Journal of Visualized Experiments

Brain Death Induction in Mice Using Intra-Arterial Blood Pressure Monitoring and Ventilation via Tracheostomy

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60831R1
Full Title:	Brain Death Induction in Mice Using Intra-Arterial Blood Pressure Monitoring and Ventilation via Tracheostomy
Section/Category:	JoVE Neuroscience
Keywords:	Transplantation, Brain Death, Organ donation, Murine, Blood pressure measurement, tracheostomy
Corresponding Author:	Paul Ritschl Charite Universitatsmedizin Berlin Berlin, Berlin GERMANY
Corresponding Author's Institution:	Charite Universitatsmedizin Berlin
Corresponding Author E-Mail:	paul.ritschl@charite.de
Order of Authors:	Paul Ritschl
	Lena Hofhansel
	Bernhard Flörchinger
	Rupert Oberhuber
	Robert Öllinger
	Johann Pratschke
	Katja Kotsch
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Berlin, Germany

CHARITÉ

CharitéCentrum für Chirurgische Medizin

Editor in Chief
JoVE

1 Alewife Center, Suite 200
Cambridge, MA 02140
tel: 617-945-9051
fax: 866-381-2236

Berlin, 27.09.2019

Cover letter: **A Murine Model of Brain Death Induction Using Intra-arterial Blood Pressure Monitoring and Ventilation via Tracheostomy**

Dear Editor-in-Chief,

Dear Aaron Berard, Ph.D.,

On behalf of my co-authors, I am submitting the enclosed manuscript for possible publication in ***Journal of Visualized Experiments.***

Solid organ transplantation remains the only cure for organ end-stage failure. With increasing success of modern transplant medicine, the desire for life-saving transplantation unmasks the lack of appropriate donors. Brain death donors are still the number one source of solid organs for transplantation. Unfortunately, this irreversible brain damage leads to a variety of pathophysiological changes with direct and indirect impact on graft quality and transplantation outcome.

In this video publication we demonstrate the murine model of brain death induction to facilitate innovate brain death related transplantation research. Due to the superiority of the mouse as animal model in immunological research, this publication is of special value. This is reflected by recently high-impact publications utilizing the presented model.

We confirm that this work is original and has not been published elsewhere, nor is currently under consideration for publication elsewhere. All of our listed authors participated meaningfully in the study and had seen and approved the final manuscript. There are no commercial affiliations or

consultancies of one of our authors that might cause a conflict of interest with respect to the submitted data.

We thank you in advance for your time and kind consideration.

Sincerely yours,

Paul Ritschl

TITLE:

Brain Death Induction in Mice Using Intra-Arterial Blood Pressure Monitoring and Ventilation via Tracheostomy

AUTHORS AND AFFILIATIONS:

Paul V. Ritschl[1,2,3,*], Lena Hofhansel[2,4,*], Bernhard Flörchinger[5], Rupert Oberhuber[2], Robert Öllinger[1], Johann Pratschke[1], Katja Kotsch[6]

[1]Department of Surgery Campus Charité Mitte/Campus Virchow-Klinikum, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

[2]Department of Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck, Austria

[3]Charité Clinician Scientist Program, Berlin Institute of Health, Berlin, Germany

[4]Department of Psychiatry, Psychotherapy and Psychosomatics, Faculty of Medicine, RWTH Aachen University, Aachen, Germany

[5]Department of Cardiothoracic Surgery, University Medical Center Regensburg, Regensburg, Germany

[6]Department of General, Visceral and Vascular Surgery, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

*These authors contributed equally.

Corresponding Author:

Paul V. Ritschl (paul.ritschl@charite.de)

Email Addresses of Co-authors:

Lena Hofhansel (lhofhansel@ukaachen.de)
Bernhard Flörchinger (bernhard.floerchinger@ukr.de)
Rupert Oberhuber (rupert.oberhuber@i-med.ac.at)
Robert Öllinger (robert.oellinger@charite.de)
Johann Pratschke (johann.pratschke@charite.de)
Katja Kotsch (katja.kotsch@charite.de)

KEYWORDS:

transplantation, brain death, organ donation, murine, blood pressure measurement, tracheostomy

SUMMARY:

We present a murine model of brain death induction in order to evaluate the influence of its pathophysiological effects on organs as well as on consecutive grafts in the context of solid organ transplantation.

ABSTRACT:
While both living donation and donation after circulatory death provide alternative opportunities for organ transplantation, donation after donor brain death (BD) still represents the major source for solid transplants. Unfortunately, the irreversible loss of brain function is known to induce multiple pathophysiological changes, including hemodynamic as well as hormonal modifications, finally leading to a systemic inflammatory response. Models that allow a systematic investigation of these effects in vivo are scarce. We present a murine model of BD induction, which could aid investigations into the devastating effects of BD on allograft quality. After implementing intra-arterial blood pressure measurement via the common carotid artery and reliable ventilation via a tracheostomy, BD is induced by steadily increasing intracranial pressure using a balloon catheter. Four hours after BD induction, organs may be harvested for analysis or for further transplantation procedures. Our strategy enables the comprehensive analysis of donor BD in a murine model, therefore allowing an in-depth understanding of BD-related effects in solid organ transplantation and potentially paving the way to optimized organ preconditioning.

INTRODUCTION:
Transplantation is currently the only curative treatment for end-stage organ failure. Until now, brain death (BD) patients have been the main source for organ donations, although living donation and donation after circulatory death are valuable alternatives[1]. BD is defined by an irreversible coma (with a known cause), the absence of brain stem reflexes and apnea[2]. Unfortunately, BD organs demonstrate inferior results in long-term graft survival independent of human leukocyte antigen (HLA)-mismatch and cold ischemic time[3]. Meanwhile, intensive research on this antigen-independent risk factor has been performed resulting in three main aspects of pathophysiological changes mediated as a consequence of BD: hemodynamic, hormonal, and inflammatory[4].

To date, experimental BD models in rodents have been mostly performed using rats. In order to gain greater insight into the immunological consequences on solid organs following BD, we aimed to establish a murine model of BD, as currently only mouse models allow for comprehensive investigations into genetic or immunological factors. In this context, the mouse system provides a larger variety of analytical tools.

The principle of BD induction as described here is based on an increase in intracranial pressure induced by the inflation of a balloon catheter inserted under the skull. Increased intracranial pressure mimics the physiological mechanism of BD by blocking the perfusion of the cerebrum, cerebellum, and brain stem[5,6]. To guarantee sufficient perfusion of peripheral organs, blood pressure measurement is obligatory during the procedure. The catheter used for this purpose at the same time serves for saline administration in order to stabilize the blood pressure by fluid substitution. As BD is accompanied by cessation of spontaneous breathing, sufficient ventilation must be ensured. An electric blanket maintains physiological core body temperature.

In summary, this model will enable in-depth studies into the influence of BD-induced injury, on

leukocyte migration[7], compliment activation[8], ischemic reperfusion injury[9], and other factors.

PROTOCOL:

Animal experiments were performed in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). All experiments were approved by the Austrian Ministry of Education, Science and Culture (BMWF-66.011/0071-II/3b/2012).

1. Arterial catheterization

1.1. Anesthetize the mouse with an intraperitoneal injection of ketamine and xylazine[10]. Pinch the hind limbs with forceps to confirm the correct depth of anesthesia.

NOTE: For this study, male C57BL/6N mice at the age of 8−12 weeks were used (weight 20−25 g). The authors have tested male BALB/c of the same age unsuccessfully but have not tried other strains (see Discussion).

1.2. Remove the hair in the regions of interest (head, neck) using an electric razor. Ensure that no loose hair remains to prevent wound contamination. Subsequently disinfect the surgical field with 70% ethanol and place the mouse supine with the head facing the surgeon.

1.3. Make a cervical midline incision with dissecting scissors.

1.4. Dissect the submandibular glands and neck muscle tissue and separate them in order to expose the common carotid artery. Use mostly blunt dissection via forceps.

1.5. Place three 8-0 silk ligatures beneath the right common carotid artery.

1.6. Place a clamp on the proximal ligature and bring tension in the artery so that the flow is suspended.

1.7. Close the most distal ligature.

1.8. Insert the arterial catheter through a small, preformed skin hole on the cranial aspect of the incision. Squeeze and deform the lumen of the catheter if it appears too large to reduce blood backflow. Fixate with all three ligatures.

1.9. Fixate the catheter to the skin to avoid dislocation. Do this by using a suture (e.g., 5-0 monofilament, non-absorbable) which connects the catheter to the skin in the area of the preformed skin hole.

2. Tracheostomy

2.1. Dissect the pre-tracheal musculature bluntly using forceps.

2.2. Place two 8-0 silk ligatures beneath the trachea.

2.3. Tracheotomize using micro scissors as proximally as possible to avoid unilateral ventilation. Use a horizontal cutting line between two tracheal cartilages.

2.4. Insert the ventilation tube and fixate with both prepared ligatures.

NOTE: The proximal end of the trachea does not need to be ligated.

2.5. Close the skin with a running suture (e.g., 6-0 monofilament, non-absorbable).

2.6. Ventilate the mouse with a frequency of 150/min and a tidal volume of 200 µL.

3. Brain death induction

3.1. Arrange the mouse to the prone position.

3.2. Remove the skin from the skull using surgical scissors and forceps to hold the skin.

3.3. Drill a 1 mm caliber borehole paramedially above the left parietal cortex. Stop drilling before breaching the inner compact bone and the dura mater.

3.4. Penetrate the final tissue bridge of the skull using blunt forceps, removing sharp edges.

3.5. Insert the balloon catheter, so that it is entirely within the cranial cavity. Ensure that the balloon is prefilled with saline and all air is evacuated.

3.6. Begin inflation at ~0.1 mL/min over a period of 10−15 min (total volume of 0.8−1.2 mL) with the help of a syringe pump.

NOTE: The mouse will exhibit myoclonus, mydriasis, further seizure activity and agonal gasps.

3.7. Pronounce the mouse brain dead once the tail of the mouse has gone stiff and erected.

NOTE: BD is confirmed by a characteristic initial blood pressure peak (Cushing reflex), the absence of brain stem reflexes and spontaneous breathing. Regular apnea testing should be avoided during experiments, as mice may become circulatory instable due to a lack of oxygen.

3.8. Stop the inflation of the balloon catheter.

3.9. Put a heating blanket over the mouse to avoid hypothermia.

4. During BD period

4.1. Monitor and document blood pressure regularly. Exclude mice with a prolonged hypotensive phase (mean arterial pressure [MAP] < 50 mmHg for >30 min).

4.2. Infuse 0.1 mL of saline every 30 min to stabilize the blood pressure of the mouse.

NOTE: In total 0.8 mL of saline was administered to each mouse in this study.

4.3. After 4 h of BD duration, harvest mouse organs/tissues. Exclude the mouse from the experiment if the heart of the mouse is not beating at the end of the experiment.

5. Sham procedure

5.1. Perform steps 1.1−3.3.

NOTE: Do not open the inner compact bone. Do not insert or inflate a balloon catheter.

5.2. Continuously observe the animal. Apply additional anesthesia subcutaneously (approximately 1/2 of the starting dose) if the mouse shows signs of awakening, especially after approximately 2−3 h post-anesthesia.

5.3. Apply the same amount of intravenous saline as in the BD mice.

NOTE: The sham mouse will regain spontaneous breathing after cessation of ventilation. The BD mouse will not. Apnea testing should be done while establishing the model, but during experiments it should be avoided due to unnecessary physiological stress.

REPRESENTATIVE RESULTS:

The murine BD model was successfully performed more than 100 times with a success rate of over 90%. Additionally, post interventional organ transplantation of heart and kidney has been safely performed[7].

BD induces a variety of pathophysiological changes that may be further investigated using this model. As shown in **Figure 1,** the blood pressure shows an initial hypertensive peak followed by a prolonged hypotensive phase. To avoid detrimental physiological effects of hypotension, mice with a prolonged insufficient blood pressure (MAP < 50 mmHg for more than 30 min) were excluded.

Another well-established observation is that BD induction leads to the activation of the immune system. After 4 h of BD duration organ-specific upregulation of immune markers at the mRNA level (**Figure 2**) as well as immune cell migration was observed[7].

FIGURE LEGENDS:

Figure 1: Invasive measurement of mean arterial pressure (MAP) following BD induction or sham procedure. At the time point of BD induction, blood pressure was significantly higher in BD animals (white squares) than in sham animals (black squares) which is explained via the well described cytokine storm; thereafter, normalization of blood pressure levels became evident followed by a period of declining pressure levels starting after 90 min of BD induction (n = 19 animals/group ± SD). Statistically significant differences were tested with two-way ANOVA and the Bonferroni post-test; $**p < 0.01$, $***p < 0.001$.

Figure 2: BD induction activates the immune system and causes direct organ damage prior to transplantation. We show representative results of the natural cytotoxicity triggering receptor 1 (NCR1 also NK-p46), which increases in kidneys as a consequence of BD at the mRNA level. No changes were observed in livers or hearts. Data are presented as mean ± SE of n = 7–8 animals/group. Statistically significant differences between BD and sham were tested using the Mann–Whitney U-test; $**p = 0.0037$.

DISCUSSION:

BD, a risk factor for allograft quality in multi-organ donors, entails a plethora of pathophysiological changes, which can only be sufficiently assessed using in vivo models. Hemodynamic changes, cytokine storm, hormonal changes and their ultimate impact on organ graft quality and survival cannot be analyzed in vitro[4]. The majority of basic transplantation as well as immunological research is dependent on sophisticated diagnostic tools, which are widely available only in mice models. Mice models have already led to a variety of new insights in BD-related research[7-9].

Whereas in the 20th century the first models for BD research were developed in baboons, dogs and pigs[11,12], it was not until 1996 when the first rat model was described[13]. It took a further 14 years until a murine model of BD was first published by the Vienna group in 2010[6]. Although each individual step is not challenging for a trained microsurgeon, the model described here remains fragile and may take some weeks to establish.

Critical steps of this protocol are as follows. Use sterile disposable items (especially the arterial catheter and connecting tubes) to avoid unwanted immune stimulation. Avoid any blood loss as hypotensive phase may become uncontrollable. Use small cannulas for blood pressure measurement or reduce the lumen to avoid blood reflux. Fix the ventilation tube as well as the arterial cannula to avoid dislocation when turning the mouse or during seizure activity. The borehole must not be sharp-edged and should not be too large. The balloon catheter should be in contact the borehole. Otherwise brain tissue may protrude and hinder the intended elevation in intra-cranial pressure. During the BD-period, administer intravenous fluid steadily. If mice become hypotensive too early in the process (MAP < 50 mmHg after 2–3 h), additional fluid resuscitation is unlikely to provide sustained maintenance of blood pressure.

Most problems occurred during BD induction via the balloon catheter itself or became obvious over the course of the BD period. The common endpoint of all inaccuracies will be hypotension despite fluid administration. Depending on the blood pressure stability, BD-duration can be prolonged or shortened. In the literature, varying periods ranging from 3−6 h have been implemented[6-8]. The success of BD induction can also vary between different mouse strains. Although anatomical variants between mice strains should be limited, we were not able to establish the same model in BALB/c mice. Although the effort was limited to only few mice and no other strains were tested, we recommend the use of C57BL/6, which has been used in most previous research[8,9]. Only one publication has so far demonstrated a BD model using large (35 g) female OF-1 mice[6].

In summary, the mouse model described here represents a valuable tool for studying a multitude of pathophysiological changes caused by BD. Though challenging, the model can be optimized within a reasonable timeframe and performed in a standardized manner with a high success rate.

ACKNOWLEDGMENTS:
n.a.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Hart, A. et al. OPTN/SRTR 2017 Annual Data Report: Kidney. *American Journal of Transplantation.* **19 Suppl 2**, 19-123 (2019).
2. Practice parameters for determining brain death in adults (summary statement). The Quality Standards Subcommittee of the American Academy of Neurology. *Neurology.* **45** (5), 1012-1014 (1995).
3. Terasaki, P. I., Cecka, J. M., Gjertson, D. W., Takemoto, S. High survival rates of kidney transplants from spousal and living unrelated donors. *New England Journal Medicine.* **333** (6), 333-336 (1995).
4. Pratschke, J., Neuhaus, P., Tullius, S. G. What can be learned from brain-death models? *Transplant International.* **18** (1), 15-21 (2005).
5. Wilhelm, M. J. et al. Activation of the heart by donor brain death accelerates acute rejection after transplantation. *Circulation.* **102** (19), 2426-2433 (2000).
6. Pomper, G. et al. Introducing a mouse model of brain death. *Journal of Neuroscience Methods.* **192** (1), 70-74 (2010).
7. Ritschl, P. V. et al. Donor brain death leads to differential immune activation in solid organs but does not accelerate ischaemia-reperfusion injury. *Journal of Pathology.* **239** (1), 84-96 (2016).
8. Atkinson, C. et al. Donor brain death exacerbates complement-dependent ischemia/reperfusion injury in transplanted hearts. *Circulation.* **127** (12), 1290-1299 (2013).
9. Oberhuber, R. et al. Treatment with tetrahydrobiopterin overcomes brain death-associated injury in a murine model of pancreas transplantation. *American Journal of Transplantation.* **15**

(11), 2865-2876 (2015).
10. Floerchinger, B. et al. Inflammatory immune responses in a reproducible mouse brain death model. *Transplant Immunology.* **27** (1), 25-29 (2012).
11. Steen, P. A., Milde, J. H., Michenfelder, J. D. No barbiturate protection in a dog model of complete cerebral ischemia. *Annals of Neurology.* **5** (4), 343-349 (1979).
12. Cooper, D. K., Novitzky, D., Wicomb, W. N. The pathophysiological effects of brain death on potential donor organs, with particular reference to the heart. *Annals of the Royal College of Surgeons of England.* **71** (4), 261-266 (1989).
13. Herijgers, P., Leunens, V., Tjandra-Maga, T. B., Mubagwa, K., Flameng, W. Changes in organ perfusion after brain death in the rat and its relation to circulating catecholamines. *Transplantation.* **62** (3), 330-335 (1996).

Figure 1

Click here to access/download;Figure;Folie1.TIF

Ritschl et al., Figure 1

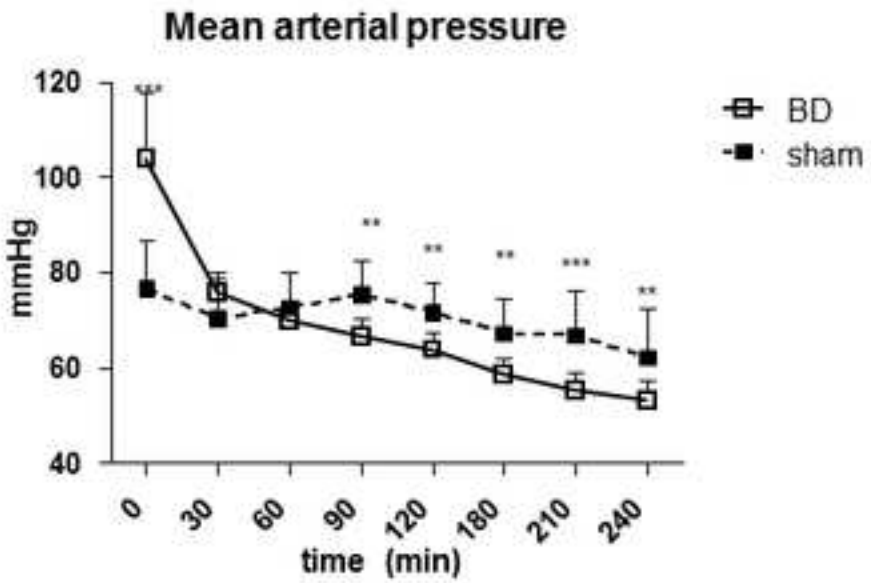
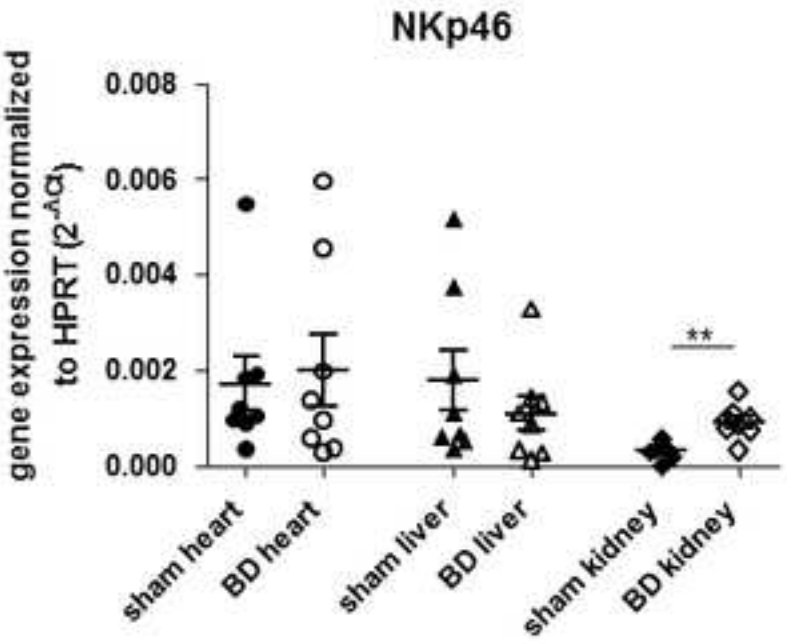


Figure 2

Click here to access/download;Figure;Folie2.TIF

Ritschl et al., Figure 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Arterial catheter (BD Neoflon 26G)	BD	391349	
Blood Pressure Transducers (APT300)	Harvard Apparatus Inc.	73-3862	
Fogarty Arterial Embolectomy Catheter N° 3	Edwards Lifesciences Corporation	120403F	
Forceps	FST	11271-30	
Homeothermic Blanket Systems with Flexible Probe	Harvard Apparatus Inc.	55-7020	
Ketansol	Graeub	6680110	
Micro scissor	FST	15018-10	
Needle holder	FST	12060-02	
Prolene 5-0	Ethicon	8698H	
Pump 11 Elite Infusion Only Single	Harvard Apparatus Inc.	70-4500	
Scissor	FST	14075-11	
Stereotactic microscope	Olympus	SZX7	
Transpore Tape	3M	1527-1	
Underpads	Molinea.A	274301	
Ventilator for mice (MiniVent Model 845)	Harvard Apparatus Inc.	73-0043	
Xylasol	Graeub	7630109	

Response Letter

Dear Editors,

Thank you for giving us the opportunity to improve our manuscript "A Murine Model of Brain Death Induction Using Intra-arterial Blood Pressure Monitoring and Ventilation via Tracheostomy" and to consider it for publication. The editor's and reviewer's comments are appreciated and we hope that we have addressed the reviewer´s concerns appropriately.

Point by point Response

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We appreciate the suggestion made by the editor and some of the reviewers. A native English speaker has now proofreaded the manuscript before its re-submission to your journal.

2. 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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: BD Neoflon, etc.

The term "BD Neoflon" was removed from the protocol section. No other trademark symbols could be found.

3. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc.

The Protocol section was changed as requested by the editor.

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

1.1: Please specify the age, gender and strain of mouse used and mention how proper anesthetization is confirmed.
1.2: Please describe how to shave and disinfect the mosue.
Please specify all surgical tools used throughout the protocol.
1.8: Please rephrase to be clear.
1.9, 2.1, 2.3, 2.4, 3.2, 3.3, etc.: Please describe how.

More details were added to the above mentioned sections and to other section where necessary.

5. 4.3: What is harvested?

Harvest is synonym for organ/tissue retrieval often referred to in animal experimentations.

6. Section 5: Please break into steps that are written in the imperative tense.

Section 5 was rewritten.

7. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

The material list was updated.

8. References: Please do not abbreviate journal titles; use full journal name.

Journal titles in the references are now in full length.

Reviewer #2:

1. I am not completely convinced that observation of tail stiffening as described in the manuscript is sufficient to be confident that brain death has occurred in a given mouse. An additional feature of brain death such as absence of spontaneous breathing efforts upon brief cessation of mechanical ventilation or a Cushing reflex should be sought.

As written in the Protocol Section each mouse will show cramps, mydriasis and agonal gasps before erection of the tail. In addition, we added that of course brain stem reflexes are missing in case of BD. On the other hand, to our experience regular apnea testing should be avoided as hypotensive phases through hypoxia cannot be countered sufficiently. When establishing the model we found that if all the above mentioned clinical signs occurred the apnea test was 100% positive and therefore not necessary. Furthermore, initial blood pressure rise (Cushing reflex) must be present in BD mice and must be monitored.

2. Figure 1 shows the mean arterial pressure trace with the peak of MAP coinciding with induction of brain death at time zero. It would be more convincing for the mean arterial pressure recording in the anaesthetised ventilated mouse to be shown at baseline, followed by the spike in mean artterial pressure upon induction of BD and the slow decline subsequently. If traces of other important physiological parameters (Heart rate, core temperature, oxygen saturation) are available, they should also be shown.

We agree with the reviewer, that a blood pressure time point prior to BD-induction in the BD group would be more convincingly show the effect of the Cushing reflex. On the other side we believe that time point 0 of the sham control is an adequate time zero, as the sham procedure ends, when the BD-induction starts. Unfortunately, we have no other vital parameters such as pulse/oxygen saturation and heart rate recorded. When we established our model, we checked arterial blood gas for oxygen partial pressure (PaO2) as well as arterial carbon dioxide partial pressure and pH, which all were in range of normal mice.

3. The authors mention that C57BL/6 were the strain of choice for tis procedure and that attempts to establish it in the Balb/c strain were not successful. Could they elaborate on reasons for the lack of success in the additional strain. Could the authors also comment on whether they used male or female C57BL/6 mice (or both), plus the age and pre-operative weight of the animals. Are any of these parameters associated with successful induction of BD and/or relatively stability of haemodynamic stutus during the maintenance phase. What was the average volume of saline admninisrered to maintain MAP? Was pulmonary oedema (neurogenic and/or related to crystalloid administration) ever observed, and if so, was this a reason for procedures to fail?

As now mentioned in the manuscript we used male C57BL/6 mice aged 8-12 weeks with a weight of 20-25 g. Unfortunately, we have not tried other strains than BALB/c and did not vary age/weight/sex of the mice. Therefore, we can just speculate why we were not able to perform this experiment in BALB/c.

On average, we administered 0.8ml of saline. As written, we applied 0.1 ml per 30 minutes. To our experience, more volume did not increase the blood pressure significantly. The question about pulmonary edema cannot final be answered here, as we did not check for lung histology or x-ray. However, to our experience clinically relevant edema would be a surprise. With an average blood volume of 1.5 ml per mouse (25g) (https://www.nc3rs.org.uk/mouse-decision-tree-blood-sampling) the volume of 0.8 ml is half of the total blood volume. Compared to humans this would mean 2.5 liter of crystalloid fluid over a period of 4 hours of surgery, which does not lead to a pulmonary edema. Similarly a study to fluid induced lung injury in rats (Bihari et al, Pflugers Arch, 2017,PMID: 28456852), shows no edema by infusing the double dose (60ml/kg) within 30 minutes. Therefore, we don't think lung edema plays a relevant role when sticking to this volume administration protocol.

4. The main measurement of inflammation shown her in figure 2 is mRNA expression for NKp46. Are there additional measurements which could be shown to support the proposition that a systemic inflammatory state has been created through the induction of BD.

Other effects of BD-induced immunomodulation can be found in former publication (Ritschl et al, J Pathol., 2016, PMID 26890577).

Reviewer #4:

1. There appear to be several mistakes in the arterial catheterization section. These need to be addressed.
2. 1.1 ketamine and xylazine are spelled incorrectly
3. 1.2 there is no need to use both terms "supine" and "upside down"
4. 1.8 there is mention of passing the catheter through a skin "whole" (should be hole), and yet a midline incision has been made. Please clarify the need for this skin hole. Protocol section 3;
5. 3.3 Please clarify inner compact bone. State which skull bone is to be drilled.
6. 3.6 What is the total volume of inflation?

The authors appreciate the comments be the reviewer and answered all of them in the revised manuscript.

Reviewer #5:

1. The changes associated with brain death are systemic, can the author explain why no changes in organs other than the kidneys are demonstrated?

2. Can the author provide more information on the inflammatory response in the organs, such as immunostaining for inflammatory markers and if possible, western blot to validate their findings?

The included figures are intended to be representative figures, which highlight the organ specific influence of BD on solid organ inflammation. For a more detailed analysis I may refer to the publication "Donor brain death leads to differential immune activation in solid organs but does not accelerate ischaemia-reperfusion injury." (Ritschl et al, J Pathol., 2016, PMID 26890577). Concurrent I would like to point towards several publications already published with the demonstrated animal model to proof its properness (Atkinson et al., Circulation, 2013; Floerichinger et al., Transplant Immunology, 2012; Floerichinger et al., Journal of Heart and Lung Transplantation, 2012)

We hope to have addressed the points properly and are grateful for reconsideration of publication of our manuscript.

Yours sincerely,

Paul Ritschl