# Supplemental digital content 1

# Materials and methods

## Anesthesia

As previously described (1), pigs were fasted for 12 hours with free access to water before the experiments. Intramuscular premedication consisted of 5 mgkg-1 azaperone and 1-2 mgkg-1 midazolam. After establishment of an intravenous access via ear vein, anesthesia was induced with propofol (1-2 mgkg-1) and ketamine (1-2 mgkg-1). The pigs were endotracheally intubated and mechanically ventilated (tidal volume 8 mlkg-1, respiratory rate 8 – 12min-1 adapted to achieve an arterial pCO2 of 35 – 40 mmHg, inspiratory/expiratory (I/E) ratio 1:1.5, fraction of inspiratory oxygen (FIO2) 30%, positive end expiratory pressure (PEEP) 10 cmH2O to prevent formation of atelectasis, peak airway pressure ≤ 40 mmHg). Anesthesia was maintained with continuous intravenous infusion of pentobarbitone (8 – 12 mgkg-1h-1). Burprenorphine was used for analgesia (30 µgkg-1 initially, further 10 µgkg-1 every 8 hours as well as prior to surgery and induction of hemorrhagic shock). Pancuronium (0.15 mgkg-1h-1) ensured muscle relaxation. Balanced electrolyte solutions (20 mlkg-1h-1, Jonosteril 1/1, Fresenius, Bad Homburg, Germany) were infused for fluid homeostasis.

## Surgical procedures

As previously described (1), both internal jugular veins were exposed and a 9F central venous catheter sheath was inserted. The central venous catheter was subsequently used for infusion therapy and application of intravenous drugs. A balloon-tipped thermodilution pulmonary artery catheter was inserted via the sheath and used for the measurement of central venous pressure (CVP), mean pulmonary artery pressure (MPAP), pulmonary artery occlusion pressure (PAOP) and cardiac output (CO). For targeted temperature management, a central venous heat exchange catheter (Icy; ZOLL Medical, Cologne, Germany) was placed and connected to a custom-made extracorporeal heat exchanger based on a FC 600s cooling system (JULABO Labortechnik, Seelbach, Germany). The left carotid and the left femoral artery were exposed for placement of a PiCCO® catheter (PULSION Medical Systems SE, Feldkirchen, Germany) for continuous cardiac output measurement and a 10F arterial catheter sheath, respectively. The latter was used for blood removal during hemorrhagic shock, blood sampling and blood pressure monitoring. In order to determine kidney blood flow, a right-sided laparotomy was performed, and a pre-calibrated ultrasonic flow probe was placed around the right kidney artery. Following exposure of the left femoral vein, a 4F catheter was advanced into the inferior vena cava and guided into the right renal vein under manual and visual control. An indwelling catheter was placed in the urinary bladder via midline mini-laparotomy for urine collection. During surgery, hydroxyethyl starch 6% 130/0.42 (Vitafusal, Serumwerk, Bernburg, Germany) was used to maintain filling pressures.

## Experimental protocol

The experimental protocol is depicted in figure 1. After surgery and post-op recovery for five hours, hemorrhagic shock was induced by passive removal of 30% of the calculated blood volume (24 mlkg-1). Mean arterial blood pressure (MAP) was titrated to 40 mmHg for three hours by further removal or retransfusion of 50 ml of blood. Shed blood was stored in citrate-phosphate-dextrose bags at 8-10°C until the start of resuscitation. With the induction of hemorrhagic shock until the start of resuscitation, ventilator settings were set to PEEP 0 cmH2O, fraction of inspiratory oxygen (FIO2) 0.21, I/E ratio 1:2, and crystalloid infusion rate was reduced to 20 mlh-1. After three hours of shock, animals were randomized into three groups: the control group, the hyperoxia group, and the hypothermia group (each n=9). The study was designed with two independent experimental groups and one control group in order to reduce the numbers of animals needed according to the “3R” principles. Standard resuscitation following hemorrhagic shock consisted of re-transfusion of shed blood, fluid administration, vasopressor support, and lung-protective mechanical ventilation. Standard respirator settings were: PEEP 10 cmH2O, FIO2 0.3 and I/E ratio 1:1.5. PEEP was increased to 12 or 15 cm H2O and I/E ratio to 1:1 when the ratio of the arterial oxygen partial pressure (paO2) to FIO2 (Horowitz index) dropped below 300 or 200 mmHg, respectively. FIO2 was stepwise adjusted to maintain an arterial hemoglobin oxygen saturation of ≥ 90%. Balanced electrolyte solutions were continuously infused at a rate of 20 mlkg-1h-1, and reduced to 10 mlkg-1h-1 if PAOP or CVP > 18 mmHg. If MAP remained below baseline values despite volume resuscitation, norepinephrine was used to stabilize MAP at baseline values. Infusion rates of norepinephrine were not further increased, if the heart rate was higher than 160 min-1, in order to avoid tachycardia-induced myocardial ischemia.

In the hyperoxic group, FIO2 was set to 1.0 for the first 12 hours of resuscitation and then modified according to the standard treatment protocol.

In the hypothermia group, active cooling was initiated with the start of the resuscitation phase at a rate of 1-1.5°Ch-1. Thus, the target core temperature of 34°C was reached after 2-3 hours and was maintained until 12 hours of resuscitation. Afterwards, animals were actively rewarmed at similar rates with the heat exchange catheter, external heating blankets and warming of fluids until a core temperature of > 37.5°C was reached. In the control group and the hyperoxic group, temperature management was aimed to avoid core temperatures < 37.5°C.

At the end of the experiment, pigs were euthanized under deep anesthesia via injection of potassium chloride and organs were collected. Experiments were prematurely terminated in case of persistent hemodynamic instability (MAP ≤ 60 mmHg) despite maximal fluid and norepinephrine resuscitation.

## Measurements and calculations

Data sets were recorded before induction of hemorrhagic shock, at the end of shock, after 12 hours of resuscitation, i.e. at the end of the hyperoxic or hypothermic treatment phase, and after 23 hours of resuscitation. Measurements included assessment of systemic and pulmonary hemodynamics (MAP, MPAP, PAOP, CVP, heart rate, CO), calculated O2 uptake and calorimetric CO2 production, arterial and mixed venous blood gases, pH, base excess (BE), glucose, lactate, troponin I, interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor alpha (TNFα), nitrite plus nitrate (NO2- + NO3-), and 8-isoprostane. Renal function was assessed by determination of the following parameters: urine output, right kidney artery flow, renal venous blood gases, plasma creatinine and neutrophil gelatinase-associated lipocalin (NGAL) concentrations and creatinine clearance as described previously (1). Plasma proteins and nitrite/nitrate levels were normalized per gram of plasma protein to correct for fluid resuscitation-related hemodilution.

Effective pulmonary compliance (CPul) was calculated as tidal volume (VT) divided by the difference between the inspiratory plateau pressure (PPlat) and PEEP (CPul= VT(PPlat-PEEP)-1). Venous admixture was calculated using the shunt equation: QS/QT = (CcO2 – CaO2)/(CcO2 – CvO2) (QS: amount of blood bypassing the lungs, QT: total cardiac output, CcO2: oxygen content of pulmonary capillary, CaO2: arterial oxygen content, CvO2: mixed venous oxygen content). For calculation of CcO2, the alveolar gas equation pAO2 = (pB – 47) \* FIO2 – paCO2 / 0.8 (pAO2: alveolar partial pressure of oxygen, pB: barometric pressure (set to 760 mmHg) FIO2: fraction of inspiratory oxygen, paCO2: arterial partial pressure of CO2) was employed.

Using a steady state approach, endogenous glucose production was calculated after measurement of plasma glucose isotope enrichment by combined gas chromatography/mass spectrometry (GC/MS) during continuous intravenous infusion of stable, non-radioactively labeled 1,2,3,4,5,6-13C6-glucose and correction for exogenous glucose administration. Additional analysis of the enrichment of 13CO2/12CO2 isotope in the expiratory gas using non-dispersive infrared (NDIR) spectrometry together with the calorimetric determination of total CO2 production allowed calculation of the aerobic glucose oxidation rate (1, 2). The NDIR measurements were corrected for increased fractions of inspiratory oxygen as described previously (3).

## Coagulation

Platelet count was performed in EDTA blood using a blood cell counter (Z2 Coulter; Beckman Coulter, Krefeld, Germany) adapted for swine blood. Activity of the von Willebrand factor (vWF) and concentrations of the thrombin-antithrombin complexes in citrated blood were measured as previously described (4). Rotational thromboelastometry (ROTEM®) was performed in blood samples using a ROTEM® device (TEM International GmbH, Munich, Germany) according to the manufacturer’s protocol set to the actual pig body temperature. Analysis comprised clotting time (CT in s), maximum clot firmness (MCF in s) and maximum lysis (%), i.e., reduction of clot firmness in relation to MCF during the course of analysis, indicating the stability of the clot in the thromboelastometry measurements. Data reported for the intrinsically activated test refer to measurements after adding heparinase (Heptem) to avoid disturbances resulting from heparin contamination at the arterial sampling site (1).

## Western blots and electrophoretic mobility shift assays

As described previously (1), kidney specimens were immediately collected post-mortem and analyzed for protein expression of heme oxygenase-1 (HO-1), B-cell lymphoma-extra large (Bcl-xL), inhibitor of nuclear factor kappa B (IκBα) and the inducible isoform of the nitric oxide synthase (iNOS) by western blotting. Activation of the nuclear transcription factor kappa B (NF-κB) was assessed by electrophoretic mobility shift assay (EMSA). Actin and vinculin served as loading controls. For comparison between individual gels, the intensity of each band was related to that of the mean of two native animals, which had not undergone surgical instrumentation and hemorrhagic shock. Western blots and EMSA were run at least in triplicates. The mean value of the individual gels for each animal was used for quantitative analysis, and data are reported as fold increase over the mean of the two native animals.

## Immunohistochemistry

As described previously (1), additional kidney specimens underwent immunohistochemical analysis for 3-nitrotyrosine formation, expression of the endogenous H2S-producing enzyme cystathionine γ-lyase (CSE), and extravascular albumin. Therefore, specimens were fixed in formalin, embedded in paraffin, dewaxed in xylene, and rehydrated with a graded series of ethanol. After incubation in citrate buffer and boiling for heat-induced antigen retrieval, samples were blocked with goat sera and subsequently incubated with a primary anti-nitrotyrosine (polyclonal rabbit; Merck Millipore, Darmstadt, Germany), anti-CSE (monoclonal mouse; Abnova, Taipei City, Taiwan) or anti-albumin antibody (polyclonal rabbit; Abcam, Cambridge, United Kingdom). Primary antibody detection was performed by using the Dako REAL Detection system/RED (Dako Deutschland GmbH, Hamburg, Germany), and followed by counterstaining with haematoxylin. Slides were visualized using a Zeiss Axio Imager A1 microscope with a x10 objective. Quantification for intensity was performed using the AxioVision 4.8 software (Zeiss, Jena, Germany). Results are presented as mean densitometric sum red.

## Histopathology

As described previously (1), renal tissue samples were examined by an experienced pathologist. Pyramid-shaped kidney specimens, showing cortex, medulla, renal papilla, and the corresponding renal calyx were taken. Tissues were fixed in paraformaldehyde, and standard 3-μm paraffin sections were stained with hematoxylin-eosin and periodic acid-Schiff staining. Specimens were analyzed for edema of tubulus cells, dilation of tubuli, tubular necrosis, infiltration of inflammatory cells, cast formation, tubularization of the Bowman’s capsule, synechiae (adherence between Bowman’s capsule and glomeruli) and dilation of the Bowman’s capsule. As the histopathological examination revealed only very minor histological damage, quantification of the histopathological sections was not carried out.

## Statistical analysis

All data are expressed as median (quartiles) unless otherwise stated. Differences within each group over time as well as between the control group and the hyperoxia or hypothermia group were analyzed with the Kruskal-Wallis test and a subsequent Dunn’s test for comparisons with baseline values or the control group, respectively. Survival curves were compared using the Log-rank (Mantel-Cox) test. A p value of less than 0.05 was considered statistically significant. In case of premature termination of the experiments, a final dataset was recorded for animals that survived at least 18 hours and pooled for the last dataset. Thus, the final datasets refer to nine animals in both the control and the hyperoxia group and six animals in the hypothermia group. For immunohistochemistry, histopathology, western blots, and EMSAs, tissue specimens were collected and analyzed from every animal. The sample size was based on the previous study from our group (1). GraphPad Prism 7 for Mac (GraphPad Software, La Jolla, California, USA) was used for statistical analysis and graphical display.

**References**

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