microvascular NO-dependent vasodilation in normal volunteers.[21] In the same manner, deficits in NO production could contribute to the characteristic alterations of microvascular perfusion already found in septic patients. Therefore, an adequate balancing of NO levels, as achieved through the administration of NO donors, is an attractive therapeutic approach. In this regard, nitroglycerin may be an appropriate medication for this purpose because it does not affect blood pressure in endotoxaemic animals.[22]

### Effects of nitroglycerin on tissue perfusion

In our study, the sheep allocated to the nitroglycerin group did not manifest any significant differences from the controls. The nitroglycerin group exhibited a non-significant trend toward lower arterial blood pressures, but in both groups the intestinal villi remained similarly hypoperfused, as indicated by similar decreases in the MFI and RBCV values and the persistent intramucosal acidosis. In addition, neither intramucosal acidosis nor lactic acidosis was affected by nitroglycerin.

The reports concerning the effects of nitrovasodilators in sepsis are controversial. Sodium nitroprusside, an NO donor, improved hepatic blood flow and microcirculation in animal models of endotoxaemia.[23,24] Assadi and colleagues studied the effects of sodium nitroprusside on experimental septic shock and found an increased perfusion in the intestinal mucosa.[25] Nevertheless, the concomitant administration of fluids and norepinephrine (noradrenaline) to avoid arterial hypotension resulted in a higher cardiac output. Consequently, the increases in mucosal perfusion could have merely reflected the elevation in systemic blood flow. In addition, mucosal perfusion was evaluated by the use of laser Doppler flowmetry. This method provides a relative signal of red blood cell flow from an unknown tissue volume and thus is unable to discriminate the capillary stopped-flow or the flow heterogeneity induced by septic state. Most significantly, sodium nitroprusside did not improve $\Delta P_{CO_2}$, a sensitive indicator of microvascular perfusion,[25] so the drug could have increased shunting without improving microcirculation.

Another NO donor, SIN-1, has been reported to increase tissue-oxygen extraction and hepatic, portal, and mesenteric blood flows without decreasing blood pressure.[26] SIN-1 also ameliorated intramucosal acidosis in endotoxaemic pigs.[27] L-Arginine, a precursor of NO, has been found to improve villi perfusion in mice with septic shock.[28] Conversely, L-arginine had proved to worsen shock and increase mortality in canine peritonitis.[29] Moreover, nitrovasodilators could be harmful because of their inhibitory effects on mitochondrial respiration.[30] In contrast, the inhibition of NO exhibited beneficial effects on intestinal oxygenation in experimental endotoxaemia.[31,32]

De Backer and colleagues showed that a topical application of acetylcholine in septic patients reversed the severe abnormalities present in sublingual mucosa.[2] Spronk and colleagues, in a small series of patients with septic shock, found that sublingual microvascular flow was normalised after a bolus dose of 0.5 mg of nitroglycerin followed by an infusion of 2 mg/h.[33] Accordingly, Jansen and colleagues showed that a therapeutic protocol aimed at decreasing lactate levels improves the outcome of critically ill patients.[34] Nitroglycerin was included in that protocol. However, a recent controlled trial of patients with severe sepsis and septic shock indicated not only a lack of improvement in the sublingual microcirculation but also detrimental effects on outcome.[35] Nitroglycerin has also failed to improve gut microvascular oxygenation in other settings such as gastric tube reconstruction, both in humans[36] and pigs.[37]

The lack of beneficial effects of nitroglycerin in our septic model could be explained by glutathione depletion. Glutathione depletion plays an important role in sepsis and could prevent the formation of NO from nitroglycerin.[38] In addition, the deficit in NO may not be central in gut microvascular dysfunction in this study, as occurred in renal failure in endotoxaemic rats.[39] In this setting, inducible nitric oxide synthase inhibition, NO donation, and their combination lack beneficial effects. Another explanation for the failure of nitroglycerin to improve mucosal perfusion is the change in regional blood flow. In the nitroglycerin group, there was a trend to a reduced superior mesenteric artery blood flow and gut fractional blood flow that suggests that some blood flow redistribution from the gut could have occurred.

To our knowledge, this is the first study that has evaluated the effects of nitroglycerin on intestinal microcirculation. Our negative results, however, are insufficient to rule out the possible usefulness of nitroglycerin at other doses or in other models of septic shock.

### Limitations of the study

Our study has several limitations. First, the model of septic shock and resuscitation comprised a short-term infusion of endotoxin. A long-term model of sepsis might have resembled more adequately the inflammatory and mitochondrial derangements that characterise human septic shock.[40,41] Second, fluid resuscitation was only aimed at restoring baseline conditions, whereas a more aggressive volume expansion might have produced different results. For example, a supranormal increase in blood flow by saline adminis-

tration had been found to prevent intramucosal acidosis in ovine endotoxaemia.[42] In addition, we only evaluated the effect of a specific dosage of nitroglycerin, which was even lower than one used by Spronk and colleagues,[33] but similar to that reported by Boerma and colleagues.[35] Finally, although reflecting some of the features of human septic shock, the ovine model may not replicate the human situation with complete precision, as the sheep is a ruminant species.

### Conclusions

In this experimental model of septic shock and resuscitation, low doses of nitroglycerin were unable to correct the subtle alterations of gut mucosal perfusion that remained present after fluid resuscitation. Nevertheless, this study does not rule out the possible usefulness of nitroglycerin at higher doses, with other vascular beds, or in other experimental models.

## Acknowledgements

This study was supported by the Agencia Nacional de Promoción Científica y Tecnológica, Argentina (grant PICT-2007-00912). This work was performed in the Cátedra de Farmacología Aplicada, Facultad de Ciencias Médicas, Universidad Nacional de La Plata. We are indebted to Donald Haggerty, PhD, for editing the manuscript.

## Competing interests

Can Ince is the inventor of SDF imaging technique and holds shares in Microvision Medical.

## Author details

**Vanina S Kanoore Edul,** Investigator[1]
**Gonzalo Ferrara,** Investigator[1]
**Mario O Pozo,** Investigator[1]
**Gastón Murias,** Investigator[1]
**Enrique Martins,** Investigator[1]
**Carlos Canullán,** Investigator[1]
**Héctor S Canales,** Investigator[1]
**Elisa Estenssoro,** Investigator[1]
**Can Ince,** Professor[2]
**Arnaldo Dubin,** Associate Professor[1]

1 Cátedra de Farmacología Aplicada, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina.
2 Translational Physiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

*Correspondence:* arnaldodubin@speedy.com.ar

## References

1 Ince C, Sinaasappel M. Microcirculatory oxygenation and shunting in sepsis and shock. *Crit Care Med* 1999; 27: 1369-77.
2 De Backer D, Creteur J, Preiser JC, et al. Microvascular blood flow is altered in patients with sepsis. *Am J Respir Crit Care Med* 2002; 166: 98-104.
3 Sakr Y, Dubois MJ, De Backer D, et al. Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock. *Crit Care Med* 2004; 32: 1825-31.
4 Dubin A, Kanoore Edul VS, Pozo M, et al. Persistent villi hypoperfusion explains intramucosal acidosis in sheep endotoxemia. *Crit Care Med* 2008; 36: 535-42.
5 Aranow JS, Fink MP. Determinants of intestinal barrier failure in critical illness. *Br J Anaesth* 1996; 77: 71-81.
6 Buwalda M, Ince C. Opening the microcirculation: can vasodilators be useful in sepsis? *Intensive Care Med* 2002; 28: 1208-17.
7 Vincent JL, Zhang H, Szabo C, Preiser JC. Effects of nitric oxide in septic shock. *Am J Respir Crit Care Med* 2000; 161: 1781-5.
8 Goedhart PT, Khalilzada M, Bezemer, R, et al. Sidestream Dark Field (SDF) imaging: a novel stroboscopic LED ring–based imaging modality for clinical assessment of the microcirculation. *Opt Express* 2007; 15: 101-14.
9 Boerma EC, Mathura KR, van der Voort PHJ, et al. Quantifying bedside-derived imaging of microcirculatory abnormalities in septic patients: a prospective validation study. *Crit Care* 2005; 9: R601-6.
10 Dobbe JG, Streekstra GJ, Atasever B, et al. Measurement of functional microcirculatory geometry and velocity distributions using automated image analysis. *Med Biol Eng Comput* 2008; 46: 659-70.
11 De Backer D, Hollenberg S, Boerma C, et al. How to evaluate the microcirculation: report of a round table conference. *Crit Care* 2007; 11: R101.
12 Trzeciak S, Dellinger RP, Parrillo JE, et al. Early microcirculatory perfusion derangements in patients with severe sepsis and septic shock: relationship to hemodynamics, oxygen transport, and survival. *Ann Emerg Med* 2007; 49: 88-98.
13 Dubin A, Pozo MO, Ferrara G, et al. Systemic and microcirculatory responses to progressive hemorrhage. *Intensive Care Med* 2009; 35: 556-64.
14 Revelly JP, Ayuse T, Brienza N, et al. Dysregulation of the veno–arterial response in the superior mesenteric artery during endotoxic shock. *Crit Care Med* 1995; 23: 1519-27.
15 Verdant CL, De Backer D, Bruhn A, et al. Evaluation of sublingual and gut mucosal microcirculation in sepsis: a quantitative analysis. *Crit Care Med* 2009; 37: 2875-81.
16 Jhanji S, Stirling S, Patel N, et al. The effect of increasing doses of norepinephrine on tissue oxygenation and microvascular flow in patients with septic shock. *Crit Care Med* 2009; 37: 1961-6.
17 Dubin A, Pozo MO, Casabella CA, et al. Increasing arterial blood pressure with norepinephrine does not improve microcirculatory blood flow: a prospective study. *Crit Care* 2009; 13: R92.
18 Morelli A, Donati A, Ertmer C, et al. Levosimendan for resuscitating the microcirculation in patients with septic shock: a randomized controlled study. *Crit Care* 2010; 14: R232.
19 Ruiz C, Hernandez G, Godoy C, et al. Sublingual microcirculatory changes during high-volume hemofiltration in hyperdynamic septic shock patients. *Crit Care* 2010; 14: R170.
20 López A, Lorente JA, Steingrub J, et al. Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock. *Crit Care Med* 2004; 32: 21-30.
21 Engelberger RP, Pittet YK, Henry H, et al. Acute endotoxemia inhibits microvascular nitric oxide-dependent vasodilation in humans. *Shock* 2011; 35: 28-34.
22 Kozlov AV, Albrecht M, Donnelly EM, et al. Release and hemodynamic influence of nitro-glycerine-derived nitric oxide in endotoxemic rats. *Vascul Pharmacol* 2005; 43: 411-4.
23 Gundersen Y, Corso CO, Leiderer R, et al. The nitric oxide donor sodium nitroprusside protects against hepatic microcirculatory dysfunction in early endotoxaemia. *Intensive Care Med* 1998; 24: 1257-63.

24 Gundersen Y, Saetre T, Scholz T, et al. The NO donor sodium nitroprusside reverses the negative effects on hepatic arterial flow induced by endotoxin and the NO synthase inhibitor L-NAME. *Eur Surg Res* 1996; 28: 323-32.

25 Assadi A, Desebbe O, Kaminski C, et al. Effects of sodium nitroprusside on splanchnic microcirculation in a resuscitated porcine model of septic shock. *Br J Anaesth* 2008; 100: 55-65.

26 Zhang H, Rogiers P, Friedman G, et al. Effects of nitric oxide donor SIN-1 on oxygen availability and regional blood flow during endotoxic shock. *Arch Surg* 1996; 131: 767-74.

27 Siegemund M, Van Bommel J, Sinaasappel M, et al. The NO donor SIN-1 improves intestinal–arterial $PCO_2$ gap in experimental endotoxemia: an animal study. *Acta Anaesthesiol Scand* 2007; 51: 693-700.

28 Nakajima Y, Baudry N, Duranteau J, et al. Effects of vasopressin, norepinephrine, and L-arginine on intestinal microcirculation in endotoxemia. *Crit Care Med* 2006; 34: 1752-7.

29 Kalil AC, Sevransky JE, Myers DE, et al. Preclinical trial of L-arginine monotherapy alone or with *N*-acetylcysteine in septic shock. *Crit Care Med* 2006; 34: 2719-28.

30 Boulos M, Astiz ME, Barua RS, et al. Impaired mitochondrial function induced by serum from septic shock patients is attenuated by inhibition of nitric oxide synthase and poly(ADP-ribose) synthase. *Crit Care Med* 2003; 31: 353-8.

31 Pittner A, Nalos M, Asfar P, et al. Mechanisms of inducible nitric oxide synthase (iNOS) inhibition-related improvement of gut mucosal acidosis during hyperdynamic porcine endotoxemia. *Intensive Care Med* 2003; 29: 312-6.

32 Siegemund M, van Bommel J, Schwarte LA, et al. Inducible nitric oxide synthase inhibition improves intestinal microcirculatory oxygenation and $CO_2$ balance during endotoxemia in pigs. *Intensive Care Med* 2005; 31: 985-92.

33 Spronk PE, Ince C, Gardien MJ, et al. Nitroglycerin in septic shock after intravascular volume resuscitation. *Lancet* 2002; 360: 1395-6.

34 Jansen TC, van Bommel J, Schoonderbeek FJ, et al. Early lactate-guided therapy in intensive care unit patients: a multicenter, open-label, randomized controlled trial. *Am J Respir Crit Care Med* 2010; 182: 752-61.

35 Boerma EC, Koopmans M, Konijn A, et al. Effects of nitroglycerin on sublingual microcirculatory blood flow in patients with severe sepsis/septic shock after a strict resuscitation protocol: a double-blind randomized placebo controlled trial. *Crit Care Med* 2010; 38: 93-100.

36 Buise M, van Bommel J, Jahn A, et al. Intravenous nitroglycerin does not preserve gastric microcirculation during gastric tube reconstruction: a randomized controlled trial. *Crit Care* 2006; 10: R131.

37 Van Bommel J, De Jonge J, Buise MP, et al. The effects of intravenous nitroglycerine and norepinephrine on gastric microvascular perfusion in an experimental model of gastric tube reconstruction. *Surgery* 2010; 148: 71-7.

38 Huet O, Cherreau C, Nicco C, et al. Pivotal role of glutathione depletion in plasma-induced endothelial oxidative stress during sepsis. *Crit Care Med* 2008; 36: 2328-34.

39 Johannes T, Mik EG, Klingel K, et al. Effects of 1400W and/or nitroglycerin on renal oxygenation and kidney function during endotoxaemia in anaesthetised rats. *Clin Exp Pharmacol Physiol* 2009; 36: 870-9.

40 Brealey D, Karyampudi S, Jacques TS, et al. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. *Am J Physiol Regul Integr Comp Physiol* 2004; 286: R491-7.

41 Brealey D, Brand M, Hargreaves I, et al. Association between mitochondrial dysfunction and severity and outcome of septic shock. *Lancet* 2002; 360: 219-23.

42 Dubin A, Murias G, Maskin B, et al. Increased blood flow prevents intramucosal acidosis in sheep endotoxemia: a controlled study. *Crit Care* 2005; 9: R66-73. ❑