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TITLE:

Standardized Hemorrhagic Shock Induction Guided by Cerebral Oximetry and Extended Hemodynamic Monitoring in Pigs

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KEYWORDS:

Hemorrhagic shock, near-infrared spectroscopy, cerebral oxygenation, blood withdrawal, pig, animal model

SUMMARY:

Hemorrhagic shock is a severe complication in seriously injured patients, which leads to life-threatening oxygen undersupply. We present a standardized method to induce hemorrhagic shock via blood withdrawal in pigs that is guided by hemodynamics and microcirculatory cerebral oxygenation.

ABSTRACT:

Hemorrhagic shock ranks among the main reasons for severe injury-related death. The loss of circulatory volume and oxygen carriers can lead to an insufficient oxygen supply and irreversible organ failure. The brain exerts only limited compensation capacities and is particularly at high risk of severe hypoxic damage. This article demonstrates the reproducible induction of life-threatening hemorrhagic shock in a porcine model by means of calculated blood withdrawal. We titrate shock induction guided by near-infrared spectrometry and extended hemodynamic monitoring to display systemic circulatory failure, as well as cerebral microcirculatory oxygen depletion. In comparison to similar models that primarily focus on predefined removal volumes for shock induction, this approach highlights a titration by means of the resulting failure of macro- and microcirculation.

INTRODUCTION:

Massive blood loss is among the main causes of injury-related deaths[1–3]. The loss of circulatory fluid and oxygen carriers leads to hemodynamic failure and severe oxygen undersupply and can cause irreversible organ failure and death. The severity level of shock is influenced by additional factors like hypothermia, coagulopathy, and acidosis[4]. Particularly the brain, but also the kidneys lack compensation capacity due to high oxygen demand and the incapability of adequate anaerobic energy generation[5,6]. For therapeutic purposes, fast and immediate action is pivotal. In clinical practice, fluid resuscitation with a balanced electrolyte solution is the first option for treatment, followed by the administration of red blood cell concentrates and fresh frozen plasma. Thrombocyte concentrates, catecholamines, and the optimization of coagulation and the acid-base status support the therapy to regain normal physiological conditions after sustained trauma. This concept focuses on the restoration of hemodynamics and macrocirculation. Several studies, however, show that microcirculatory perfusion does not recover simultaneously with the macrocirculation. Especially, cerebral perfusion remains impaired and further oxygen undersupply may occur[7,8].

The use of animal models allows scientists to establish novel or experimental strategies. The comparable anatomy, homology, and physiology of pigs and humans enable conclusions on specific pathological factors. Both species have a similar metabolic system and response to pharmacologic treatments. This is a great advantage in comparison to small models where differences in blood volume, hemodynamics, and overall physiology make it almost impossible to mimic a clinical scenario[9]. Furthermore, authorized medical equipment and consumables can be easily used in porcine models. Additionally, it is easily possible to obtain pigs from commercial suppliers, which allows a high diversity of genetics and phenotypes and is cost reducing[10]. The model of blood withdrawal via vessel cannulation is quite common[11–15].

In this study, we extend the concept of hemorrhagic shock induction via arterial blood withdrawal with an exact titration of hemodynamic failure and cerebral oxygenation impairment. Hemorrhagic shock is achieved if the cardiac index and mean arterial pressure drops below 40% of the baseline value, which has been shown to cause considerable deterioration of the cerebral regional oxygenation saturation[8]. Pulse contour cardiac output (PiCCO) measurement is used for continuous hemodynamic monitoring. First, the system has to be calibrated by transpulmonary thermodilution, which enables the calculation of the cardiac index of the extravascular lung water content and the global end-diastolic volume. Subsequently, the continuous cardiac index is calculated by pulse contour analysis and also provides dynamic preload parameters like pulse pressure and stroke volume variation.

This technique is well established in clinical and experimental settings. Near-infrared spectroscopy (NIRS) is a clinically and experimentally established method to monitor changes in cerebral oxygen supply in real-time. Self-adherent sensors are attached to the left and right forehead and calculate the cerebral oxygenation noninvasively in the cerebral frontal cortex. Two wavelengths of infrared light (700 and 900 nm) are emitted and detected by the sensors after being reflected from the cortex tissue. To assess the cerebral oxygen content, contributions of arterial and venous blood are calculated in 1:3 relations and updated in 5 s intervals. The

sensitivity in depth of 1–4 cm is exponential decreasing and influenced by the penetrated tissue (e.g., skin and bone), although the skull is translucent to infrared light. The technique facilitates quick therapeutic actions to prevent patients from adverse outcomes like delirium or hypoxic cerebral injury and serves as the target parameter in case of impaired cardiac output[16,17]. The combination of both techniques during experimental shock enables an exact titration of macrocirculation, as well as cerebral microcirculatory impairment, to study this life-threatening event.

PROTOCOL:

The experiments in this protocol were approved by the State and Institutional Animal Care Committee (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany; Chairperson: Dr. Silvia Eisch-Wolf; reference number: 23 177-07/G 14-1-084; 02.02.2015). The experiments were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. The study was planned and conducted between November 2015 and March 2016. After extended literature research, the pig model was chosen as a well-established model for hemorrhagic shock. Seven anesthetized male pigs (*Sus scrofa domestica*) with a mean weight of 28 ± 2 kg and an age of 2–3 months were included in the protocol. The animals were cared for by a local breeder that was recommended by the State and Institutional Animal Care Committee. The animals were kept in their known environment as long as possible to minimize stress. Food, but not water was denied 6 h before the experiment was scheduled, to reduce the risk of aspiration. The representative time course is displayed in **Figure 1**.

1. Anesthesia, intubation, and mechanical ventilation

1.1. Sedate pigs with a combined injection of ketamine (4 $mg \cdot kg^{-1}$) and azaperone (8 $mg \cdot kg^{-1}$) in the neck or the gluteal muscle with a needle for intramuscular injection (1.2 mm). Ensure that the animals remain stable until the sedation sets in.

CAUTION: Gloves are absolutely necessary when handling animals.

1.2. Transport the sedated animals to the laboratory.

NOTE: The animals fall deeply asleep and do not awake during normal handling, like when they are lifted into the transport cage. In this setting, the transport time was about 20 min with a special van for animal transport.

1.3. Monitor the peripheral oxygen saturation (SpO_2) with a sensor clipped to the pig's tail or ear directly after arrival.

1.4. Disinfect the skin with colorless disinfection tincture and wait for 3 min before inserting a peripheral vein catheter (1.2 mm) into an ear vein. Then, induce anesthesia by an intravenous injection of fentanyl (4 $\mu g \cdot kg^{-1}$) and propofol (3 $mg \cdot kg^{-1}$).

1.5. When all reflexes are absent and spontaneous breathing expires, place the pigs in supine

position on a stretcher and fix them with bandages.

NOTE: Adequate levels of anesthesia have to be confirmed by an experienced researcher by the absence of an eyelid reflex and other reactions to external stimuli.

1.6. Immediately start noninvasive ventilation with a dog ventilation mask (size 2). Use the following ventilation parameters: inspiratory oxygen fraction (FiO_2) = 1.0; respiratory rate = 14–16 min^{-1}; peak inspiratory pressure < 20 cm H_2O, positive end-expiratory pressure (PEEP) = 5 cm H_2O.

1.7. Maintain anesthesia via a continuous infusion of fentanyl (0.1–0.2 $\mu g \cdot kg^{-1} \cdot h^{-1}$) and propofol (8–12 $mg \cdot kg^{-1} \cdot h^{-1}$) and start an infusion of balanced electrolyte solution (5 $mL \cdot kg^{-1} \cdot h^{-1}$).

1.8. Facilitate endotracheal intubation by the application of a muscle relaxant (atracurium 0.5 $mg \cdot kg^{-1}$).

1.9. Secure the airway via intubation with a common endotracheal tube (ID 6–7) and an introducer. Use a common laryngoscope with a Macintosh blade (size 4). Two persons facilitate the procedure.

1.9.1. Person 1: Fix the tongue outside with a piece of tissue and open the snout with the other hand.

1.9.2. Person 2: Perform a laryngoscopy.

1.9.3. Person 2: When the epiglottis comes into view, move the laryngoscope ventrally. Lift up the epiglottis and make sure the vocal cords are visible.

NOTE: If the epiglottis does not move dorsally, it sticks to the soft palatine and can be mobilized by the tip of the tube. Alternatively, a blade with another size (3 or 5) or type (Miller blade) can be used.

1.10. Move the tube carefully through the vocal cords.

NOTE: The narrowest point of the trachea is not on the level of the vocal cords but subglottic. If tube insertion is not possible, try to rotate the tube or use a smaller tube.

1.11. Pull the introducer out of the tube, use a 10 mL syringe to block the cuff with 10 mL of air, and control the cuff pressure with a cuff manager (30 cm H_2O).

1.12. Start mechanical ventilation after the tube is connected to a ventilator (PEEP = 5 cm H_2O; tidal volume = 8 $mL \cdot kg^{-1}$; FiO_2 = 0.4; inspiration-to-expiration ratio = 1:2; respiratory rate = variable to achieve an end-tidal CO_2 of <6 kPa).

NOTE: Avoid fluctuation of the CO_2 to minimize any respiratory effects on the cerebral perfusion.

1.13. Make sure that tube position is correct by regular and periodic exhalation of CO_2 via capnography, and check the double-sided ventilation through auscultation.

NOTE: If the tube is placed incorrectly, air inflation into the stomach rapidly forms a visible bulge in the abdominal wall, even before the capnography is installed. In this case, replacement of the tube and the insertion of a gastric tube are absolutely necessary.

1.14. With two persons, place a gastric tube into the stomach to avoid reflux and vomiting.

1.14.1. Person 1: Fix the tongue outside with a piece of tissue and open the snout with the other hand.

1.14.2. Person 2: Perform a laryngoscopy of the porcine larynx.

1.14.3. Person 2: Visualize the esophagus.

1.14.4. Person 2: Push the gastric tube inside the esophagus with a pair of Magill forceps until gastric fluid is drained.

NOTE: Sometimes, visualization is not easy. In this case, move the laryngoscope dorsally to the tube and push it ventrally to open the esophagus. During the procedure, the animal body is covered with blankets to avoid hypothermia. If the animal's body temperature decreases, use a heating system to stabilize the temperature on a physiological level (see the **Table of Materials**). The body temperature is displayed on the screen of the PiCCO.

2. Instrumentation

2.1. Use bandages to pull back the hind legs to smoothen the folds in the femoral area for vessel catheterization.

2.2. Prepare the following materials: one 5 mL syringe, one 10 mL syringe, one 50 mL syringe, one Seldinger needle, introducer sheaths (2 mm, 2.7 mm, 2.7 mm), guidewires for the sheaths, a central venous catheter with three ports (2.3 mm, 30 cm) with guidewire, and a PiCCO catheter (1.67 mm, 20 cm).

2.3. Disinfect the inguinal area with colored disinfection, wait for 2 min, and wipe the disinfection off with a sterile tissue. Repeat this procedure 3x. After the third time, do not remove the disinfection.

2.4. Fill all catheters with saline solutions.

2.5. Apply ultrasound gel to the ultrasound probe. Cover the inguinal area with a sterile

fenestrated drape and scan the right femoral vessels with ultrasound. Use the Doppler technique to distinguish between the artery and the vein[18].

2.6. Bright red pulsating blood confirms the aspired needle position. Disconnect the syringe and insert the guidewire into the right femoral artery.

2.7. Visualize the longitudinal axis of the right femoral vein and insert the Seldinger needle under permanent aspiration with the 5 mL syringe.

2.8. Aspirate dark red nonpulsating venous blood.

2.9. Visualize the right femoral artery axially and switch to a longitudinal view of the artery by rotating the probe 90°.

2.10. Puncture the right femoral artery under ultrasound visualization with the Seldinger needle under permanent aspiration with the 5 mL syringe.

NOTE: The ultrasound-guided Seldinger technique is associated with significantly lower blood loss, tissue trauma, and time consumption than other methods of vascular access[19,20].

2.10.1. If the correct position of the needle in the different vessels cannot be established for certain, take the blood probes and analyze the blood gas content with a blood gas analyzer (see the **Table of Materials**). A high oxygen level is a good sign of arterial blood, and a low oxygen level is a sign of venous blood.

2.11. Insert the guidewire for the central venous catheter into the right femoral vein after disconnecting the syringe and retracting the Seldinger needle.

2.12. Visualize both right vessels with ultrasound to control the correct wire position.

2.13. Push the arterial introducer sheath (2 mm) over the guidewire into the right artery and secure the position with blood aspiration.

2.14. Use the Seldinger technique to position the central venous line into the right femoral vein. Aspirate all ports and flush them with saline solution.

2.15. Perform the same procedure on the left inguinal side to insert the other introducer sheaths in the Seldinger technique into the left femoral artery (2.7 mm) and femoral vein (2.7 mm).

2.16. Connect the right arterial introducer sheath and the central venous catheter with two transducer systems for the measurement of invasive hemodynamics, and position both transducers on the heart level to get appropriate values.

2.17. Switch the three-way-stopcocks of both transducers open to the atmosphere to calibrate

the systems to 0 as is prescribed in the operation instructions.

NOTE: It is absolutely necessary to avoid any air bubbles and bloodstains in the systems to generate plausible values.

2.18. Switch all infusions for maintaining anesthesia from the peripheral vein to the central venous line.

2.19. Take baseline values (hemodynamics, spirometry, NIRS (see section 4) and PiCCO (see section 3) after 15 min of recovery.

2.20. Initiate hemorrhagic shock (see section 5).

3. PiCCO measurement

NOTE: For the PiCCO equipment, see the **Table of Materials**.

3.1. Insert the PiCCO catheter into the right arterial introducer sheath.

NOTE: In clinical medicine, PiCCO catheters are directly placed by the Seldinger technique. However, placement via an introducer sheath is feasible as well.

3.2. Connect the catheter with the arterial wire of the PiCCO system and the arterial transducer directly with the PiCCO port. Then, recalibrate as described in step 2.17.

3.3. Connect the venous measuring unit of the PiCCO system with the left venous introducer sheath.

NOTE: It is necessary to connect the venous and arterial probes at some distance from each other. Otherwise, the measurement will be disturbed, because the application of cold saline solution into the venous system will influence the arterial measurement. For more details on PiCCO, see Mayer and Suttner[21].

3.4. Turn on the PiCCO system and confirm that a new patient is measured.

3.5. Enter the animal's size and weight and switch the category to adults.

3.6. Enter the protocol name and ID and enter **Exit**.

3.7. Set the injection volume to 10 mL.

NOTE: The volume of the chosen injection solution can be varied. A higher volume makes the measured values more valid. Chose a small volume to avoid any hemodilution effects through repetitive application.

3.8. Enter the central venous pressure.

3.9. Open the three-way stopcock to the atmosphere, click on **Zero** for system calibration, and click on **Exit**.

3.10. Calibrate the continuous cardiac output measurement as described next and click on **TD** (Thermodilution). Prepare physiological saline solution with a temperature of 4 °C in a 10 mL syringe and click on **Start**.

3.11. Inject 10 mL of the cold saline solution quickly and steadily into the venous measuring unit and wait until the measurement is completed and the system requests a repetition.

3.12. Repeat this procedure until three measurements are completed.

3.13. Let the system calculate the mean of all parameters and click on **Exit**.

3.14. After complete calibration, immediately start the measurement. To monitor shock induction, focus on the PiCCO-derived parameter cardiac index.

4. Cerebral regional oxygenation saturation

NOTE: For the equipment to monitor cerebral regional oxygenation, see the **Table of Materials**.

4.1. Shave the forehead of the pig with a disposable razor and water and stick two self-adherent sensors (see the **Table of Materials**) for NIRS to the forehead of the pig.

4.2. Connect the preamplifier to the monitor and connect the sensor cable connectors color-coded to the preamplifier.

4.3. Close the preamp locking mechanism and attach the sensors to the sensor cables.

NOTE: In order to record real-time data, a USB flash drive has to be connected to the NIRS monitor.

4.4. Switch on the monitor, click on **New Patient**, enter the study name, and click on **Done**.

4.5. Check the incoming signal. When the signal is stable, click on **Baseline Menu** and click on **Set Baselines**. If the baseline has already been entered, confirm the new baseline by clicking **Yes** and click on **Event Mark**.

4.6. Chose the event with the arrow buttons on the keyboard and with **Next Event**; select the event **3 Induction** and press **Select Event**.

NOTE: If further information is necessary, consult the operation manual of the NIRS system[22].

5. Hemorrhagic shock induction

5.1. Connect left introducer sheath with a tree-way stopcock. Connect one port of the three-way stopcock with a 50 mL syringe and one with an empty infusion bottle.

NOTE: Alternatively, the withdrawn blood may be collected in citrated bags for later autotransfusion. This is a major advantage of controlled blood withdrawal.

5.2. Measure and document the exact hemodynamic parameters and calculate 40% of the cardiac index and mean arterial pressure as hemodynamic targets. Set the event **93 Blood Loss** in the NIRS system as described in step 4.6.

NOTE: Hemorrhagic shock is achieved if the cardiac index and mean arterial pressure drops below 40% of the baseline value. A considerable cerebral regional oxygenation saturation ($crSO_2$) decline of 20% is preferable to depict microcirculatory impairment. The average blood loss to achieve this lies within a range of 25–35 $mL \cdot kg^{-1}$.

5.3. Aspirate 50 mL of blood into the syringe and switch the three-way stopcock. Push the blood into the empty bottle.

5.4. Note the removed blood volume.

5.5. Monitor the arterial blood pressure, the cardiac index, and the $crSO_2$ closely. Repeat the blood withdrawal until the target blood pressure and cardiac index are achieved (after 20–30 min).

5.6. Set the event **97 Hypotension** in the NIRS device as described in step 4.6.

NOTE: Do not withdraw the blood too quickly, because this bears the risk of immediate cardio-circulatory failure. After finishing the shock induction procedure, the animals can be used for various therapeutic interventions.

6. End of the experiment and euthanasia

6.1. Inject 0.5 mg of fentanyl into the central venous line and wait for 5 min.

6.2. Inject 200 mg of propofol into the central venous line and euthanize the animal with 40 mmol potassium chloride.

REPRESENTATIVE RESULTS:

After starting the shock induction, a short time of compensation can be registered. With ongoing blood removal, the aforementioned cardio-circulatory decompensation, as monitored by a

significant decrease of $crSO_2$, the cardiac index, the intrathoracic blood volume index, and the global end-diastolic volume index (**Figure 2**, **Figure 3**, and **Figure 4**), occurs. Furthermore, significant tachycardia and a decrease of arterial blood pressure are observed as common manifestations of hemorrhagic shock (**Figure 2**). Stroke volume variation increases significantly (**Figure 3**). Extravascular lung water content and systemic vascular resistance are usually unaffected (**Figure 3**). After ending the blood withdrawal (28 ± 2 $mL \cdot kg^{-1}$), the hemodynamic values remain on a critically low level. Parallelly, $crSO_2$ also drops down significantly. These sensors do not regularly start on the same level, but the percental dropdown is comparable. **Figure 4** shows a representative recording from one animal. Hemoglobin content and hematocrit do not directly decrease in the process, but lactate levels rise and the central venous oxygen saturation decreases (**Figure 5**).

FIGURE LEGENDS:

Figure 1: Experimental flow chart. The baseline is set after preparation and a 30 min stabilization. Shock is induced for 30 min. Pulse contour cardiac output parameters and cerebral regional oxygenation are measured during the whole experiment. The times of measurement are termed as **Preparation**, **Baseline**, and **Shock**.

Figure 2: Development of hemodynamics during hemorrhagic shock. Effects over time are analyzed by ANOVA and post hoc Student-Newman-Keuls method. $^{\#}p < 0.05$ to baseline. Data are presented as mean and standard deviation. (**A**) Heart rate (**B**) mean arterial pressure, and (**C**) central venous pressure are considerably influenced in this model.

Figure 3: Development of the pulse contour cardiac output and thermodilution-derived parameters during hemorrhagic shock. Effects over time are analyzed by ANOVA and post hoc Student-Newman-Keuls method. $^{\#}p < 0.05$ to baseline. Data are presented as mean and standard deviation. (**A**) Cardiac index decreases, (**B**) Stroke volume variation increases, (**D**) intrathoracic blood volume index and (**E**) global end-diastolic volume index decrease, **(C)** systemic vascular resistance index and **(F)** extravascular lung water index remain unaffected.

Figure 4: $crSO_2$ flow chart during hemorrhagic shock in one representative animal. The left panel shows a schematic presentation of the $crSO_2$ during hemorrhagic shock. The right panel shows the display of the NIRS system. $crSO_2$ significantly breaks down through shock induction and remains at a low level after the blood withdrawal is ended.

Figure 5: Development of hematologic parameters during hemorrhagic shock. Effects over time are analyzed by ANOVA and post hoc Student-Newman-Keuls method. $^{\#}p < 0.05$ to baseline. Data are presented as mean and standard deviation. (**A**) Hemoglobin and **(D)** base excess remain unaffected, (**C**) lactate level rises significantly, (**B**) central venous oxygen saturation decreases.

DISCUSSION:

The protocol describes one method of inducing hemorrhagic shock via controlled arterial bleeding in pigs that is guided by systemic hemodynamics, as well as by cerebral microcirculatory

impairment. Shock conditions were achieved by a calculated blood withdrawal of 25–35 $mL \cdot kg^{-1}$ and confirmed by the mentioned composite of surrogate parameters indicating considerable cardio-circulatory failure. If untreated, this procedure was lethal within 2 h in 66% of the animals, which underlines the severity and reproducibility of the model. Adequate fluid resuscitation, on the other hand, restabilized the circulation and approved the patency to mimic a clinical scenario[8]. However, less blood loss may not lead to the hemodynamic instability that also affected $crSO_2$ leading to experimental failure. The amount of removed blood needed to be adapted to the animal's body weight, which corresponded with the total blood volume[8].

This method allows scientists to examine different aspects of this life-threatening condition and opens up the opportunity to study a wide array of therapeutic interventions in a pseudoclinical scenario. In this context, it is important to note that during manifest hemorrhagic shock, the macrocirculation alone hardly indicates an intact or impaired microcirculation and organ oxygen supply[7]. The advantage of the procedure lies in its simple design and usability. The transfer to other medium-sized mammals appears uncomplicated, although different species may exhibit specific challenges. The design provides high flexibility as different levels of cardio-circulatory impairment can be easily chosen by titrating the effect variables. The combination with NIRS provides information about the otherwise unrecognized microcirculatory oxygen supply during hemorrhagic shock.

Some of the model's critical steps have to be highlighted and require attention. Adequate sedation prior to transport is essential to avoid stress that could complicate the animal handling and falsify results by endogenic catecholamine release. The porcine snout, with its long oropharyngeal cavity, complicates intubation and makes the assistance of a second person reasonable. Regularly, the epiglottis sticks to the palate and has to be mobilized with the tip of the tube. The narrowest part of the airway is not at the level of the vocal cords but subglottic, like in pediatric patients[23]. These aspects make adequate muscle relaxation essential because intubation is facilitated. Ultrasound-guided vessel catheterization is preferable, although surgical access can also be used in reproducible fashion. The minimally invasive technique needs special training and experience but can minimize uncontrolled bleeding, tissue damage, complication rates, access time, and pain[24]. The induction of the hemorrhagic shock itself appears to be very simple, but the user should be aware of several pitfalls. It is important to reduce the blood removal speed to recognize hemodynamic instability. Arterial removal is efficient, but when it is performed too fast, it can lead to unplanned cardio-circulatory and experimental failure. The calculation of the approximate extraction volume helps to manage the removal and avoids critically low cardio-circulatory levels[25–27]. Other published protocols vary in terms of targeted hemodynamic failure, amount of removed blood volume, and period of blood withdrawal. The punctuated vessel can differ as well[27,28].

NIRS enables real-time measurements of the $crSO_2$. In several clinical settings, this method has been used to recognize an impaired cerebral oxygen supply: particularly during cardiac and major vascular surgery, NIRS represents a valuable tool. NIRS-derived parameters can predict a worse neurological outcome and patient survival caused by insufficient tissue oxygenation[29]. Interestingly, the intracerebral lactate level decreases in correlation with the NIRS values. Studies

have shown that, during oxidative stress, lactate can be utilized as a source of pyruvate, and the intracranial lactate level decreases[10]. These findings and measurements are not considered in this basic model description. Changes of mean arterial pressure that influence the cerebral perfusion, PaO_2, $PaCO_2$, or the hemoglobin directly affect NIRS-derived $crSO_2$[30,31]. NIRS has a prognostic value in patients suffering from hemorrhagic shock and hemodynamic instability as well[32-39]. However, several limitations and disadvantages have to be noted. Extracranial tissue below the sensors, like skin, muscles, and fat, may influence the measurements and can lead to false negative results. The spatial resolution is low, and the penetration depth is limited[32–34,40–43]. The method neither differentiates between arterial and venous blood nor between oxygen delivery and demand[41,44,45]. The device is primarily approved for human application. The used sensors are designed for human adults. Smaller sensors for children and newborns exist, but these were not available for this protocol. In pigs, the technique is widely accepted, and $crSO_2$ correlates with a partial pressure of oxygen, quantitative electroencephalography, and cerebral venous oxygen saturation[46,47]. Several devices directly measure the oxygen partial pressure in the cerebral tissue. For this purpose, the probes have to be inserted surgically into the brain. This enables unaffected measurements in the respective region of interest and avoids disturbances by surrounding noncerebral tissue. Although this approach is highly invasive, it is rather suitable for special scenarios, like neurosurgical procedures[48–51]. The use of porcine models to simulate human pathomechanisms is a very common approach[11–13,15]. The advantage lies in the physiologic comparability between both species. Experiments that simulate life-threatening clinical conditions require fundamental expertise in intensive care medicine and anesthesia but also in specific species-related features. This allows mimicking clinical scenarios in realistic fashion for the translational testing of novel devices or therapeutic regimes on the threshold to clinical application[8,52]. However, we have to be aware that direct or immediate conclusions concerning clinical application can hardly be drawn from experimental models. Some relevant differences and limitations have to be noted: regarding shock or hemorrhage, the porcine coagulation system appears to be more effective and the hemoglobin content is significantly lower. Also, lactate and succinate plasma levels differ[53]. The porcine blood consists of an “A0” blood group system, compared to the human “AB0” system[54]. Some studies discuss if splenectomy should be performed to exclude the occurrence of intrinsic autotransfusion in porcine shock models. On the other hand, during splenectomy, oxidative stress, pain, and sympathetic stimulation occur, and the procedure is associated with autotransfusion reactions by itself. For these reasons, splenectomy is not recommended[55,56]. The use of clinically approved devices has some systemic sources of error. The PiCCO system requires calculation of the body surface area, which differs between pigs and humans. This can cause a systemic error, but the trending ability of the device will be unaffected. Other methods of cardiac output measurement, like echocardiography or a pulmonary arterial catheter, can be discussed in this setting.

In conclusion, this protocol presents a standardized hemorrhagic shock model initiated by arterial blood withdrawal and controlled by extended hemodynamic monitoring, as well as $crSO_2$. In comparison to similar models that primarily focus on predefined removal volumes for shock induction, this approach highlights a titration by means of the resulting failure of macro- and microcirculation.

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Figure 1

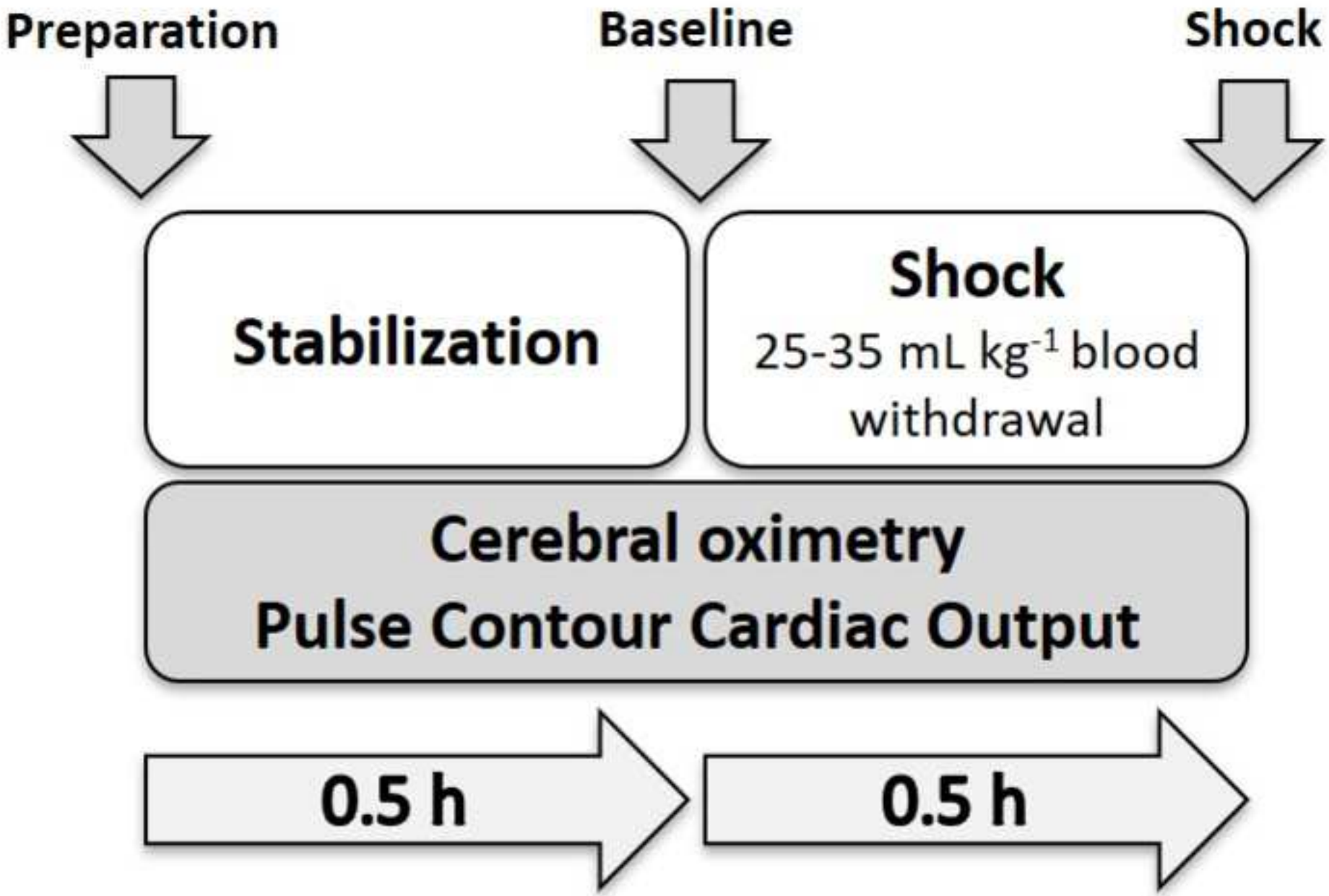
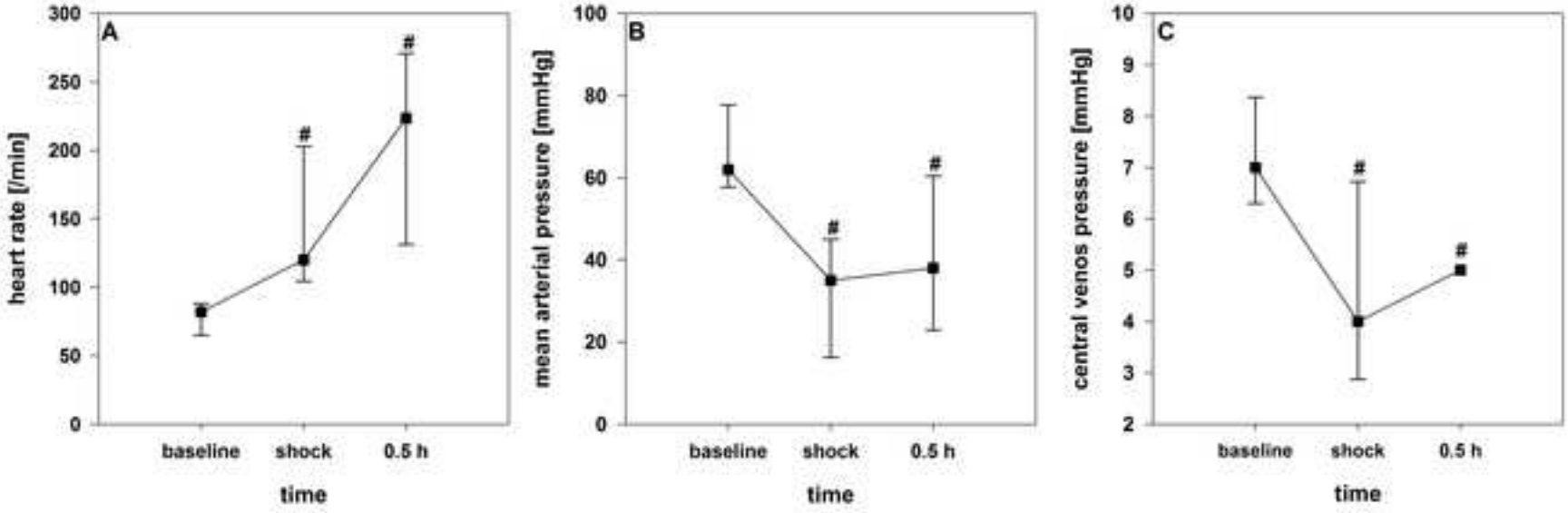


Figure 2



Figure

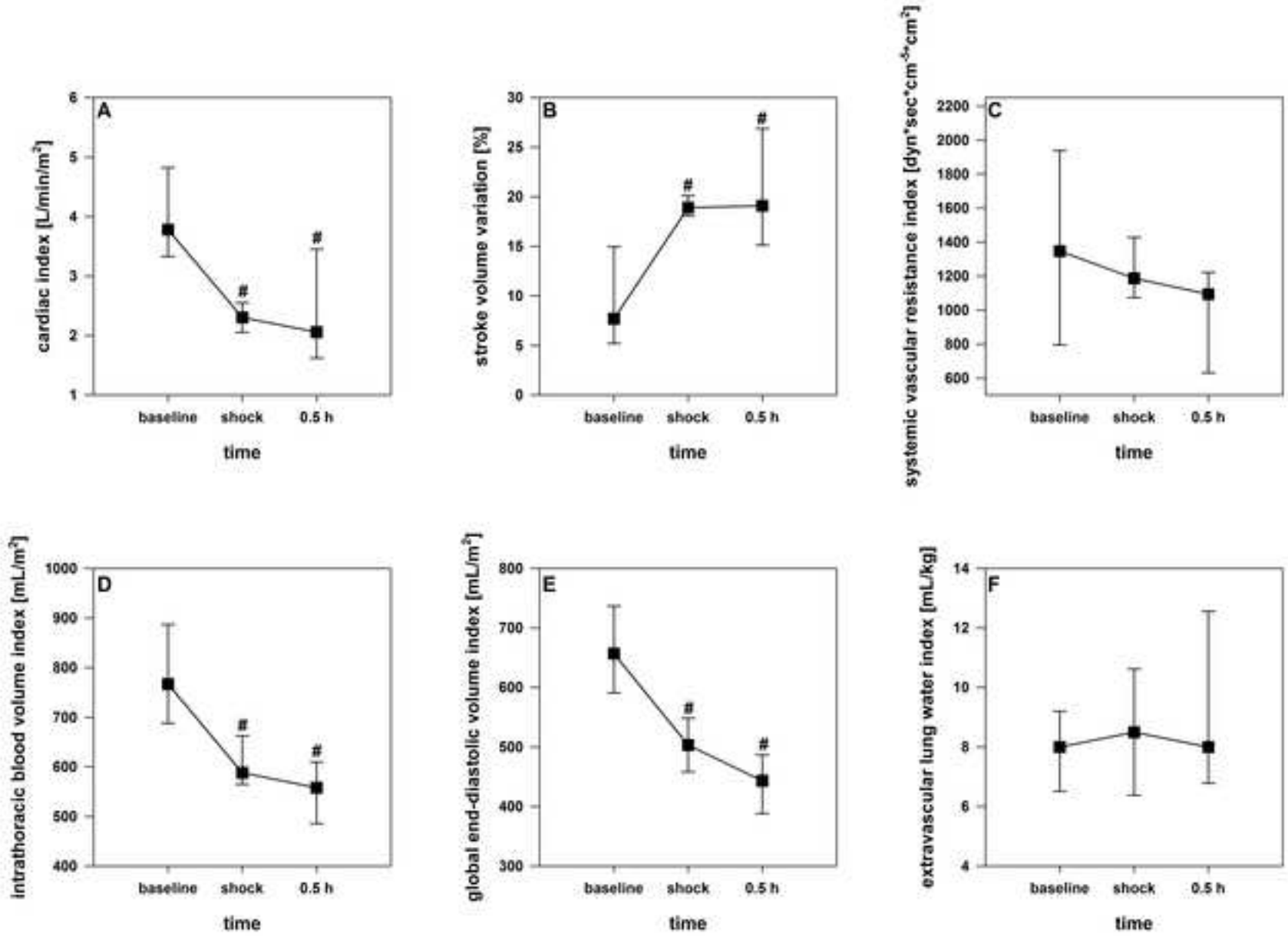


Figure 4

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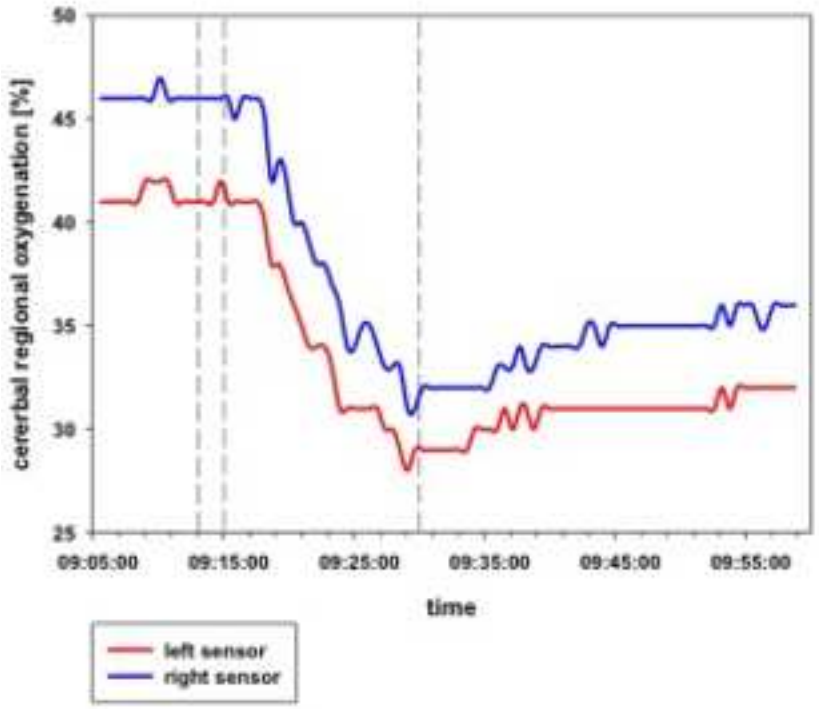

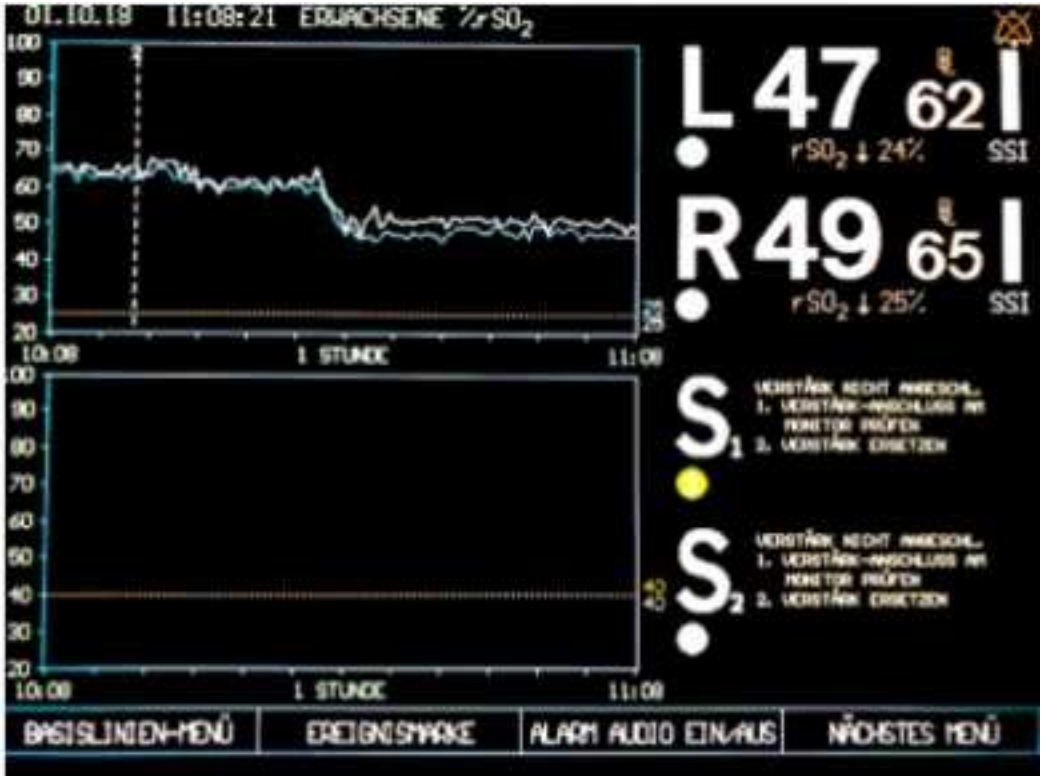

Figure 5

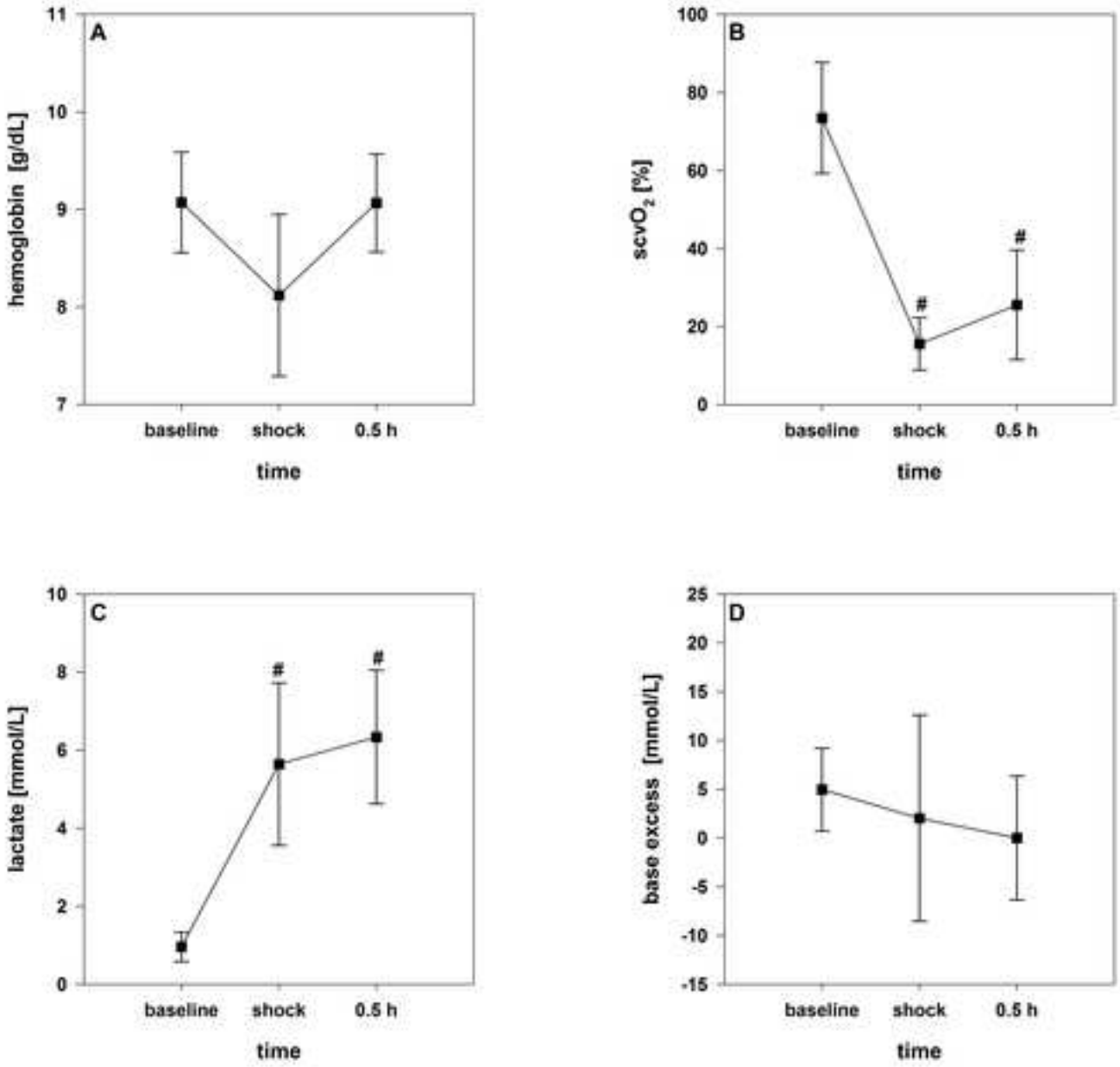

Table of Materials

Name of Material/ Equipment	Company	Catalog number	Comments/Description
3-way-stopcock blue	Becton Dickinson Infusion Therapy AB Helsingborg, Sweden	394602	Drug administration
3-way-stopcock red	Becton Dickinson Infusion Therapy AB Helsingborg, Sweden	394605	Drug administration/Shock induction
Atracurium	Hikma Pharma GmbH , Martinsried	AM03AC04*	Anesthesia
Canula 20 G	Becton Dickinson S.A. Carretera	301300	Vascular access
Datex Ohmeda S5	GE Healthcare Finland Oy, Helsinki, Finland	-	Hemodynamic monitor
Desinfection	Schülke & Mayr GmbH, Germany	104802	Desinfection
Heidelberger Verlängerung 75CM	Fresenius Kabi Deutschland GmbH	2873112	Drug administration/Shock induction
INVOS™ 5100C Cerebral	Medtronic PLC, USA	-	Monitore for cerebral regional oxygenation
INVOS™ Cerebral/Somatic Oximetry Adult Sensors	Medtronic PLC, USA	20884521211152	Monitoring of the cerebral regional oxygenation
Endotracheal tube	Teleflex Medical Sdn. Bhd, Malaysia	112482	Intubation
Endotracheal tube introducer	Wirutec GmbH, Sulzbach, Germany	5033062	Intubation
Engström Carestation	GE Heathcare, Madison USA	-	Ventilator
Fentanyl	Janssen-Cilag GmbH, Neuss	AA0014*	Anesthesia
Gloves	Paul Hartmann, Heidenheim, Germany	9422131	Self-protection
Incetomat-line 150 cm	Fresenius, Kabi GmbH, Bad Homburg, Germany	9004112	Drug administration
Ketamine	Hameln Pharmaceuticals GmbH, Zofingen, Schweiz	AN01AX03*	Sedation
Laryngoscope	Teleflex Medical Sdn. Bhd, Malaysia	671067-000020	Intubation
Logical pressure monitoring system	Smith- Medical GmbH, Minneapolis, USA	MX9606	Hemodynamic monitor

Logicath 7 Fr 3-lumen 30cm	Smith- Medical GmbH, Minneapolis, USA	MXA233x30x70-E	Vascular access/Drug administration
Masimo Radical 7	Masimo Corporation, Irvine, USA	-	Hemodynamic monitor
Mask for ventilating dogs	Henry Schein, Melville, USA	730-246	Ventilation
Original Perfusor syringe 50ml Luer Lock	B.Braun Melsungen AG, Melsungen, Germany	8728810F	Drug administration
PICCO Thermodilution. F5/20CM EW	MAQUET Cardiovascular GmbH, Rastatt, Germany	PV2015L20-A	Hemodynamic monitor
Percutaneous sheath introducer set 8,5 und 9 Fr, 10 cm with integral haemostasis valve/sideport	Arrow international inc., Reading, USA	AK-07903	Vascular access/Shock induction
Perfusor FM Braun	B.Braun Melsungen AG, Melsungen, Germany	8713820	Drug administration
Potassium chloride	Fresenius, Kabi GmbH, Bad Homburg, Germany	6178549	Euthanasia
Propofol 2%	Fresenius, Kabi GmbH, Bad Homburg, Germany	AN01AX10*	Anesthesia
Pulse Contour Cardiac Output ($PiCCO_2$)	Pulsion Medical Systems, Feldkirchen, Germany	-	Hemodynamic monitor
Sonosite Micromaxx Ultrasoundsystem	Fujifilm, Sonosite Bothell, Bothell, USA	-	Vascular access
Stainless Macintosh Size 4	Teleflex Medical Sdn. Bhd, Perak, Malaysia	670000	Intubation
Sterofundin	B.Braun Melsungen AG, Melsungen, Germany	AB05BB01*	balanced electrolyte infusion
Stresnil 40mg/ml	Lilly Germany GmbH, Wiesbaden, Germany	QN05AD90	Sedation
Syringe 10 mL	Becton Dickinson S.A. Carretera Mequinenza Fraga, Spain	309110	Drug administration

Syringe 2 mL	Becton Dickinson S.A. Carretera Mequinenza Fraga, Spain	300928	Drug administration
Syringe 20 mL	Becton Dickinson S.A. Carretera Mequinenza Fraga, Spain	300296	Drug administration
Syringe 5 mL	Becton Dickinson S.A. Carretera Mequinenza Fraga, Spain	309050	Drug administration
venous catheter 22G	B.Braun Melsungen AG, Melsungen, Germany	4269110S-01	Vascular access

***ATC: Anatomical Therapeutic Chemical / Defined Daily Dose Classification**

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Author(s): Alexander Ziebart1, Jens Kamuf1, Robert Ruemmler1, René Rissel1, Moritz Gosling1, Andreas Garcia-Bardon1, Erik K. Hartmann1

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Article Title:	Standardized hemorrhagic shock induction in pigs guided by cerebral oximetry and extended hemodynamic monitoring
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Date:	30.10.2018

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Dear Reviewers,

thank you for the constructive comments to the present manuscript / video protocol.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was proofread again and all remaining spelling and grammar issues were corrected.

2. Abstract: Please also include an overview of the advantages and limitations of the method.

An overview of the advantages and limitations of the methods was included.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

SI abbreviations were used in the manuscript.

4. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

A space was included between all numerical values and their units.

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns were removed.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Pulsion Medical Systems, INVOS™, Medtronic PLC, Somanetics Corporation, etc.

All contents of commercial language are removed from the manuscript.

7. 1.6, 2.3: How to disinfect the skin/inguinal area? Please specify.

The exact disinfection procedure of the skin and inguinal area was specified in the manuscript and the used products were added to the table of materials.

8. *1.3 and 1.8: Please mention how proper anesthetization is confirmed*

General anaesthesia was confirmed by an experienced clinical anaesthetist using a combination of clinical (i.e. lack of spontaneous breathing efforts, movement, reflexes or response to handling or invasive stimuli like vascular access) and monitoring (hemodynamics, lack of vegetative stress reaction) parameters.

9. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

With combination and summarization of shorter steps, protocol was truncated.

10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

13. Figures 1, 2, and 4: Please change "Stabilisation" to "Stabilization" and change "ml" to "mL". Please include a space between all numerical values and their corresponding units (35 mL, 0.25 h).

All figures were changed into the proper formatting.

14. Figures 2 and 4: Please label and describe each panel in the figure legend.

The figure legend was revised and each panel of the figures was described.

15. Figure 3: Please include a title for this figure in the figure legend.

A title for figure 3 was included in the figure legend.

16. Table of Materials: Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.

All trademark and registered signs were removed.

17. References: Please do not abbreviate journal titles.

Reviewer #1:

Major Concerns:

None

Minor Concerns:

1.

Line 72

"… via thermodilution and pulse contour analysis…" - The device used is based on calibrated pulse contour analysis technology. Calibration is undertaken by transpulmonary thermodilution. This should be described clearly.

Thank you very much for this hint. The technical description and the functionality of thermodilution and pulse contour analysis was added.

2.

Line 355

Cardiac index is definitely not a preload parameter. The technology used provides ΔPP (also known as PPV) and SVV, well-established clinical parameters to assess cardiac preload. Were these parameters monitored throughout the study? If so, showing the results e.g. in Figure 2 would be interesting.

Thank you for this comment, the author agrees that the cardiac index is no preload parameter. The passage was changed. SVV was measured in this study as well as intrathoracic blood volume index and systemic vascular resistance. These parameters are displayed in the new figure 3.

Line 415-447 (Hemorrhagic shock induction)

This part of the methodology remains somehow unclear. Presumably, the parameters CI, crSO2, and MAP were used to titrate blood removal (line 426). Was blood removed until all of these three parameters declined below 40% of baseline? Was blood removal terminated when one of these parameters declined below 40% of baseline? Why had a level of 40% of baseline been chosen? How did the amount of removed blood (in absolute numbers) correspond to the weight of the animals?

As blood removal is the crucial intervention in the study, and all results depend on a precise approach of almost identical amount of blood withdrawal in relation to blood volume for all animals, the authors are encouraged to describe their methodology in this section in more detail.

Thank you for this suggestion: the manuscript was edited to explain this important step more detailed. Hemorrhagic shock is achieved, if cardiac index AND mean arterial pressure drop below 40% of the baseline values. A significant drop of the cerebral oxygenation of 20% is preferable though not mandatory. The average blood loss to achieve this lies within a range of 25-35 mL kg^{-1}.

It is the authors experience that aiming for even lower parameters or larger blood loss causes high dropout rates through immediate cardio-circulatory failure. In one of our present studies that made use of this model only 3 of 9 animals from the untreated control group survived longer than two hours (Ziebart et al. SJTREM 2018). On the other hand less blood loss may not lead to hemodynamic

instability that affects cerebral regional oxygenation leading to experimental failure. The amount of removed blood is adapted to the animals´ body weight, which corresponds with the total blood volume.

4.

Line 515

Reference 14 describes a sepsis model. Results from sepsis models are not necessarily transferable to hemorrhage models.

Thank you for this hint, the error was corrected.

5.

Figure 3

Presumably, the figure shows an example of measurements in one animal - This should be pointed out in the figure legend.

We clarified that figure 4 depicts one representative animal as example.

6.

Table of materials

Stresnil® is azaperone (as described correctly in line 110). Atracurium is used for neuromuscular blockade, fentanyl is used for analgesia, propofol is used for induction of anesthesia/hypnosis.

Thank you very much, the error was corrected.

7.

The discussion is to be focused on specific aspects of the present study. E.g.: It is not necessary to discuss aspects of surgical intervention to induce hemorrhagic shock (lines 537-8), as this technique was not used.

Thank you very much for this suggestion. We removed the mentioned aspects.

8.

Thorough proofreading, perhaps by a native English speaker, is encouraged. E.g.: line 430 remains a mystery.

The sentence in line 430 was rephrased and the manuscript proofread.

Reviewer #2:

1. The dose of the continuously infused fentanyl seems very high (100-200 mikrog/kg/h). In this reviewer's experience it is 10-50 times the needed dose. Please compare with existing publications in pigs.

Thank you very much for this hint. This was a typing error. The permanent infusing dose of quantity was 0.1-0.2 **µg** kg^{-1} h^{-1})

2. Glucose infusion in addition to the balanced electrolyte solution seems not to be given. Do the authors believe that a continuous infusion of glucose (e.g. 5%, 1 ml/kg/h) is necessary in a longer protocol?

Thank you for this comment: the authors agree that glucose infusion can be necessary in the case of hypoglycaemia and long-term experiments. Our experience with various porcine models shows that glucose administration is not necessary in most scenarios. Increased need of glucose administration can be necessary particularly in porcine sepsis models. Furthermore, the applied blood gas analyser automatically provides blood glucose levels with every measurement, which prevents unrecognized episodes of hypoglycaemia.

3. Muscle relaxants may facilitate endotracheal intubation but depending on the drugs used for induction of anaesthesia it may not be necessary. Since it is more difficult to evaluate anaesthetic depth while using muscle relaxants it may be problematic.

Thank you for this note. The authors agree that muscle relaxants are not absolute necessary. However, endotracheal intubation without muscle relaxation is a risk factor for complications and especially intubation failure. For this reason, adequate muscle relaxation is an internationally accepted standard for endotracheal intubation in clinical anaesthesia. Furthermore, depending on the surgical procedure muscle relaxation needs to be maintained during the whole operation.

Our approach is to facilitate endotracheal intubation by only one single shot that lasts about 30 minutes for safety reasons. Depth of anaesthesia can be maintained and monitored by a wide array of clinical and technical parameters, and trained and experienced personal has to be able to maintain adequate anaesthesia and closely monitor the animals.

4. Spontanous breathing can be kept by using another induction regimen with no opioid.

Thank you for this hint. The author agrees that spontaneous breathing can be kept by using another anaesthetic regime, i.e. by using ketamine instead of an opioid (i.e. Ziebart et al. Respir Res 2014), if this is part of the experimental approach. In the present model, however, there is no reason to waive the use of opioids. From a clinical perspective, which this model aims to mimic, one would not aim for spontaneous breathing in an intubated patient suffering from life-threatening shock to optimize pulmonary oxygen supply. Opioids are an indispensable part of general anaesthesia, cost effective, and provide sufficient analgesia and stress reduction. In this setting spontaneous breathing was undesirable to secure stable level of $paCo_2$. Different level of $paCo_2$ can affect cerebral perfusion

5. Prone or supine position during intubation? Probably supine since the authors state that epiglottis is lifted upwards, but it should be stated.

The supine position offers the advantage that decent overstretching of the head opens the airway sufficiently. Additionally, the animals have to place in supine position for instrumentation and vascular access anyway.

6. Two persons are not needed for intubation of the pig according to this reviewer's experience.

Thank you for this hint. The author agrees that intubation with only one person is possible for experienced researchers, but it is much more elegant to perform this procedure with an assisting person to minimize the risk of intubation failure.

7. In this reviewer's experience a gastric tube is normally not necessary since the pigs normally do not vomit or have reflux.

Thank you for this hint. We routinely apply a gastric tube to minimize the danger of microaspiration especially in models focussing on pulmonary pathophysiology like ARDS.

8. Is 15 min of recovery enough for stable baseline values? This reviewer believes a longer recovery period (30-60 min) is advantageous.

Thank you for this hint the recovery period was to 30 min.

9. Using PiCCO2-system for cardiac output measurements is not golden standard. This issue must be discussed. Perhaps this also explains the large variation in cardiac index at the 0.5 h time point.

The author agrees that the PiCCO2-system is not the only standard system to evaluates cardiac index in clinical practise. The pulmonary artery catheter and the transthoracic echocardiography are well established devices for cardiac output assessment. Especially the last technique gets even more popular in the last years, but it needs a high skill level for adequate performance. The PiCCO2-system is often used especially on critical care units. Utilization is learned quickly and it is less invasive than pulmonary artery catheter. The disadvantage of this technique is the need for repetitive calibration, especially in hemodynamic instable situations.

10. The formula for calculating body surface area in pigs is not similar in humans and pigs, using the in-built formula in the PiCCO2-system may introduce a systematic error.

Thank you for this hint. We have included this into the limitations of the protocol.

11. Large-bore introducer may completely occlude the femoral arteries, which may affect the thermodilution calibration of the PiCCO2-system.

Thank you for this hint, the author agrees that theoretically the introducer may occlude the femoral arteria, but no signs of ischaemia were ever detected into the legs. The length of the PiCCO2-catheter is 20cm. That means that the tip of the probe lies in the iliac artery or distal aorta. The diameter of these vessels is much greater than 1.667 mm of the probe. Additionally, the pulse curve is displayed into the system and the guide-wire is visualized in ultrasound guided techniques, so that dislocations and dissections can be excluded.

12. The withdrawn blood may be collected in citrated bags for later autotransfusion. This is one of the major advantages of using controlled blood withdrawal for induction of haemorrhagic shock.

We fully agree that the collection of the blood for later autotransfusion is one great advantage of controlled blood withdrawal. Here, we describe the principles of this model. Depending on the experimental questions that leads to the use of this model, re-transfusion can be implemented in the protocol and we have already performed this alternative method in other experimental settings as well.

13. Did the authors check body temperature and a heating system for maintenance of normal body temperature? This is very important.

Body temperature is controlled permanently with the PiCCO-catheter. In case of hypothermia a heating blanket system was used to stabilize the temperature on physiological level.

14. How was arterial blood analyzed?

We used a commercially available blood gas analyzer that is validated for porcine blood.

15. In haemorrhagic shock models some researchers remove spleen or precontract it with external adrenaline to avoid autotransfusion. According to this reviewer it is not necessary in piglets due to the small volume of the spleen, but this aspect may be included in the discussion.

Thank you for this very controversial question. Studies argue that after shock inducing the autotransfusion mechanism increase the hematocrit and the blood volume. On the other side oxidative stress, pain and sympathetic stimulation of the spleen is associated with autotransfusion reactions by itself and for this reason, splenectomy is not recommended. Hence, we did not perform splenectomy in this protocol to avoid a two-hit model that takes influence on the study results.

16. It is not clear to this reviewer the advantage of using cerebral oximetry for guidance of the shock induction.

Thank you for this request. We clarified this issue: hemorrhagic shock orientated on mean arterial pressure and cardiac index. This drop down of about 40% of both parameters is necessary to achieve significant hemorrhagic shock that affects the cerebral regional oxygenation. The use of the cerebral regional oxygenation as complementary parameter makes sense, because the coherence of macro- and microcirculation is not linear.

17. Although this reviewer finds the described technique of catheter insertion elegant, insertion of femoral introducers on dissected and exposed vessels not necessarily cause extensive tissue damage and blood loss if using a correct surgical technique.

Our research group has fully shifted from the surgical approach to minimal-invasive seldinger technique some years ago. The use of Seldinger-technique and ultrasound is a common procedure and state-of-the-art in clinical practise. The authors are convinced that this technique is less time-consuming and invasive. Furthermore stress, need for analgesics, tissue damage and inflammatory response may be mitigated.

18. Parametric statistical tests are used, but data seem to be presented with median and interquartile range (?).

We again checked all figures and presented data to assure that all data are presented with mean and standard deviation.

19. The discussion needs to be revised according to the raised concerns.

We modified the discussion according to the raised concerns as highlighted in the revised version.

20. Figure legends need to be improved.

- The figure legends are revised.

Reviewer #3:

Major Concerns:

Limitations and conclusions should be added

Thank you for this hint. We added a conclusion section at the end. We modified the discussion according to the raised concerns and discuss limitations of the model as highlighted in the revised version.

Reviewer #4:

Major Concerns:

It would be good to provide a bit more in-depth information on the recordings and correlation validity data for the NIR spectroscopy recordings and how they correlate with systemic cardiovascular impairment (MABP) and further assessment of model severity (e.g. lactates, BD values, PO2, PCO2) which are generally used in these models. It would also be very valuable to have further reflection on the reproducibility of the model and the relevance for transparent and detailed reporting of the methodology. It would also be an important reminder to include some notes on the feasibility and challenges for implementing the ARRIVE guidelines in these complex ICU models. Finally, this should also be linked to the need for large animals' models in the context of face validity model and/or its benefits for construct validity (mechanisms) and the importance of choosing the right model with most suitable outcomes for the predictability value of these studies. It is crucial to understand the translational validity provided by the proposed IR brain imaging approach.

Thank you for these hints. The author reworks the section of the NIRS-system and included studies that compare the values of regional cerebral oxygenation with heamodynamic parameters like lactate and BD values. Additionally, the manuscript explains the advantages of a pig model especially in the concordance of the ARRIVE guidelines.

Minor Concerns:

Lines 64-66: "The use of animal models is indispensable to establish novel or experimental strategies. The comparable anatomy and physiology of pigs and humans allow conclusions on special pathological effects and enables the evaluation of new therapy concepts."

Authors should provide more detail on why the pig model may be of benefit compared to other preclinical models; also if it also addressing mechanistic (construct validity) approaches, why other models would not be used?

Thank you for this question. The use of animal models is indispensable to establish novel or experimental strategies. The similar anatomy, homology and physiology of pigs and humans allow conclusions on specific pathological effects. Both species have similar metabolic systems and the pharmacological effects are comparable that can be transferable into humans and enables the evaluation of new therapy concepts. This is a great advantage in comparison for example to rodent models were the blood volume and overall hemodynamic response differs significantly. Furthermore, medical equipment and products can be easily used in porcine models. All devices, consumables and pharmaceutics are clinically authorized for human application. Additionally, it is easily possible to obtain pigs form the commercial economy, which allows a high diversity of genetics and phenotypes and allows cost reducing.

Lines 74-75: Near-infrared spectroscopy is a clinically and experimentally established method to monitor changes in cerebral oxygen supply.

Feasibility of this imaging approach will also depend on the penetration range and the ability to image across the bony skull-which is remarkably think on the pig (and its age)

Thank you for this hint, we edit the near-infrared spectroscopy passage for more technical details.

Line 97: 2The experiments were conducted in accordance with the ARRIVE guidelines."

Reviewer would like to see more detailed information on this; e.g. planning of study following appropriate revision of literature, selection of suitable model, full information on animal supplier, strain, housing/feeding regimes, acclimatization period.

Thank you for this hint: The experiments were conducted in accordance with the ARRIVE guidelines. The study was planned and conducted between November 2015 and March 2016 after extended literature research and the choice of pig model, a well-established model for hemorrhagic shock. Seven anesthetized male pigs (sus scrofa domestica) with a mean weight of 28 ± 2 kg and an age of two to three months were included in the protocol. The animals are cared for and delivered to the laboratory by a local breeder recommended by the State and Institutional Animal Care Committee for his high hygienic standards. The animals maintain in their known environment as long as possible to minimize stress. Six hours before experiment was scheduled food but water was not denied to reduce risk of aspiration. Hence, none of the members of our group is involved in breeding or housing conditions, which all comply with the German laws on animal welfare.

Line 104: 1.1. Maintain the animals in their known environment as long as possible to minimize stress.

Please provide further detail on their housing conditions

Please see above.

Line 110 1.3. Sedate pigs with a combined injection of Ketamine (4 mg kg-1) and Azaperone.

Please provide supplier information for Azaperone

The information is added the table of materials

Line 116 1.4. Transport the sedated animals to laboratory.

Would be good to have an idea of travelling time/distance

The animals are transported by the breeder in a special transport box for animals. The drive to our laboratory lasts about 20 minutes.

Line 118 1.5. Monitor the peripheral oxygen saturation (SpO2) with

Using which device? Provide more detail please

We use the Masimo Radical 7 (Masimo Corporation, Irvine, USA) for the peripheral oxygen saturation. The probe is placed at the animals' tail.

Line 138 1.12. Start continuous infusion of balanced electrolyte solution (5 mlkg-1 h-1).

Provide further detail on supplier please

Further details of the supplier are added.

Line 216 2.4. Fill all catheters with saline solutions.

Sterile and warm saline solution (provide supplier)

The catheters are filled with the mentioned balanced electrolyte solution with room temperature. The supplier information is added to the table of materials.

Lines 232-234: NOTE: In our opinion, ultrasound guided Seldinger technique is associated with significantly lower blood loss and tissue trauma than other methods of vascular access

Maybe add REF if available

We added a reference.

Lines 270-271: Connect the right arterial introducer sheath and the central venous catheter with two transducer systems for measurement of invasive hemodynamics

Please provide further details on transducer and system used as this is crucial information.

The used system is the logical pressure monitoring system of Smith- Medical GmbH, Minneapolis, USA.

Lines 275-276: Switch the tree-way-stopcocks of both transducer open to the atmosphere to calibrate the system to zero.

Would this be better than using a manometer to record two known levels of pressure that can then be validated as standards?

The operation instructions of the manufacturer describe this calibration method.

Lines 442- 443: 5.13. Repeat blood withdrawal until target blood pressure and cardiac index is reached

What is the targeted blood pressure?

Hemorrhagic shock was achieved if cardiac index and mean arterial pressure drops down under 40% of the baseline values. A significant drop down of the cerebral microcirculation of 20% is aspired. The average amount of blood volume corresponds with 25-35 mL kg^{-1}.

517 Line: The transfer of the technique into other species appears uncomplicated.

Different species will posed different challenges!

Thank you for this important aspect that is added to the manuscript.

Lines 567-568: The experimental setting of the pig model requires a fundamental expertise in intensive care medicine, application of anaesthesia and large animal research.

This is very important point and highlights the relevance of need of a multidisciplinary team for running these pig models, including staff well trained in animal physiology and veterinary care.

We fully agree. This point is extremely relevant and will be highlighted as key aspect of the video.

Reviewer #5:

37 - 39: how do you reconcile that circulatory shock does not invariably lead to organ failure, and that the organ failures are not necessarily irreversible.

Thank you for this note. We agree that hemorrhagic shock does not lead to invariably to orange failure and that the organ failures are not necessarily irreversible. Different compensation mechanisms exist that depended on the individual status (i.e. comorbidities), but also handling, duration and treatment of the circulatory shock. Independently of the ethology, if the cause of shock is not treated or in case of acute blood loss the bleeding is not controlled cardio-circulatory failure, organ failure and death is inevitably. On the other hand, the presented model is not designed to assess long-term recovery from shock-related organ failure.

39: the brain is at risk "of what?", please specify

The brain is particularly at high risk of severe irreversible damage due to high oxygen consumption and limited ischemia tolerance.

50: it is confusing to read collapse, when previously you introduced the terms circulatory shock, circulatory failure or loss of circulatory volume. Please limit the variability of the terms used.

Thank you for this hint. We changed to manuscript accordingly.

50: is "can cause" irreversible organ failure rather than "it causes"

Thank you for this note, the author edited the manuscript in the suggested way.

52: what compensatory mechanisms do you want to refer to? You were speaking of coagulopathy etc., but I would rather think you are referring to compensation for the lack of oxygen and the microcirculatory failure resulting from severe blood losses.? Please specify.

The extended blood loss does not affect the oxygen supply exclusively. It additional takes influence on many physiological parameters like e.g. the acid-base balance, the coagulation and the temperature. All this parameters of the patients have to recover to avoid hazardous complications.

58*: physiological levels of what? Please be more specific and to the point.*

The manuscript is specified and the term “physiological conditions “replace “level”.

77: in this context it is not at all clear why you refer to delirium in this context. It also remains obscure what kind of actions would be used to prevent delirium associated with hemorrhagic shock.

Thank you for this question. Near-infrared spectroscopy is a clinically and experimentally established method to monitor changes in cerebral oxygen supply. A decrease of the NIRS level under 20% of the baseline is e.g. associated with delirium. This section describes the NIRS in general and does not only focus the use during haemorrhagic shock.

81: you may want to consider a more detailed description of the mechanisms of cerebral oximetry, what exactly is being measured, and why do you think this represents cerebral microcirculation (of what brain areas? Generalizability? Caveats? Limitations? Advatages?)

We author edit this section and descripted the mechanism of cerebral oximetry more detailed.

69: traditional hemorrhagic shock models focus on volume target (of whithdrawn blood) or pressure targets (of the resulting arterial pressure). Please specify in what way your protocol differs from this strategy and why the protocol is/may be a better approach. When targeting a specific cardiac output, how do you reconcile that pulse contour cardiac output results drift significantly with rapidly changing blood volumes?

Thank you for this question. Our model focuses on a combination of different targets: a significant decrease of the mean arterial pressure and the cardiac index. This ensures adequate hemorrhagic shock and reduces the possibility of distortion. Protocols focused only on one aspect can be influenced by different initial volume status or compensation mechanisms. The well-known drift of continuous cardiac output method, which is described after significant hemodynamic changes, is equalised by periodic recalibration.

118: please advise the optimal time point when to initiate monitoring in a sedated pig

Non-invasive monitoring (pulseoximetry) is initiated directly after the arrival of the animal.

126-130: please advise on the optimal course of actions: from induction, to supine, to non-invasive ventilation: what if propofol shuts down spontaneous ventilation? What are the pros and cons for non-invasive ventilation in the prone position. The "dog" mask, is not necessarily a dog mask.

Thank you for this hint. After positioning the sedated pigs in prone position, anaesthesia is induced and non-invasive ventilation is started. This is followed by endotracheal intubation. Accordingly this procedure follows the clinical standard of inducing general anaesthesia in humans. From a clinical perspective, which this model aims to mimic, one would not aim for spontaneous breathing in an intubated patient suffering from life-threatening shock to optimize pulmonary oxygen supply. Furthermore, cerebral blood flow and perfusion is influenced by different CO2 level requiring constant ventilation or changes in intrathoracic pressure. The authors have no experience with

another kind of mask for non-invasive ventilation in pigs, but it is possible that the mask is invented for all animals with long snouts.

152: what do you mean by "lift up" epiglottis? With the use of the blade directly? Or via lifting up the tongue with the laryngoscope? Is a Miller blade a useful alternative?

Thank you for this question. The tongue is pulled out and down by one person. The laryngoscope has to be placed in front of the epiglottis and not directly on it. We use both laryngoscope blades, but have the best experience with the Macintosh blade.

166-168: do you need both strategies? Why?

The blocking of the cuff is much faster with a syringe, but in the most cases the cuff pressure is too high after this procedure. With the extra step the procedure is more secure and complications are limited.

174: by observation (!) of regular and periodic exhalation…

Thank you for this hint. After intubation the animal is connected with the ventilator. The ventilator works irrespective if the tube is placed correctly or not. Measurement of expiratory CO_2 is the method to secure correct tube location.

236: I guess "striven" is not the intended wording

The error was corrected.

266: what is the reason you chose to puncture the arteries first, and the veins. In case of malposition or failure to puncture the artery this causes a lot more problems when subcutaneous hematoma is present during the subsequent venous puncture attempts. Please comment.

From our experience sonography often shows that the femoral vein lies quite below and not strict medial of the artery: femoral vein puncture might bear the risk to puncture of through the artery. Hence, we decided to puncture the (easier accessible) artery first to secure the access by the guide wire for exactly the reason you noted.

306: I doubt that water is being injected intravenously!

Thank you for this question. The pulse contour cardiac output measures the time and the amount of an intravenous injected cold saline solution bolus after heart passage at an arterial probe to calculate e.g. cardiac output.

428: what is the rational for naming this event "93 blood loss!?

The NIRS system has a database of prescribed events. There exists no event “shock initiation” so the author chose “blood loss” instead.

430: please revise and rephrase

The sentence is revised and rephrased

465: does the pic possess specific compensatory mechanisms for acute blood loss? Can additional blood volume be recruited form the spleen into the systemic circulation?

Thank you raising up this very controversial question. Studies argue that after shock inducing the autotransfusion mechanism increases the hematocrit and the blood volume. On the other side oxidative stress, pain and sympathetic stimulation of the spleen is associated with autotransfusion reactions by itself and for this reason splenectomy is not recommended. For this reason, splenectomy was not performed in this protocol to avoid a two-hit model that takes influence on the study results.

517: what other species do you think of? I doubt it can be easily transferred to a rabbit or rat or mouse, or fish, or bird. You may want to name the species that are relevant and actually transferrable.

Thank you very much for this question (We were quite amused about it!). The authors agree that the technique is not transferable into fish, birds or maybe dinosaurs (last one because they vanished). The study is designed for medium size mammals and it is imaginable that the protocol can be used in sheep, goats or dogs as well. Elephants, Rhinos or Wales are too big for this study; especially their blood volume would be a great challenge. Tigers, Lions and Bears would be challenging in another way.

528: I do not see the connection between the epiglottis sticking to the palate and the use of neuromuscular blocking agents

Thank you for this hint. Intubation of a pig is a challenging task. Especially for physicians that are used to intubate humans the differences can be confusing. For this reason application of relaxations is very useful and avoid dangerous complications like laryngospasm.

531-533: I am sorry, this description is rather vague and does not help the reader anticipate relevant problems during shock induction. Please consider rewriting to include more specific advise for the readers.

We rewrote this section to help the reader to identify pitfalls and avoidable mistakes.

535: what does "hemodynamic level" refer to?

- Thank you for this question. Different protocols vary in terms of aspired cardiac index and mean arterial pressure.

541: I disagree that NIRS can be referred to as an "extended" cerebral monitoring because it is literally the ONLY cerebral monitoring that has been described in you experimental setting. In this respect, it is "a" and "the" cerebral monitoring in this setting, and it is not in any way more extended than any other method that you are NOT using in your setting.

We agree and delete the word “extended” from this section.

549: manipulating an oximetry sensor with scissors is a tough advise for readers, and I doubt this is a recommendation that can be printed and called a standard. Regulatory affairs and warranty issues make this a statement that should absolutely removed from the manuscript at all cost!

- Thank you for this hint. This sentence is removed from the manuscript.

567-571: These conclusions have very little grounds, and I doubt that it is of any help for the reader to claim that this model allows for direct translation to the bedside. In fact, I completely disagree because any translation depends on the specific results and therapeutic interventions that are being

studied. The mere methodology, which admittedly is relatively similar to a patient ICU setting, does not confer scientific translation per se. Rather that making this conclusion which are too far fetched, you may want to discuss the scalability of you experimental setting, the future advance that are possible, and the possible advantages over other previously published hemorrhagic shock models in large mammals.

We revised the conclusion section and agrees that translation of these specific results onto the bedside is limited.

Figure 1: a withdrawal volume of 30-35 ml/kg is mentioned in the figure. In the text, you mention 28 ml/kg???

Thank you for this hint. This error is corrected.

Figure 2: 6th inlet: what is endvascular volume???

Thank you again. The spelling error is corrected. author.

Figure 3: what is the purpose of the screenshot?

This figure shows the typical course of a hemorrhagic shock, when measured by like it will be displayed into the video.