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A reproducible intensive care unit-oriented endotoxin model in rats

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Title:
A reproducible intensive care unit-oriented endotoxin model in rats

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Summary:
Here, we present a reproducible intensive care unit-oriented endotoxin model in rats.

Abstract:
Sepsis and septic shock remain the leading cause of death in intensive care units. Despite significant improvements in sepsis management, mortality still ranges between 20 and 30%. Novel treatment approaches in order to reduce sepsis-related multiorgan failure and death are urgently needed. Robust animal models allow for one or multiple treatment approaches as well as for testing their effect on physiological and molecular parameters. In this article, a simple animal model is presented.

First, general anesthesia is induced in animals either with the use of volatile or by intraperitoneal anesthesia. After placement of an intravenous catheter (tail vein), tracheostomy, and insertion of an intraarterial catheter (tail artery), mechanical ventilation is started. Baseline values of mean arterial blood pressure, arterial blood oxygen saturation, and heart rate are recorded.

The injection of lipopolysaccharides (1 milligram/kilogram body weight) dissolved in phosphate-buffered saline induces a strong and reproducible inflammatory response via the toll-like receptor 4. Fluid corrections as well as the application of norepinephrine are performed based on well-established protocols.

The animal model presented in this article is easy to learn and strongly oriented towards clinical sepsis treatment in an intensive care unit with sedation, mechanical ventilation, continuous blood pressure monitoring and repetitive blood sampling. Also, the model is reliable, allowing for reproducible data with a limited number of animals in accordance with the 3R (reduce, replace, refine) principles of animal research. While animal experiments in sepsis research cannot easily replaced, repetitive measurements allow for a reduction of animals and keeping septic animals anesthetized diminishes suffering.

Introduction:
Sepsis and its more severe form, septic shock, are syndromes on the ground of an infection, resulting in an overshooting inflammatory reaction with the release of cytokines, leading to physiological and biochemical changes with a suppressed immune defense and fatal results[1,2].

This unbalanced inflammatory reaction results in organ dysfunction and organ failure in various vital organs such as lung, kidney and liver. With 37%[3], sepsis is one of the most common reasons for a patient to be admitted to an intensive care unit (ICU). Mortality of sepsis currently ranges around 20-30%[4]. Early and effective antibiotic treatment is of utmost importance[5]. Fluid and vasopressor resuscitation need to be installed early, other than that, treatment is purely supportive[6].

Sepsis is defined as a proven or suspected infection with bacteria, fungi, viruses, or parasites, which is accompanied by organ dysfunction. Septic shock criteria are met when a further cardiovascular collapse irresponsive to fluid treatment alone, and a lactate level of more than 2 millimole/liter is present[2]. Sepsis related organ failure may occur in any organ, but is very common in the cardiovascular system, the brain, the kidney, the liver, and the lung. Most patients suffering from sepsis require endotracheal intubation to secure the patient's airway, to protect from aspiration, and to apply positive end expiratory ventilation with a high fraction of inspired oxygen to prevent or overcome hypoxia. In order to tolerate a tracheal tube and mechanical ventilation, patients usually require sedation.

Endotoxins, such as lipopolysaccharides (LPS) as a component of the membrane of gram negative bacteria induce a strong inflammatory reaction via the toll-like receptor (TLR) 4[7]. Activation of a defined pathway ensures a stable inflammatory reaction. Cytokines like cytokine induced neutrophil chemoattractant protein 1 (CINC-1), monocyte chemoattractant protein 1 (MCP-), and interleukin 6 (IL-6) are known as prognostic factors for severity and outcome in this model[8]. Intravenous LPS application has been successfully used to study various aspects of sepsis in rats[8,9].

Treatment of sepsis is still a challenge, particularly due to the lack of predictive animal models. If endotoxemia with activation of systemic inflammation is an adequate model for the development of pharmacological therapies is debatable. However, with the well-known LPS-induced TLR 4 pathway important knowledge can be gained.

Protocol:

All experiments presented in this protocol were approved by the Veterinary Authorities of the Canton Zurich, Switzerland (approval numbers 134/2014 and ZH088/19). Moreover, all steps performed in this experiment were in accordance with the Guidelines on Experiments with Animals by the Swiss Academy of Medial Sciences (SAMS) and Guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

1. Anesthesia induction and animal monitoring

1.1. Keep male Wistar rats with a weight of 250-300 gram (g) in ventilated cages under pathogen-free conditions. Provide a 12-12-hour light/dark cycle at an ambient temperature of 22 $\pm$ 1 °C, and free access to food and water.

1.2. Induce general anesthesia either by volatile induction with isoflurane (concentration of 3-5%) in an anesthesia induction box for 30 seconds (**Figure 1A**) or alternatively, induce

anesthesia with a single-shot injection of ketamine/xylazine (10/1 milligram (mg) per 100 g body weight).

1.3. Transfer the animal to a working place and lay the animal on a heating-mat throughout the entire experiment. Keep the body-temperature between 36.5 and 37 °C.

1.4. Use a nosecone to provide oxygen (600 mL/minute). Add isoflurane 2-3% if volatile anesthesia was chosen for anesthesia maintenance. Make sure that the animal is spontaneously breathing.

1.5. Confirm the level of anesthesia by the absent of the toe-pinch reflex prior to the installation of tracheostomy and arterial and venous catheters.

1.6. Verify a sufficient oxygenation by peripheral oxygen saturation monitoring (normal oxygen saturation 98 – 100%).

1.7. Use an ointment (Vitamin A ointment) to protect the eyes.

1.8. Prepare sterile surgical instruments and catheters on a side table as displayed in **Figure 1B**.

1.9. Additionally, prepare pressure and oxygen saturation monitoring as displayed in **Figure 1C**.

2. Intravenous access

2.1. Apply a tourniquet at the rat's proximal tail to facilitate venous access (**Figure 2A**).

2.2. Disinfect the tail 3 times with alcohol.

2.3. Induce a G26 intravenous catheter into one of the two lateral tail veins.

NOTE: From our experience it is easier to place the intravenous access at the distal part of the rat's tail, because the vein here is located closer to the skin. In addition, in case of a failed cannulation, there is enough space to move proximally.

2.4. Strictly avoid air injection.

2.5. Untie the tourniquet after placing the intravenous catheter.

2.6. Fix the intravenous catheter in place with adhesive tape (**Figure 2B**).

2.7. Connect syringe-pumps to the intravenous access for continuous fluid and drug application.

2.8. Use 3-way stopcocks for bolus fluid, drug application, and venous blood sampling.

3. Tracheostomy

3.1. Shave the animal's anterior neck-area.

3.2. Disinfect the shaved skin 3 times with providone-iodine solution.

3.3. Perform a circa 2 cm longitudinal incision using a scalpel (with a blade number 10).

3.4. Retract the skin with 2-0 silk sutures.

3.5. Bluntly prepare the larynx and the trachea with surgical scissors (**Figure 3A**).

3.6. Make sure to open the trachea with surgical microscissors at the 3-5th tracheal clasp.

3.7. Insert a sterile tracheal cannula into the trachea. Be careful, do not insert the cannula too deeply in order to avoid unilateral ventilation.

3.8. Fix the cannula in place using a 2-0 silk suture.

3.9. Connect the cannula to a ventilator for pressure or volume-controlled ventilation (**Figure 3B**).

4. Arterial access

4.1. Disinfect the rat tail 3 times with povidone-iodine solution.

4.2. Cut the skin using a scalpel (with a blade number 10) circa 1 cm longitudinally at the ventral side

4.3. Take care, do not cut too deeply to avoid an injury of the tail artery.

4.4. Use a surgical microscope to expose the artery carefully. Cut the fascia surrounding the artery with surgical micro scissors.

4.5. Ligate the distal part of the artery using a 6-0 silk suture.

4.6. Prepare a proximal 6-0 silk suture but do not tighten the silk (**Figure 4A**).

4.7. Introduce a G-26 catheter into the artery between the distal and proximal silk suture.

4.8. Once the catheter is in the artery, tighten the proximal silk suture and fix the catheter in place (**Figure 4B**).

4.9. Connect the catheter to a pressure transducer to provide continuous arterial pressure measurement (normal mean arterial pressure: 60 - 100 mmHg) (**Figure 4C**).

4.10. Additionally, place a 3-way stopcock between the catheter connected to the pressure transducer and the G-26 catheter for arterial blood sampling.

5. Baseline measurement, sepsis induction and follow-up measurements

5.1. After the animal reached a steady state, inject the LPS.

5.2. Collect blood samples when a steady state is reached (usually after 15-30 minutes).

5.3. Replace fluid loss from blood samples by Ringer's solution in a ratio of 1:4.

5.4. To induce sepsis, inject the LPS as a bolus or as a continuous LPS application.

5.5. For the bolus application, inject 1 mg of LPS/kilogram body weight (kg) dissolved in phosphate buffered saline (PBS) at a concentration of 1 mg/mL.

5.6. For continuous application, inject 300 µg of LPS/kg/hour throughout the entire experiment using a syringe pump (stock solution of LPS: 1 mg/mL in PBS).

5.7. Avoid air-injection at all times in order to prevent air embolism.

5.8. Define fluid replacement protocols, vasoconstrictor application protocols, and abortion criteria (for example hypotension defined as a mean arterial blood pressure below 50 mmHg for more than 30 minutes despite fluid replacement) before setting up the experiment.

NOTE: We suggest a continuous infusion of Ringer's solution at a rate of 10 mL/kg/hour.

5.9. Subtract any continuous administration of fluids (e.g., for continuous LPS application) from the amount infused so that the results are comparable with those of the control groups.

NOTE: At the end of the experiment, and prior to harvesting any organs such as liver, kidney or spleen for further analyses such as histological or biochemical examination animals can be euthanized by an incision of the inferior cava vein. To verify sepsis-related organ failure, pro-apoptosis marker like caspase-3 may be analyzed as well as α1-microglobuline to verify tubular damage in the kidneys. The organ specific analyze of markers like CINC-1, MCP-1 and IL-6 may also provide information about the organ specific inflammatory response.

Representative results:

The system presented allows for endotoxemia with hemodynamically stable animals as reported previously[9]. While the mean arterial pressure remains stable in animals with and without LPS stimulation LPS treated animal develop characteristics of sepsis such as a negative base excess and a strong inflammatory reaction measured by plasma cytokines (6 hours after application) such as CINC-1 (867 ng/mL), MCP-1 (5027 ng/mL), and IL-6 (867 ng/mL)[8], **Figure 5**.

Figure 1: Preparation of equipment: Anesthesia induction box and nosecone for anesthesia/oxygen application (**A**). Sterile material to be prepared prior surgery: 26G intravenous catheters, a scalpel with a blade number 10, 2 curved forceps, 1 needle-holder, 2 surgical clamps, 2-0 and 6-0 silk ties, q-tips, tracheal cannulas, 3-way stop-cocks (**B**). Monitoring equipment: Anesthesia monitoring with pressure transducer and a saturation of peripheral oxygenation (SpO2) sensor for continuous monitoring (**C**).

Figure 2: **Venous access**: A tourniquet is applied to the proximal rat's tail (**A**). The venous access should be introduced at the distal part of the tail and fixed in place (**B**). Air embolism should be strictly avoided.

Figure 3: **Tracheostomy**: The larynx and trachea are bluntly prepared using surgical scissors and exposed using 2-0 silk sutures (**A**). After opening the trachea using surgical microscissors at the 3-5th tracheal clasp, a tracheal cannula is introduced, fixed in placed, and connected to a ventilator (**B**)

Figure 4: **Arterial access**: After surgical exposure of the tail artery using a scalpel and surgical microscissors, a distal silk 6-0 ligature is tightened, and a proximal ligature is prepared (**A**). After insertion of the G-26 catheter into the artery, it is fixed in place (**B**). The arterial catheter allows for repetitive blood sampling as well as for continuous blood pressure monitoring (**C**).

Figure 5: Representative results: While animals remain hemodynamically stable in the LPS as well as in the sham-group (A), they develop characteristics of endotoxemia such as a negative base excess (B) and increased inflammatory mediators such as cytokine induced neutrophil chemoattractant protein 1 (CINC-1) (C), monocyte chemoattractant protein 1 (MCP-) (D), and interleukin 6 (IL-6) (E). The figure is reproduced with permission from Wolters Kluwer Health Inc., Beck-Schimmer et al, Eur J Anaesthesiol 2017; 34:764–775[9].

Discussion:

The protocol described here allows for a highly reproducible, yet simple to learn sepsis model, which can be adapted according the research question. Essential in vivo data referring to organ function such as heart rate, blood pressure, and peripheral arterial oxygen saturation may be collected continuously, and blood sampling may be performed repetitively throughout the experiment. In addition, modifications with regard to fluid replacement protocols and vasopressor support can be installed. Given the hemodynamic stability of the animals, experiments can be carried out over several hours[8].

It has to be pointed out, that an appropriate sepsis-model has to be chosen in order to answer a specific research question[10,11]. All sepsis models have their advantages, but also their drawbacks. In the current article, an endotoxemia model is presented, which induces a strong, but sterile inflammation. Key characteristics of sepsis, such as the development of a strong inflammatory response[12], endothelial dysfunction and damage[13] and multi-organ failure[14] are present. Therefore, the model presented is in accordance with the previously published definition of sepsis models in animals by the "International Expert Consensus for Pre-Clinical Sepsis Studies"[15]. Other key elements may be different than in bacterial sepsis. The LPS bolus application, for example, induces a hypodynamic cardiovascular response[16], which does not correspond to the hyperdynamic response observed in human sepsis. The latter, however,

may be induced by a continuous LPS infusion as also suggested in the current article[16]. It has to be considered, that LPS only represents one toxin and may be over-simplified for certain research questions – on the other hand, simplification increases reproducibility of the data. Another characteristic of endotoxemia models is a different cytokine response in comparison to bacterial models - endotoxemia induces higher, yet shorter lasting cytokine elevations[10]. Although, the model enables repetitive measurements over several hours, the tracheostomy is not ideal for survival experiments. In case of survival experiments, tracheal intubation or spontaneous breathing via a mask may be preferred.

Three fundamentally different classes of sepsis models are currently applied in laboratory sepsis research: toxemia models (e.g., LPS), bacterial infection models (e.g., intravenous Escherichia coli), and host barrier disruption models (e.g., cecal ligation and puncture, CLP)[17]. Even if toxemia models with LPS were proposed as an inappropriate model for a replication of human sepsis[15], it has to be emphasized that characteristics of these fundamental classes of sepsis models have been described in detail[8,12-14] and been critically reviewed in a recent article[17].

There is no final answer, of what humane animal experiments are, but the most common sense is the 3R principle, by their definition, animal experiments should be reduced, replaced, and refined[18]. While in sepsis research replacement of animal experiments is difficult, repetitive blood sampling and continuous measurements of vital data may reduce the number of animals necessary. Moreover, keeping septic animals anesthetized refines the experimental setup as animal suffering is diminished.

In summary, we present a well-characterized and reproducible model of endotoxemia, a setting similar to that of an intensive care unit with the possibility of generating a high data density, and at the same time limiting the animal burden. In addition, this model can be easily modified depending on the research question needs to be answered.

Acknowledgments:
The authors would like to thank Beatrice Beck-Schimmer (MD) and Erik Schadde (MD) for their critical examination and their valuable contribution for this manuscript.

Disclosures (<36 months):
The authors have no conflicts of interests with regard to the presented study. Martin Schläpfer has submitted a patent to mitigate the negative effects of surgery and/or anesthesia for patients using medical gases, particularly oxygen (O_2) and carbon dioxide (CO_2). He has received unrestricted research grants from Sedana Medical, Sweden, and from Roche, Switzerland, not related to this work.

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Figure 1

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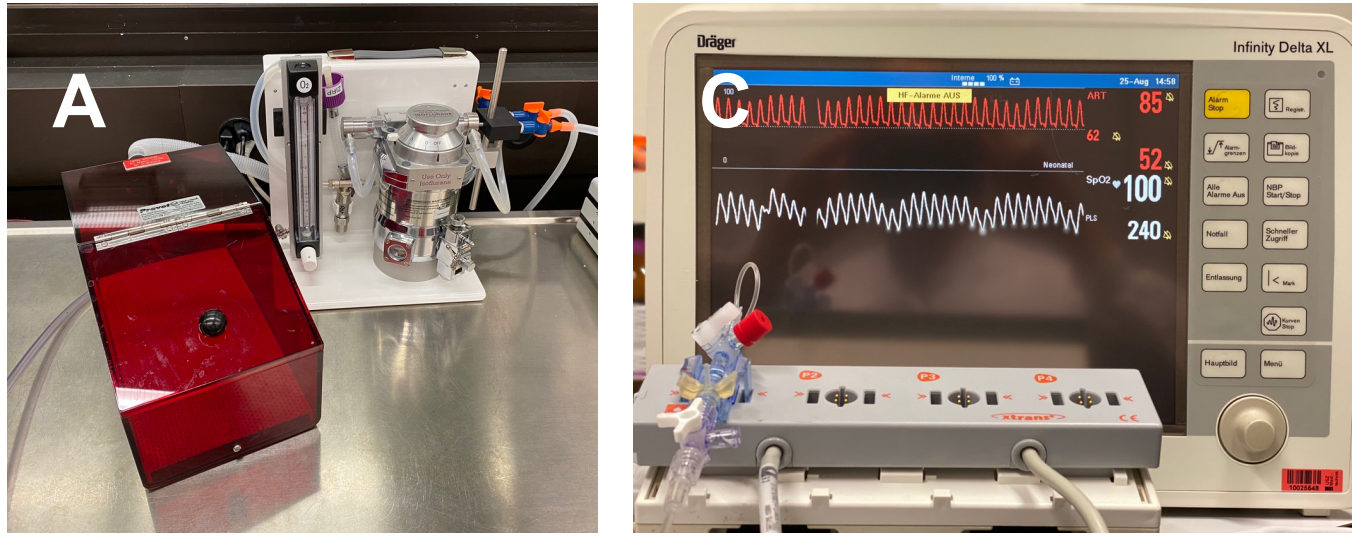

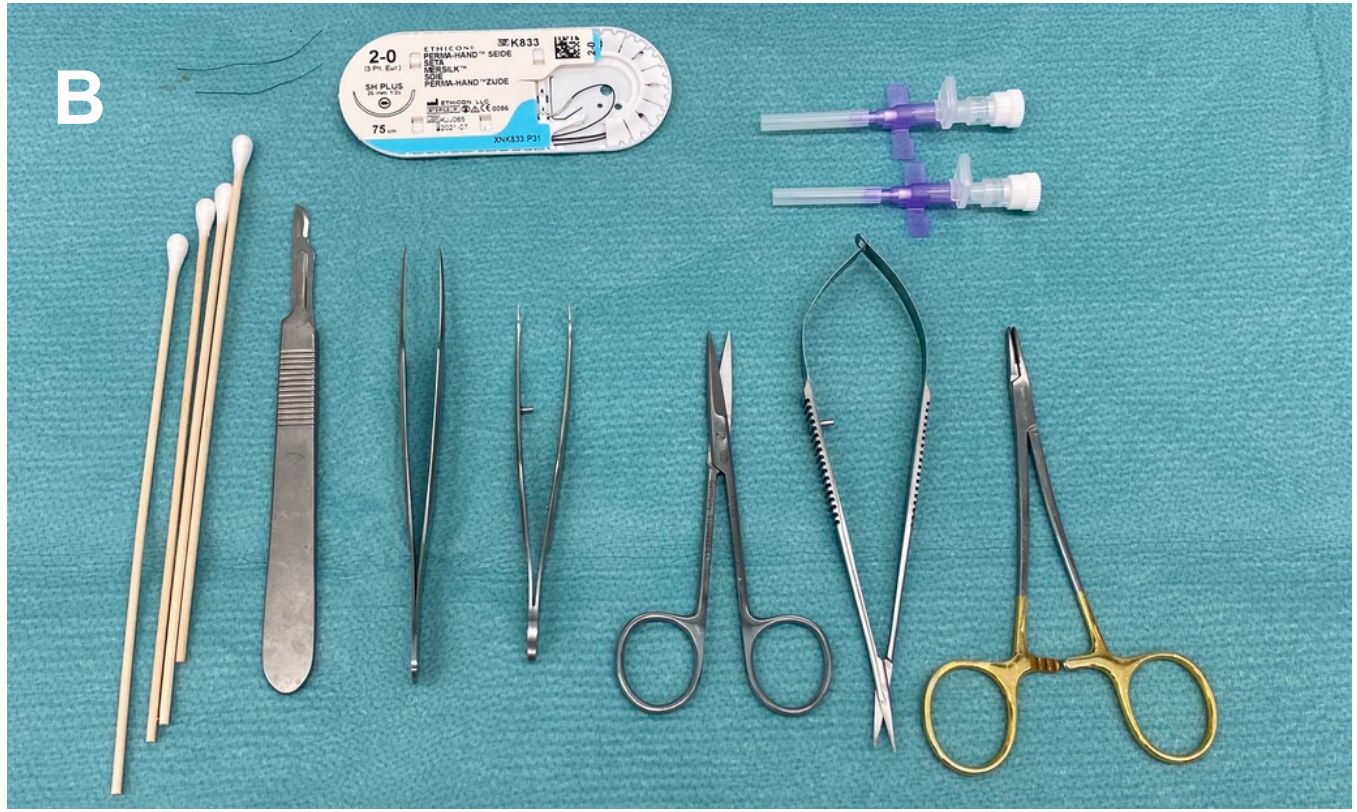

Figure 2

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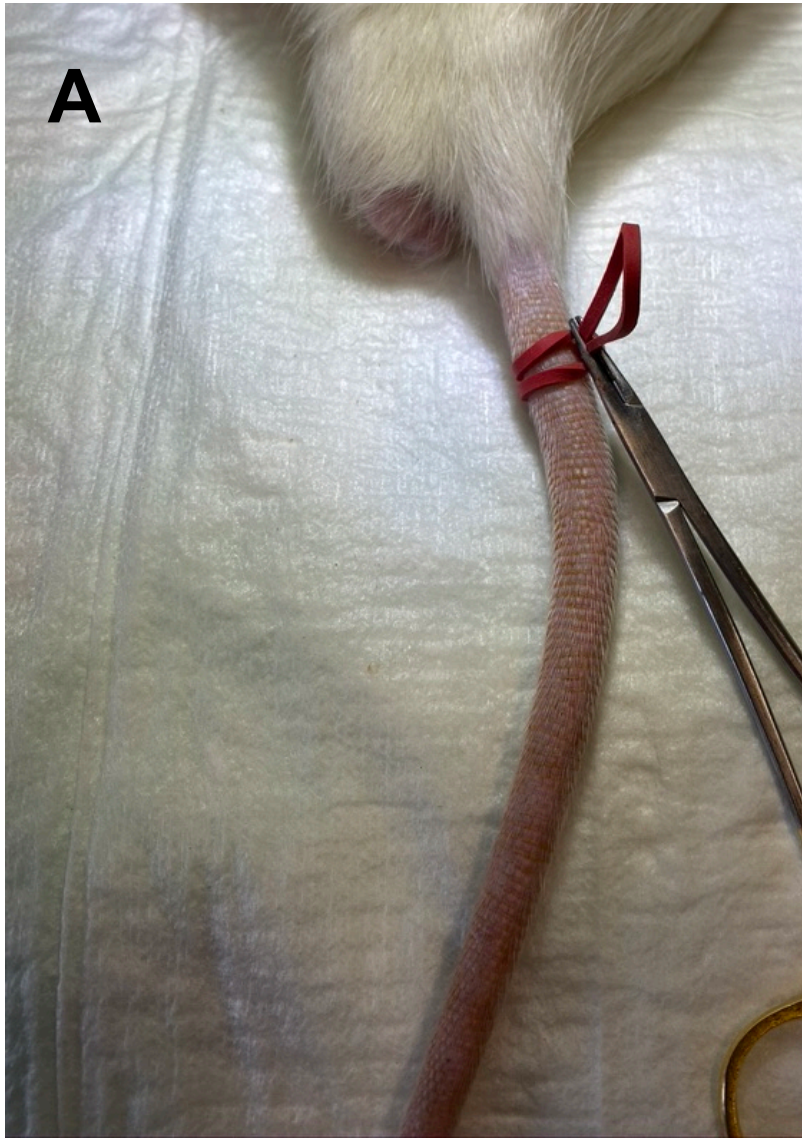

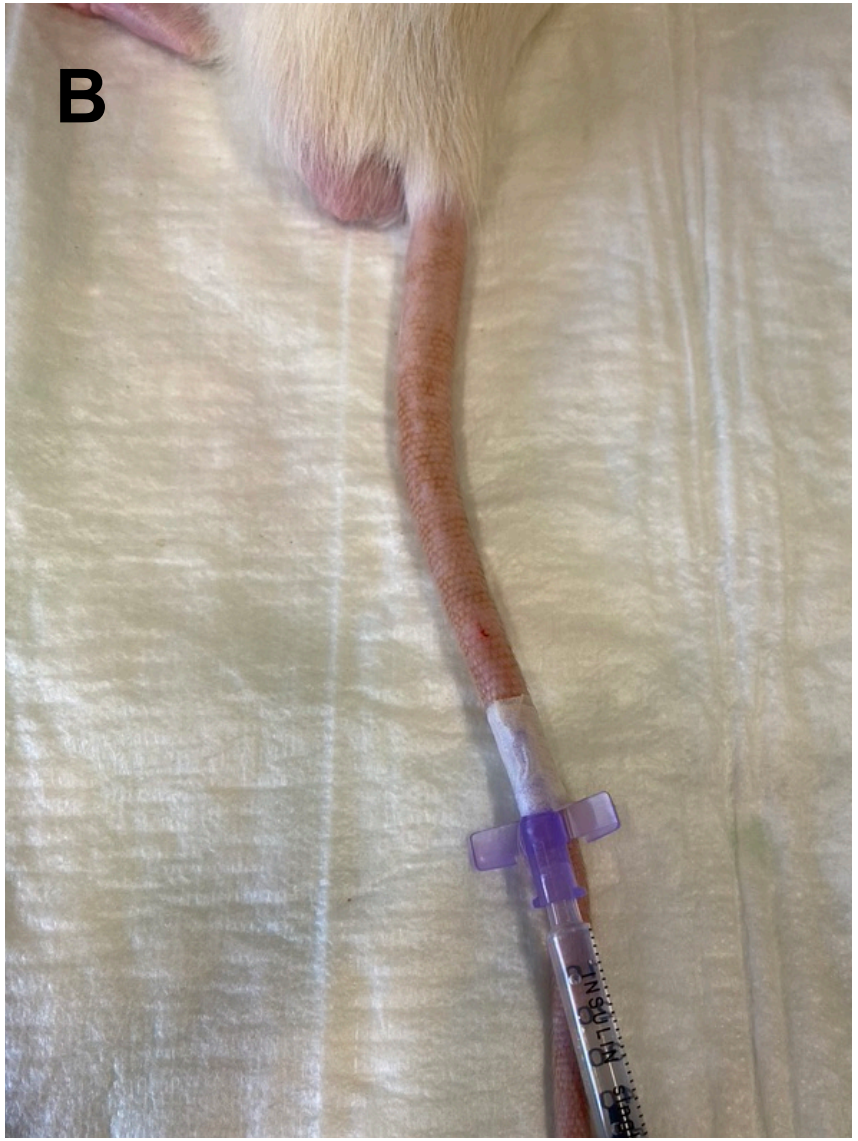

Figure 3

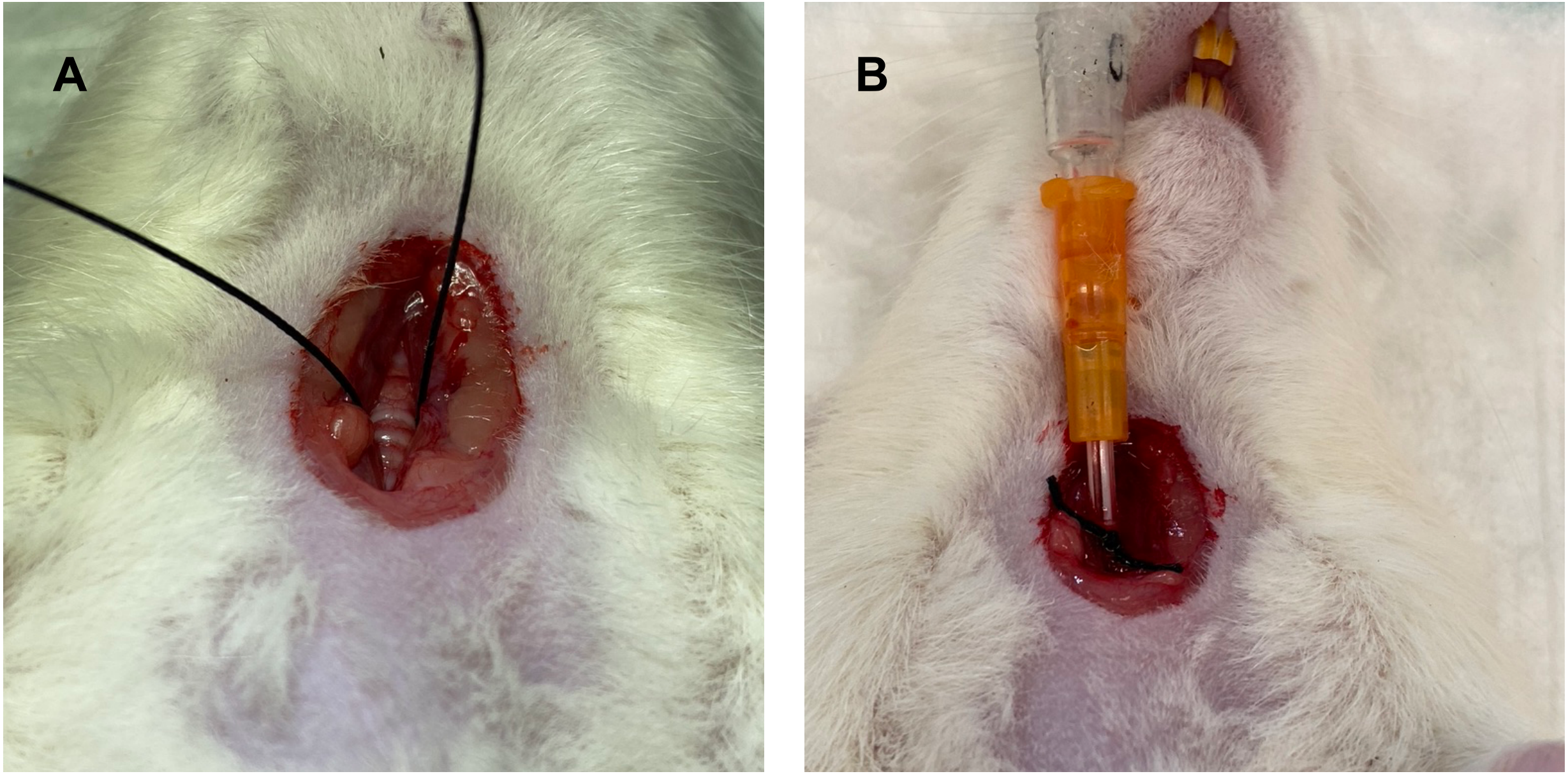

Figure 4

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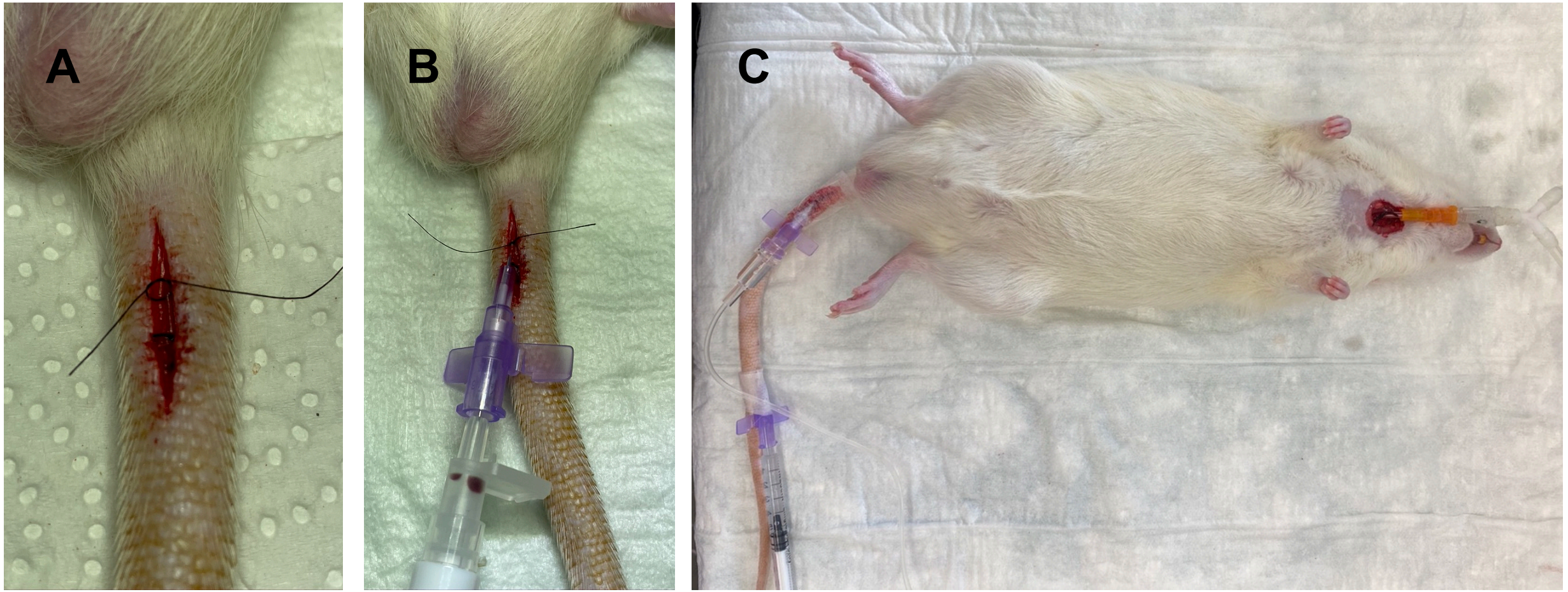
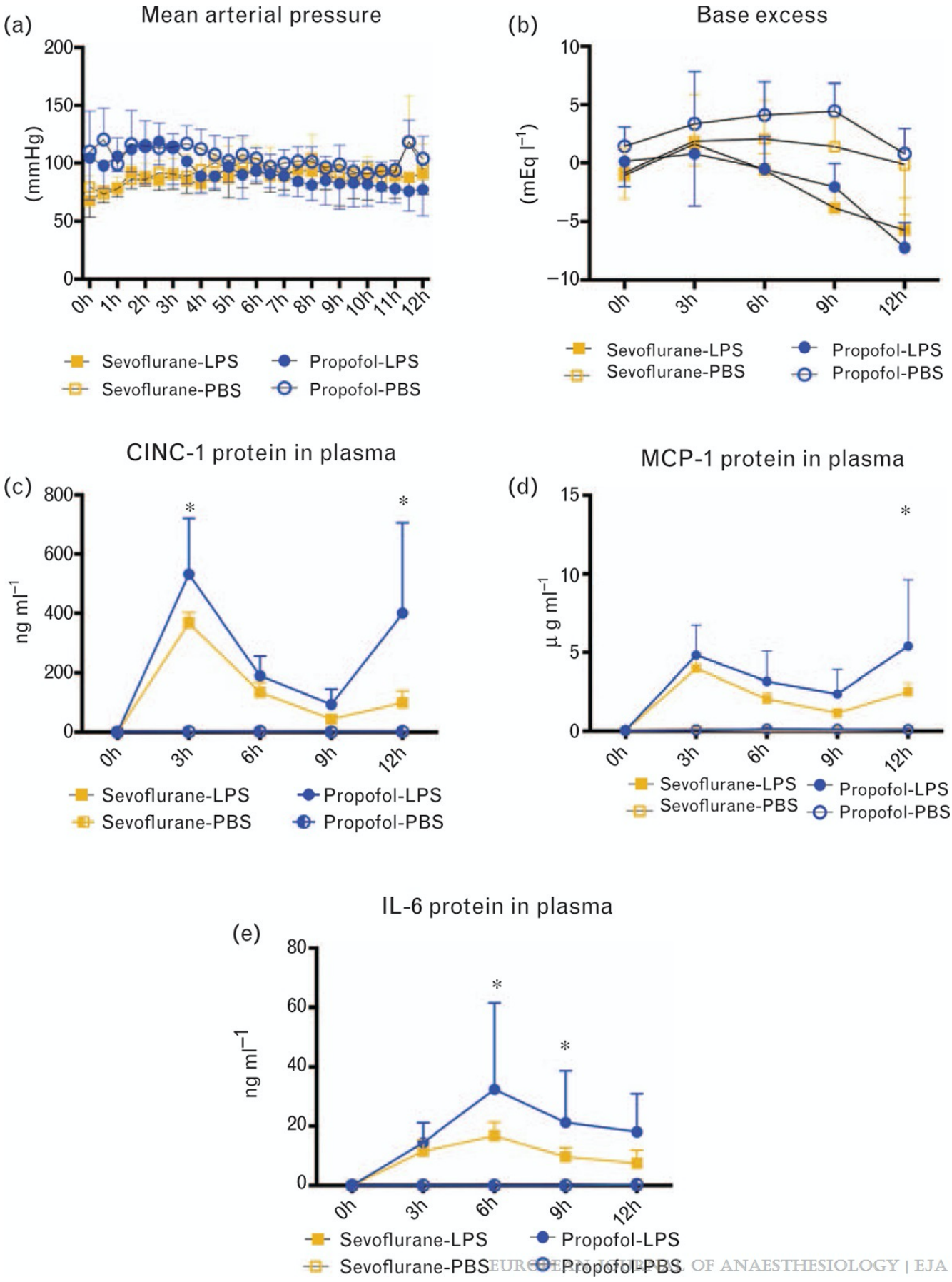


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-0 silk sutures	Ethicon, Sommerville, NJ	K833	Standard surgical
26 intravenous catheter	Becton Dickinson, Franklin Lakes, NJ	391349	Standard anesthesia equipment
6-0 LOOK black braided silk	Surgical Specalities Corporation,	SP114	Standard surgical
Alaris Syringe Pump	Bencton Dickinson		
Betadine	Mundipharma, Basel, Switzerland	7.68034E+12	GTIN-number
Curved fine tips microforceps	World precision instruments (WPI), Sarasota, FL	504513	Facilitates vascular preparation
Fine tips microforceps	World precision instruments (WPI), Sarasota, FL	501976	Tips need to be polished regularly
Infinity Delta XL Anesthesia monitoring	Draeger, Lübeck, Germany		
Isoflurane, 250 mL bottles	Attane, Piramal, Mumbai, India	LDNI 22098	Standard vet. equipment
Ketamine (Ketalar)	Pfitzer, New York, NY		
Lipopolysaccharide (LPS) from *Escherichia coli* , serotype 055:B5	Sigma, Buchs, Switzerland		
Q-tips small	Carl Roth GmbH, Karlsruhe, Germany	EH11.1	Standard surgical
Ringerfundin	Bbraun, Melsungen, Germany		
Tec-3 Isofluorane Vaporizer	Ohmeda, GE-Healthcare, Chicago, IL	not available anymore	Standard vet. equipment
Xylazine (Xylazin Streuli)	Streuli AG, Uznach, Switzerland		

Dear Dr. Nguyen

We would like to thank you and the reviewers for their time and helpful comments. We are convinced that the manuscript improved thanks to the reviewer's suggestions. Several changes and additions have been made. Please find a point to point reply letter below. We hope, that our manuscript now meets the high standards of JoVE. Best regards

Martin Schläpfer

A proofread was done again. Abbreviations were defined at the first use. Headings were removed from the abstract and references were set as numbered superscripts after the appropriate statements, but before the punctuation. Additionally, references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).]

An ethic statement was added including an approval number as well as a confirmation that the steps were performed in accordance with the local animal guidelines. A suggestion how to euthanize the animals was added at the end of the protocol. The application of a Vitamin A ointment was mentioned at the beginning of the protocol. All advices in the protocol how to perform the sepsis model were changed into the imperative tense. Additionally, we tried to explain more in detail how each step has to be performed. Therefore, reference values were given for oxygen saturation and arterial blood pressure. Also, the information was added how big the incision should be for preparing the trachea and the tail artery, and what kind of instrument should be used.
We also specified which variates should be analyzed for histological and biochemical tests, and expanded the "representative results" section.
Furthermore, we included a one-line space between each protocol step and highlighted the sections (blue marker in markup version) which should be included in the protocol section of the video.
A figure and table legend section was added after the section "representative results". In addition, materials were alphabetically sorted in table 1.
Regarding the section discussion, we put more attention on critical steps, limitations, modification and troubleshooting as well as future application of the technique.
The text about copyright permission of figure 5 was changed into "This figure has been modified from…". We also uploaded a letter regarding the copyright permission.

Reviewer 1

Reviewer states (1): "The authors did not clearly show the characteristics of sepsis in this model. In deed, the LPS model itself carries many disadvantages being the model with the least resemblance to human sepsis. Some other drawbacks of this model are: 1- High, rapid and transient increase in cytokines, which differs from human sepsis, 2- Rodents are endotoxin resistant, whereas humans are very sensitive, 3- Different hemodynamic response compared to human sepsis (Lien Dejager, Iris

Pinheiro, Eline Dejonckheere, Claude Libert, Cecal ligation and puncture: the gold standard model for polymicrobial sepsis?,, Trends in Microbiology,Volume 19, Issue 4, 2011)."

Our reply: *Thank you for this comment. As we mentioned in the discussion (page 6), all models have their advantages, but also their drawbacks. For example, the lipopolysaccharide (LPS) bolus application induces a hypodynamic cardiovascular response in rats which is contrary to the response in humans[1]. Additionally, the cytokine response differs between the sepsis models depending on the sepsis stimulus[2]. LPS induced sepsis results in a rapid cytokine expression as mentioned by reviewer 1. However, the LPS-induced sepsis model is well-established in rats, since key characteristics are present, such as a strong inflammatory response, endothelial dysfunction and multiorgan failure[3-6]. Characteristics of the cytokine expression of this model are displayed in* ***Figure 5 C, D*** *and* ***E****.*
The model presented is simple and highly reproduceable. Therefore, we think that this LPS-triggered sepsis model in rats still is an important sepsis model.

Reviewer 2 states (2): "Why did not the authors measure serum creatinine for kidney function assessment, or serum liver function parameters to demonstrate examples of multiple organ failures?

Our reply: *Thank you for this important note. One main advantage of the model presented is the ability of repetitive blood sampling (liver enzymes, creatinine, etc.) among other measurements. This may reduce the number of animals necessary (in accordance with the 3R principle) and is one of the main advantages of this sepsis model. This current article aims to focus on technical aspects and is based on the most recent article of our group. However, organ function was assessed in similar, previous article of our group, where kidney damage, but no liver damage could be demonstrated[7].*

Reviewer 2 states (3): "Is there mortality in this model? How to test the efficacy of any proposed treatment?"

Our reply: *Thank you for this important question. Mortality rate depends on the dose and biological activity of the used endotoxin[2]. From our experience, rats do tolerate lipopolysaccharide (LPS) injection of 1 milligram / kilogram body weight well[8]. However, mortalities due to technical limitations (air embolism or bleeding) or other reason cannot be excluded and depends more or less on the personal experience.*
To measure the systemic inflammation induced and also the efficacy of any treatments, several markers like monocyte chemoattractant protein (MCP)-1, IL (interleukine)-6 and CINC (cytokine-induced neutrophil chemoattractant protein)-1 have proved to be prognostic markers for severity and outcome of sepsis[8]. We added this information in the introduction on page 2.

Reviewer 2 states (4): "Figure legends are not informative."

Our reply: *Thank for this note. We revised the figure legends.*

Reviewer 2 states (5): "Figure 5a: mean arterial pressure: the figure legend sevoflurane-LPS is repeated!"

Our reply: *This has now been corrected.*

Reviewer 2

Reviewer 2 states (1): " Although, the model appears feasible and easy to reproduce, it is not a clinical relevant model of sepsis. As such, the title is misleading since the authors show only a model of endotoxemia, which does not reproduce the clinical sepsis. The title should be changed to reflect the model."

Our reply: *Thank you for this comment. We have adapted the title of our manuscript.*
As mentioned in the discussion, depending on the question needs to be answered the choice of the sepsis model really matters[2,9]. We do agree with reviewer 2 that for some specific questions the model presented is not appropriate. However, several published articles already confirmed the usefulness of this sepsis model[3-6,8].

Reviewer 2 states (2): "Introduction and Discussion. The authors acknowledge the limitation of their model of LPS injection in reproducing sepsis. The references listed in the manuscript to refer to other models of sepsis are not up to date and miss several important initiatives of the scientific community (for example, Osuchowski et al., 2018 Shock. 2018 Oct;50(4):377-380), including major governmental agencies (for example, the "NIGMS Priorities for Sepsis Research" of the USA National Institute of Health). Also, the authors should discuss the utility or advantages of a rat model in comparison to a mouse model. With the availability of genetically modified mouse models, the use of a rat model has become obsolete in the sepsis research field."

Our reply: *Thank you for this important comment. We implemented the definition of sepsis models in animals which was proposed by the "International Expert Consensus for Pre-Clinical Sepsis Studies"[10]. Furthermore, we expanded the critical reflection of our sepsis model (page 6). However, we would like to mention that the LPS endotoxin model in rats has been used in several peer-reviewed articles in recent years[3-6,8]. Moreover, this model can be easily modified depending on the research question needs to be answered. We are aware, that according the "NIGMS Priorities for Sepsis Reseach" states rodent models are of low priority, there is no suggestion of alternative models. Not all promising approaches can be tested in humans in the first place.*

Reviewer 2 states (3): "Methods: The authors should be more precise on the age of the rats since size of catheters may change accordingly. Also, the model is not adaptable to different ages of rats since cannulation of tail veins and arteries may not feasible in juvenile models."

Our reply: *Thank you for this important note. At the beginning of the protocol (paragraph 1.0) we added the advice to use rats with a weight of 250 – 300g (or not older than 17 weeks). In our experience, the intravenous and -arterial access are easily to perform in this weight category. Albeit, the cannulation is more a challenge in juvenile rats, we know from our own experience that the cannulation is indeed feasible in juvenile rats.*

Reviewer 2 states (4): "Methods: The model appears feasible only for short-term monitoring of hemodynamics. Can the authors suggest modification of their model for long-term monitoring, for example in survival studies? Can the tracheostomy be avoided or replaced by the oral intubation?

Our reply: *As demonstrated in a recently published article of our group, the experiment can be performed for several hours[8].*

The tracheostomy indeed is not allowed for survival experiment – in case survival experiments are planned, tracheostomy should be replaced by spontaneous breathing or tracheal intubation. The current model is our standard model to have a situation – monitoring wise – like in an intensive care unit or in an operating theater. As we are not experts for cardiovascular monitoring in survival experiment, we feel, we may not be able to provide a definitive answer on this question.

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