Supplemental digital content (SDC) 2

Supplementary Material – Methods

Anesthesia

Pigs were fasted for 12 hours with free access to water before the experiments. Intramuscular premedication consisted of 5 $mg \cdot kg^{-1}$ azaperone and 1-2 $mg \cdot kg^{-1}$ midazolam. After establishment of an intravenous access via ear vein, anesthesia was induced with propofol (1-2 $mg \cdot kg^{-1}$) and ketamine (1-2 mg kg^{-1}). The pigs were endotracheally intubated and mechanically ventilated (tidal volume 8 $ml \cdot kg^{-1}$, respiratory rate 8 – 12 min^{-1} adapted to achieve an arterial pCO_2 of 35 – 40 mmHg, inspiratory/expiratory (I/E) ratio 1:1.5, fraction of inspiratory oxygen (F_IO_2) of 0.3, positive end expiratory pressure (PEEP) 10 cmH_2O to prevent formation of atelectasis, peak airway pressure ≤ 40 mmHg). Anesthesia was maintained with continuous intravenous infusion of pentobarbitone (8 – 12 $mg \cdot kg^{-1} \cdot h^{-1}$). Burprenorphine was used for analgesia (30 $\mu g \cdot kg^{-1}$ initially, further 10 $\mu g \cdot kg^{-1}$ every 8 hours as well as prior to surgery and induction of hemorrhagic shock). Pancuronium (0.15 $mg \cdot kg^{-1} \cdot h^{-1}$) ensured muscle relaxation. Balanced electrolyte solutions (10 $ml \cdot kg^{-1} \cdot h^{-1}$, Jonosteril 1/1, Fresenius, Bad Homburg, Germany) were infused for fluid homeostasis.

Surgical procedures

The right internal jugular vein was 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). Following exposure of the left carotid artery, an 8F catheter sheath was introduced for the placement of a pressure-conductance catheter (CD Leycom, Hengelo, The Netherlands). The catheter was advanced into the left ventricle (LV) under control of the

pressure curve for the analysis of cardiac function. Both femoral arteries were exposed for placement of a 4F 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 for induction of 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 and insertion of an 8F catheter sheath, 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, hemorrhagic shock was induced by passive removal of 30% of the calculated blood volume. Mean arterial blood pressure (MAP) was titrated to 40 mmHg for three hours by further removal or re-transfusion of 50 ml of blood. Shed blood was stored in citrate-phosphate-dextrose bags at 8°C. One hour before the induction of the hemorrhagic shock, ventilator settings were set to PEEP 0 cmH_2O, F_IO_2 of 0.21, I/E ratio 1:2. Crystalloid infusion rate was reduced to 20 $ml \cdot h^{-1}$ during the shock phase. After three hours of shock, animals were randomized into the normoxia (control) group or the hyperoxia group. While the control group received standard resuscitation, the F_IO_2 was set to 1.0 for the first 24 hours of resuscitation in the hyperoxia group: this duration of hyperoxia was chosen in analogy to our previous study in swine with CAD(1). Moreover, in mechanically ventilated patients, hyperoxia-induced lung damage was demonstrated only after two or more days of mechanical ventilation and probably without using lung-protective ventilator settings(2, 3). Thereafter, ventilator settings were modified according to the standard treatment protocol. We used F_IO_2 of 1.0 in analogy in our previous studies(1, 4, 5), because the “ideal p_aO_2“ is unknown especially for the acute resuscitation from

hemorrhagic shock. In addition, we assumed that an F_IO_2 of 1.0 would allow for a both a strong signal as well as to identify possible deleterious side effects of hyperoxia. Standard resuscitation consisted of re-transfusion of shed blood, fluid administration, vasopressor support, and lung-protective mechanical ventilation. Standard respirator settings were: PEEP 10 cmH_2O, F_IO_2 of 0.3 and I/E ratio 1:1.5. PEEP was increased to 12 or 15 cm H_2O and I/E ratio to 1:1 when the ratio of the arterial oxygen partial pressure (p_aO_2) to F_IO_2 dropped below 300 or 200 mmHg, respectively. F_IO_2 was stepwise adjusted to maintain an arterial hemoglobin oxygen saturation S_aO_2 of ≥ 90%. Balanced electrolyte solutions were continuously infused at a rate of 20 $ml \cdot kg^{-1} \cdot h^{-1}$, and reduced to 10 $ml \cdot kg^{-1} \cdot h^{-1}$ if PAOP or CVP > 18 mmHg. If MAP remained below baseline values despite volume resuscitation, norepinephrine was used to restore baseline MAP. 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. Temperature management was aimed to achieve normothermia.

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 and swine considered dead in case of persistent hemodynamic instability (MAP ≤ 60 mmHg) despite maximal fluid and norepinephrine resuscitation or in case of suspected severe ARDS, when target values of S_aO_2 ≥ 90%, p_aO_2 ≥ 60 mmHg could no longer be achieved despite maximally invasive ventilation (PEEP 15 cmH_2O, P_{peak} 40 cmH_2O, F_IO_2 of 1.0).

Biomarkers

The biomarker panel assessed were chosen for the availability of porcine specific test kits in analogy to our previous studies(1, 4, 5).

Biomarkers of inflammation and apoptosis

Several studies reported hyperoxia to induce hyper-inflammation(6-9) as indicated by increased plasma cytokine levels (e.g. tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-10(6, 10, 11)). Consequently, these plasma concentrations were measured using commercially available species-specific enzyme-linked immunosorbent assay (ELISA)

kits(1). Since hyperoxia alone was reported to increase nuclear transcription factor kappa B (NF-κB)[1] but reduce its activation during sustained ischemia and reperfusion(12), we collected post mortem heart specimens for western blotting analysis of protein expression of the inhibitor of nuclear factor kappa B (IκBα)(1). Finally, hyperoxia was reported to have pro-inflammatory properties with cell proliferation, hypertrophy and activation of apoptosis(13). Since high glucose-induced oxidative stress was reported to decrease anti-apoptotic regulator B-cell lymphoma extra-large (Bcl-xL) expression, we analyzed post-mortem heart specimens for Bcl-xL expression using western blotting.

Mitochondrial respiration, glucose production and oxidation

We previously reported in swine with resuscitated, fecal peritonitis-induced septic shock that hyperoxia increase carbohydrate oxidation, suggesting improved yield of the mitochondrial respiration(14). Therefore, the rate of glucose production and oxidation were calculated from the rate of appearance of stable, non-radioactively labelled 1,2,3,4,5,6-$^{13}C_6$-glucose during continuous isotope infusion after gas chromatography-mass spectrometry and non-dispersive infrared spectrometry measurement of the mixed expiratory $^{13}CO_2$ isotope enrichment, respectively, after correction for whole body VCO_2 (5, 14). Cardiac tissue mitochondrial respiratory activity was analyzed using “high-resolution respirometry” using the Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) in homogenized cardiac tissue specimens collected at the end of the experiment(1). After supplementation of substrates for complexes I and II and ADP, the respiratory capacity in the state of oxidative phosphorylation (coupled state, OXPHOS) was assessed. Maximal respiratory capacity of the electron transfer system (ETS) in the uncoupled state was measured after addition of 4-(trifluoromethoxy) phenylhydrazone (FCCP). Additionally, LEAK respiration compensating for proton leakage or slipping was reported as percentage of ETS capacity. Data is normalized for tissue wet weight.

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