Sepsis is associated with altered cerebral microcirculation and tissue hypoxia in experimental peritonitis

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(Supplementary Digital Content)
Complete Material and Methods Section

Experimental animals. The Institutional Review Board for Animal Care of the Free University of Brussels (Belgium) approved the study protocol. Care and handling of the animals were in accord with National Institutes of Health guidelines (Institute of Laboratory Animal Resources). Experiments were performed on 15 female sheep (weight: 28-37 kgs), which had ad libitum access to water for the 24 hours prior to the experiment. Based on findings from our previous study (1), we allocated animals to sepsis or a sham procedure using a 2:1 randomization plan. On the day of surgery, the animals were premedicated with intramuscular injection of midazolam (0.25 mg/kg - Dormicum, Roche SA, Beerse, Belgium) and ketamine hydrochloride (20 mg/kg - Imalgine, Merial, Lyon, France). The cephalic vein was then cannulated using a peripheral venous 18-G catheter (Surflo I.V Catheter, Terumo, Leuven, Belgium). Tracheal intubation (Tracheal Tube, 8.0; Hi-Contour, Mallinckrodt Medical, Athlone, Ireland) was performed after intravenous injection of fentanyl (30 μg/kg - Fentanyl, Janssen Pharmaceutica, Beerse, Belgium) and rocuronium (0.1 mg/kg - Esmeron, Organon, Oss, the Netherlands). Mechanical ventilation (Servo ventilator 900 C, Siemens-Élema, Solna, Sweden) was initiated using volume-controlled mode as follows: tidal volume of 10 ml/kg; respiratory rate of 12-16 breaths/min; positive end-expiratory pressure of 5 cmH2O; inspired oxygen fraction (FiO2) of 1; inspiratory time/expiratory time of 1:2. Respiratory rate was adjusted to maintain end-tidal carbon dioxide pressure (PetCO2, 47210 A Capnometer; Hewlett Packard GmbH, Boeblingen, Germany) between 35 and 45 mm Hg before arterial cannulation and blood gas analyses were available. General anesthesia was maintained with a continuous intravenous infusion of ketamine hydrochloride (10 mg/kg/h), morphine (0.5 mg/kg/h) and midazolam (0.5 mg/kg/h). Muscle block was achieved using 10 μg/kg/h of rocuronium. Boluses of fentanyl (3 mg/kg) were given when needed in case of tachycardia and/or hypertension because of insufficient anesthesia. The stomach was emptied with a 60 cm plastic tube (inner-diameter 1.8 cm) and a Foley catheter (14F, Beiersdorf AG, Hamburg, Germany) was placed in the bladder for continuous urine output monitoring.

Abdominal surgery. The right femoral artery and vein were surgically exposed to place the arterial catheter (6F Vygon, Cirencester, UK) and the venous introducer. A 7F pulmonary artery catheter (Edwards Life Sciences, Irvine, CA) was advanced into a pulmonary artery via the introducer under monitoring pressure waveforms. The catheters were connected to pressure transducers (Edwards Life Sciences) with the zero pressure reference at mid-thorax level; core temperature, cardiac output and pressures were measured by Sirecust 404 (Siemens, Germany) and Vigilance monitor (Edwards Life Sciences). Both catheters were flushed intermittently with a heparinized solution (2UI/mL heparin sodium in 500 mL 0.9% saline solution). In the sepsis group, through a midline laparotomy, a 2-cm incision was made in the cecum for feces collection (1.5 g/kg of body weight) and was then closed by a double suture and the cecum replaced in the abdominal cavity after careful local disinfection. A plastic 25-cm tube (Beldico SA, Marche-En-Famenn, Luxembourg) was inserted through the abdominal wall among the intestinal loops for later feces injection and the abdomen wall was closed in two layers using end-knot continuous sutures. After the surgical procedure, the animal was placed prone and allowed to stabilize. In the sham group, abdominal surgery was not performed to reduce the risk of post-operative infection.

Cerebral surgery. The scalp was opened with a cruciate incision, using an electric scalpel (eFigure 1). The skin was then sutured to maintain full access to the head bones. A large left craniectomy was performed, coagulation of bleeding bone verified, the dura mater opened and blood or cerebrospinal fluid gently removed by saline solutions and gauze. The brain...
cortex was protected by covering with the dura mater and hourly local administration of saline solution. This left craniotomy was used to assess the brain microcirculation. On the right side, a 2-cm craniotomy was performed in the same area as that on the left; the dura mater was punctured to insert a microdialysis (MD) catheter (CMA 20, cut-off membrane 20 kDa, membrane length 10 mm, CMA Microdialysis AB, Solna, Sweden) and a Clark electrode (Licox catheter, Integra Lifesciences, Zaventem, Belgium) for tissue oxygen pressure (PbO2) measurement. Both catheters were placed under sterile conditions at a depth of 0.8-1 cm in the brain parenchyma so that their tips were located in the frontal gray matter. Finally, the catheters were sutured to the skin to avoid displacement during the experiment. Correct positioning of both catheters was further confirmed in post-mortem examination.

Monitoring and measurements. Mechanical ventilation was adjusted to ensure PaO2 of 100-130 mmHg and PaCO2 of 35-45 mmHg. Blood gas analyses were performed hourly (ABL500; Radiometer, Copenhagen, Denmark) and hemoglobin concentration and oxygen saturation were measured with an analyzer calibrated for ruminant animals (OSM3; Radiometer). The total amount of blood withdrawn for analyses was around 60 mL (i.e., around 3% of a sheep’s estimated total blood volume). Peak airway pressure, plateau airway pressure, expiratory gas flow and FiO2 were recorded from ventilator. Thoraco-pulmonary compliance (TPC), respiratory system resistance and mean airway pressure were calculated using standard formulas. All these variables were recorded hourly. Measurements of mean arterial pressure (MAP), pulmonary arterial pressure, right atrial pressure, and pulmonary arterial occlusion pressure (PAOP) were obtained at end expiration. Core temperature and cardiac output were continuously monitored. Body surface area was calculated as previously reported [7]. Cardiac index (CI), stroke volume index, systemic vascular resistance, pulmonary vascular resistance, left ventricular stroke work index (LVSWI), oxygen delivery, oxygen consumption and oxygen extraction were calculated using standard formulas.

Fluid management. Ringer’s lactate solution (RL) and 6% hydroxyethyl starch solution (HES, Voluven, Fresenius Kabi, Schelle, Belgium) were initially infused at a rate of 2 ml/kg/hour via the cephalic vein catheter; fluid administration was then titrated to maintain PAOP similar to baseline and to prevent hypovolemia. In case of hypotension (defined as a drop of ≥15% of MAP from the previous value or MAP ≤ 70 mmHg), increased lactate levels (>2.0 mEq/L) or mixed venous saturation reduction (<70%), fluid challenge (100 mL RL + 100 mL of HES) was performed if hypovolemia was suspected. When hypotension (MAP ≤ 70 mmHg) occurred and failed to respond to a fluid challenge (defined as “refractory hypotension”), the fluid infusion rate was gradually reduced to 2 ml/kg/h, to avoid the rapid occurrence of severe respiratory failure. Vasopressor agents and inotropic agents were not used. Potassium chloride and glucose were administered in case of hypokalemia (<3.5 mEq/L) or hypoglycemia (<40 mg/dL), respectively.

Cerebral microcirculation. The microvascular network of the cerebral cortex was visualized using a sidestream dark field (SDF) videomicroscopy (MicroScan, MicroVisionMedical, Amsterdam, The Netherlands), with a 5x imaging objective giving 326x magnification. The lens of the imaging device was covered with a disposable sterile cap and was applied without pressure to the cerebral frontal cortex. Because of brain pulsatility, this was best accomplished by placing the device on a metallic arm for stabilization (GiEsseCi, Avellino, Italy). At specific time-points (see Experimental protocol), five videos from different areas were recorded on disk, using a computer and a video card (MicroVideo; Pinnacle Systems, Mountain View, CA), minimum duration of 20 seconds each. The images were stored by random number designation and an investigator blinded to the data analyzed.
these sequences semi-quantitatively off-line (2). The type of flow was scored from 0 to 3 (continuous = 3; intermittent = 2; markedly reduced = 1; absent = 0). The vascular density was calculated as the number of vessels crossing the lines identifying the 16 different areas of analysis divided by the total length of the lines (2). Vessel size was determined with the aid of a micrometer scale and vessels were separated into large and small using a cut-off value of 20 μm. Small vessel perfusion was defined as the proportion of perfused vessels (PSPV), calculated as the number of small vessels continuously perfused during the 20-second observation period divided by the total number of small vessels. Functional capillary density (FCD) was calculated as the product of vascular density and perfused capillary density. In each animal, the data from the different areas were averaged.

Cerebral oxygenation and metabolism. Intracranial PbO₂ catheter was connected to a specific monitor (Brain Tissue Oxygen Monitoring, AC31, Integra Lifesciences, Zaventem, Belgium), in which temperature was manually adjusted to blood temperature. Probe function was confirmed by an oxygen challenge (FiO₂ 1.0 for 2 min). To allow probe equilibration, data from the first hour after placement were not used. The CMA 20 catheter was perfused with CNS perfusion fluid (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂ and 0.85 mM MgCl₂; osmolality 305 mOsm/kg; pH 6) at a flow rate of 1.0 μL/min by a miniaturized infusion pump (CMA 107, CMA Microdialysis AB, Solna, Sweden). This flow rate guarantees an almost 50% recovery rate for molecules of less than 20 kDa and was selected to provide additional fluid for further research on brain metabolites. After one hour of stabilization, the perfusate was collected every 60 min in specific microvials. Samples were analyzed for lactate, pyruvate, glycerol, glutamate and glucose by an automatic analyzer (CMA 600 Microdialysis Analyzer, CMA Microdialysis AB, Stockholm, Sweden). The lactate/pyruvate ratio (LPR) was automatically calculated.

Experimental protocol. After the surgical procedures, baseline measurements, including cerebral microcirculation, oxygenation and metabolism were obtained. Feces were then spilled into the abdominal cavity (sepsis group). Cerebral microcirculation was assessed at baseline and 6, 12 and 18 hours thereafter. All animals were observed until 18 hours after baseline.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 for Windows (2004 SPSS, Chicago, IL). Data are presented as mean ± SD or median (range). Normal distribution was confirmed with the Kolmogorov-Smirnov test. Variables were compared with a parametric Student’s t test or a Mann-Whitney U test for nonparametric data. The significance of differences in the measured variables between groups was analyzed using a two-way (time and groups) analysis of variance for repeated measure, followed by a Bonferroni post-hoc analysis. To estimate the correlation (expressed as “r” coefficient) between different variables in the presence of repeated measurements, we used a mixed model with SAS system (version 9.2, SAS Institute Inc.). A P value of < .05 was considered statistically significant.

References
Electronic Figure Legends.

**eFigure 1**: Schematic preparation for brain surgery. *Dotted lines* indicate the cruciate cutaneous incision for skull exposure. Left craniectomy was performed to evaluate brain microcirculation. Right craniectomy was performed to insert the Licox and microdialysis catheters into the cortex.

**eFigure 2**: Evolution over time of cardiac index in sham (n = 5) and septic (n = 10) animals. ANOVA analysis: p < 0.001. * p <0.05 with Bonferroni post-hoc analysis.

**eFigure 3**: Evolution over time of mean arterial pressure in sham (n = 5) and septic (n = 10) animals. ANOVA analysis: p < 0.01. * p <0.05 with Bonferroni post-hoc analysis.

**eFigure 4**: Evolution over time of PaO$_2$/FiO$_2$ ratio in sham (n = 5) and septic (n = 10) animals. ANOVA analysis: p < 0.01. * p <0.05 with Bonferroni post-hoc analysis.

**eFigure 5**: Evolution over time of lactate levels in sham (n = 5) and septic (n = 10) animals. ANOVA analysis: p < 0.01. * p <0.05 with Bonferroni post-hoc analysis.

**eFigure 6**: Changes (%) in mean arterial pressure (MAP), cerebral glucose (cGLU) and functional capillary density (FCD) from baseline (expressed as 100%) to 18 hours. Values are shown are mean (±SE).

**eFigure 7**: Changes (%) in lactate (Lac), cardiac index (CI), cerebral glycerol (cGLY), cerebral glutamate (cGLT) and cerebral lactate/pyruvate ratio (LPR) from baseline (expressed as 100%) to 18 hours. Values are shown are mean (±SE).