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Review Editor
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JoVE63502 "A model of reverse vascular remodeling in pulmonary hypertension due to left heart disease by aortic debanding in rats," by Pengchao Sang, Mariya M. Kucherenko, Juquan Yao, Qiuhua Li, Szandor Simmons, Wolfgang M. Kuebler, Christoph Knosalla

Dear Dr. Manoj,

We would like to thank the editor and reviewers for the careful evaluation of our manuscript and the helpful and pertinent comments. We have made changes to the manuscript as requested. Please find attached below a detailed point-by-point response to all comments. All modifications in the manuscript have been highlighted in red.

Sincerely,
Mariya Kucherenko, PhD
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Charitéplatz 1, 10117 Berlin

Response to the Editor:

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

2. Please provide an email address for each author.
Email addresses for each author are provided in the manuscript.

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

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts without brackets after the appropriate statement(s), but before the punctuation.
References are reformatted.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), catalog numbers, and company names before or after an instrument, reagent, or tool. 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 (For example: Janvier labs, Vevo®, Vevo LAB, FUJIFILM VisualSonics, Millar, PowerLab, ADInstruments, F.S.T item No.14568-12, Ethicon, EH7830, AE-BV010R, Sugi Saugtupfer tissue, REF 30601).
All commercial language has been removed from the text, and the Supplementary table was adjusted accordingly.

6. Please ensure that the Introduction also includes the following:
a) The advantages over alternative techniques with applicable references to previous studies
b) A description of the context of the technique in the wider body of literature
c) Information to help readers to determine whether the method is appropriate for their application
In response to this comment, the Introduction has been rewritten to include these points (pages 3-4). The revised passages now read as follows:
Page 3, Lines 93-101: "In comparison to similar heart failure due to pressure overload in the murine model of transverse aortic constriction (TAC) [17], banding of the ascending aorta above the aortic root in AoB rats does not produce hypertension in the left carotid artery as the banding site is proximal of the outflow of the left carotid artery from the aorta. As a result, AoB does not cause left-sided neuronal injury in the cortex as is characteristic for TAC [18], and which may affect study outcome. As compared to other rodent models of surgically induced PH-LHD, rat models in general and AoB in particular prove to be more robust, reproducible, and replicate the remodeling of the pulmonary circulation characteristic for PH-LHD patients, while perioperative lethality is low (reviewed in [19])."

Pages 3-4, Lines 113-126: "Moreover, a limited number of earlier studies have explored the effects of aortic debanding on PH-LHD in rats and showed that aortic debanding may reverse medial hypertrophy in pulmonary arterioles, normalize the expression of pre-pro-endothelin 1 and improve pulmonary hemodynamics [27,28], providing evidence for the reversibility of PH in rats with heart failure. Here, we optimized and standardized the technical procedures of the debanding surgery, e.g. by applying a

tracheotomy instead of endotracheal intubation, or by using titanium clips of a defined inner diameter for aortic banding instead of polypropylene sutures with a blunt needle (as reported in [26,27]), thus providing for better control of the surgical procedures, increased reproducibility of the model and an improved survival rate.

From a scientific perspective, the significance of the PH-LHD debanding model does not solely lie on demonstrating the reversibility of the cardio-vascular and pulmonary phenotype in heart failure, but more importantly in the idenfitication of molecular drivers that trigger and/or sustain reverse remodeling in pulmonary arteries as promising candidates for future therapeutic targeting."

7. Please consider including more figures (besides Figure 3A) to illustrate the surgical steps as the Protocol mainly involves surgery.

Two new figures (new Figure 3 and 5) have been incorporated to better illustrate surgical procedures. References to these and the other figures in the text are readjusted accordingly. New figure legends are added (please also see below).

8. Step 5.1: Please mention if the animal is kept alone in the cage during recovery.

This has been clarified as follows:

Page 6, Line 234: "Only keep one animal per recovery cage at any time".

9. Lines 238-239: The details of median thoracotomy, cardiac catheterization and euthanasia are not given in the present Protocol. Please provide the experimental details or refer to previously published protocol, if any.

In response to this comment, we have included a reference to a published protocol for the thoracotomy, and included additional details on cardiac catheterization and euthanasia into the text as follows:

Page 7, Lines 268-273: "In brief, rats were again anesthetized with ketamine (87 mg/kg bw) and xylazine (13 mg/kg bw), tracheotomized and ventilated as described above. Cardiac catheterization was performed after median thoracotomy [32] through the apex of (first) the left and (second) the right ventricle, respectively, as direct catheterization of the left ventricle via the vascular route is prevented by the aortic band in AoB animals. Following euthanasia by an overdose of ketamine/xylazine, …"

10. Figure 4C-F: The labels "flow before/after the clip" are not discussed in the legend. Please discuss. Also, mention the time point of analyses in the legend.

Changes in figure legend are introduced as follows:

Page 8, Lines 319-324: "Pulsed-wave Doppler echocardiographic images show blood flow before the clip in an AoB rat (C) and blood flow in the corresponding aortic segment in a Deb rat (D) taken one day prior and one day after the aortic debanding surgery, respectively. E-F, Analogously, images show blood flow in the aortic segment after the clip in an AoB rat (E) and in the corresponding aortic segment in a Deb rat (F) taken one day prior and one day after the aortic debanding surgery, respectively".

11. Figure 5B: Please include a scale bar

Done.

12. Figure 5C-F: Please specify with respect to what are the parameters in deb group normalized? Also, please define the error bars.

Done.

Page 9, Lines 348-349: "(in comparison to 3- and 5-week AoB groups) in Deb rats. Boxes show median, 25 and 75 percentiles, respectively; whiskers indicate minimum and maximum values."

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

In response to this comment, the Discussion has been rewritten to include these points. The revised passages now read as follows:

Pages 10-11, Lines 384-424: "The success of the technically challenging aortic debanding procedure in AoB rats depends not only on surgical skills but also on precise perioperative procedures. Outlined in the following are critical steps of the surgical procedure that may cause perioperative lethality by either excessive bleeding (critical steps 1-5) or insufficient respiration (critical step 6) and recommendations on how to avoid these complications:

1. During thoracotomy the midsternal line should be approached carefully with the scissors to avoid injury to the internal mammary artery.
2. In order to visualize the heart and the conduit arteries, the thymus should be mobilized and carefully relocated in cranial direction. In the debanding surgery, the thymus tissue is often found connected to the heart and arteries via post-operative adhesions from the original AoB surgery. These adhesions should be carefully separated with a pair of blunt forceps to avoid injury of the cardiovascular structures.
3. In the debanding surgery, the aorta with the clip is frequently embedded in connective tissue. To visualize the clip, this connective tissue must be gently dissected with a blunt forceps. Here, the transthoracic echocardiography performed prior to the surgery is a helpful preparation step, allowing to identify in advance whether the clip is located close to the aortic root, in the middle of the ascending aorta, or close to the brachiocephalic artery. This knowledge saves precious time for clip allocation during the surgery.
4. The orientation of the clip is a critical step that must be considered carefully during the initial aortic banding surgery. To facilitate optimal assessment and rapid opening of the clip during aortic debanding, the part of the clip that needs to be compressed by the needle holder (Figure 4B) should be oriented ventrally. Clip reorientation during debanding surgery is feasible, although at the risk of injury to the aorta. For clip reorientation, clips should be held by a forceps while surrounding connective tissue is carefully removed, then the clip should be mobilized and turned. Holding the aorta with the forceps is to be avoided.
5. For debanding the clip should be held by a forceps with one hand and opened with a needle holder by the other hand. The aorta should not be lifted ventrally.
6. After successful completion of the debanding procedure, extubated PH-LHD rats are at considerable risk of respiratory failure, with animals commonly dying within 10-20 min after surgery while still under the anesthesia. Atelectasis of the left lung is the most common cause of death in this period, and prolonged mechanical ventilation prior to chest closure helps to recruit the lung and to warrant sufficient respiration after surgery.

The present study reports an aortic debanding technique performed 3 weeks after initial aortic banding in rats. For studies aiming to compare reverse remodeling of the pulmonary vasculature and the RV at different stages of PH, the described procedures may also be performed at later time points after aortic banding, yet caution is warranted as scar and connective tissue surrounding the aorta is likely to become more abundant with time, further complicating the procedure and necessitating additional troubleshooting and refinement, while the basic principles of the reported protocol still apply."

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 5 authors, list only the first author then et al. Please expand journal names.
Done.

Response to Reviewer 1:

Manuscript Summary:
In this manuscript, the authors report a surgical procedure to remove ascending-aortic constriction in rat model of pulmonary hypertension due to left heart diseases by aortic banding. This technique allows
studying endogenous mechanisms of reverse remodeling in the pulmonary circulation and the right heart, informing strategies to reverse pulmonary hypertension and/or right ventricular dysfunction.

Major Concerns:
No major concerns. The methods are clearly explained and well organized. Readers should be able to follow the protocol and reproduce the results presented in the discussion.
We thank the reviewer for the positive evaluation of our manuscript and the encouraging comments.

Minor Concerns:
Introduction can be simplified and focused on how this model compares to other available models of PH-LHD.
We thank the reviewer for this constructive comment. In response, we have revised the introduction to discuss the current model in the context of other models of heart failure and debanding, respectively. The revised passages now read as follows:

Page 3, Lines 93-101: "In comparison to similar heart failure due to pressure overload in the murine model of transverse aortic constriction (TAC) [17], banding of the ascending aorta above the aortic root in AoB rats does not produce hypertension in the left carotid artery as the banding site is proximal of the outflow of the left carotid artery from the aorta. As a result, AoB does not cause left-sided neuronal injury in the cortex as is characteristic for TAC [18], and which may affect study outcome. As compared to other rodent models of surgically induced PH-LHD, rat models in general and AoB in particular prove to be more robust, reproducible, and replicate the remodeling of the pulmonary circulation characteristic for PH-LHD patients, while perioperative lethality is low (reviewed in [19])."

Pages 3-4, Lines 113-126: "Moreover, a limited number of earlier studies have explored the effects of aortic debanding on PH-LHD in rats and showed that aortic debanding may reverse medial hypertrophy in pulmonary arterioles, normalize the expression of pre-pro-endothelin 1 and improve pulmonary hemodynamics [27,28], providing evidence for the reversibility of PH in rats with heart failure. Here, we optimized and standardized the technical procedures of the debanding surgery, e.g. by applying a tracheotomy instead of endotracheal intubation, or by using titanium clips of a defined inner diameter for aortic banding instead of polypropylene sutures with a blunt needle (as reported in [26,27]), thus providing for better control of the surgical procedures, increased reproducibility of the model and an improved survival rate.

From a scientific perspective, the significance of the PH-LHD debanding model does not solely lie on demonstrating the reversibility of the cardio-vascular and pulmonary phenotype in heart failure, but more importantly in the idenfitication of molecular drivers that trigger and/or sustain reverse remodeling in pulmonary arteries as promising candidates for future therapeutic targeting."

Response to Reviewer 2:

I. Manuscript Summary:

Firstly, I do thank you for giving me the important chance to read the interesting article, in which the debanding was well written. In fact, that there were already some articles in which various debanding procedures were reported in rat models with pulmonary hypertension secondary to left heart failure by banding aorta. For example, it has been reported earlier that debanding could result in both reversal of PH and attenuation of the expression of ET-1 in the aortic banded group (Exp Biol Med (Maywood). 2006 Jun;231(6):954-9).

We thank the reviewer for the positive evaluation of our manuscript and the reference to the 2006 paper by Chou and coworkers, which has now been included into the revised manuscript as reference **28** Chou, S. H. et al. The effects of debanding on the lung expression of ET-1, eNOS, and cGMP in rats with left ventricular pressure overload. Exp Biol Med (Maywood) 231, 954-959 (2006).

II. Major concerns:

I have some suggestions as follow;

1) I just wonder whether there could be different hemodynamic effects of banding between 1 cm above aortic root and transvere aorta.

We thank the reviewer for raising this relevant question. In the revised manuscript, we have added new text to address this point as follows:

Page 3, Lines 93-98: "In comparison to similar heart failure due to pressure overload in the murine model of transverse aortic constriction (TAC) [17], banding of the ascending aorta above the aortic root in AoB rats does not produce hypertension in the left carotid artery as the banding site is proximal of the outflow of the left carotid artery from the aorta. As a result, AoB does not cause left-sided neuronal injury in the cortex as is characteristic for TAC [18], and which may affect study outcome."

2) I would suggest to do directly endotracheal intubation instead of tracheostomy while debanding.

We thank the reviewer for this suggestion. Indeed, previous studies on aortic debanding performed direct endotracheal intubation instead of tracheostomy during the debanding surgery (References **26**,**27**). We suggest, however, that in comparison to endotracheal intubation tracheostomy provides for better control of appropriate ventilation during the surgical procedures, which is specifically relevant during aortic debanding. This notion is based on the following rationale:

- Tracheostomy, which is routinely perform in our lab for perioperative lung ventilation, is a straightforward and safe technique with no perioperative or postoperative complications.
- Tracheostomy eliminates the risk of esophageal intubation or tracheal injury; it enables precise positioning and fixation of the tracheal cannula and constant visual control of the cannula during all steps of the surgical procedure.
- At the time of aortic debanding, AoB animals are already in heart failure and as such more sensitive to additional stress; as a result the potential risks that come with endotracheal intubation may add to increased lethality.
- Finally, when the operated animal is weaned from the ventilator but fails to develop spontaneous breathing, a tracheostomy allows for rapid reintubation and reconnection with the ventilator, thus potentially saving lives due to the ability for prolonged postoperative ventilation.

Based on these rationales, we would advocate for tracheostomy rather than endotracheal intubation for this specific and complex surgical intervention. As such, we have left the respective text passages referring to tracheostomy unchanged in the revised manuscript.

III. Minor concerns:

Please check the alterations of brain natriuretic peptide (BNP) in plasma and left ventricle for approving the improvement of left ventricular dysfunction.

We thank the reviewer for this insightful suggestion to verify the improvement of left ventricular function after debanding. We have gladly followed the reviewer´s advice and measured BNP expression in the left ventricle by Western blotting, which revealed an increase in BNP in AoB as compared to sham animals, which was, however, normalized by debanding. Unfortunately, we could not perform BNP measurements in corresponding plasma samples, as plasma had originally not been collected in our study. However, in addition to BNP data, we now provide additional data on left ventricular ejection fraction and volume as assessed by echocardiography in sham, AoB and Deb groups. These new results confirm the improvement of left ventricular function following debanding, and are now presented in new Figure 7 and the corresponding legend (New Figure and corresponding legends is below), and incorporated in the Results section as follows:

Page 7, Lines 256-265: "To probe for reversal of left heart failure by aortic debanding, we assessed the expression levels of brain natriuretic peptide (BNP), a clinical routine parameter for the assessment of heart disease[8], in the LV myocardium. At week 3 and 5 after aortic banding AoB animals showed a significantly increased production of BNP in comparison to sham operated controls, while Deb rats at week 5 expressed BNP at levels comparable to sham animals, indicating the reversal of LV failure by aortic debanding (Figure 7A-C). In parallel, evaluation of LV function by transthoracic echocardiography revealed an increased LV ejection fraction and LV volume in Deb animals as compared to AoB rats (Figure 7D-E). While LV ejection fraction in Deb animals was comparable to sham rats, LV volume in Deb rats failed to fully normalize to values seen in the sham group, indicating that reversal of LV function is incomplete."

New Figures:
Figure 3

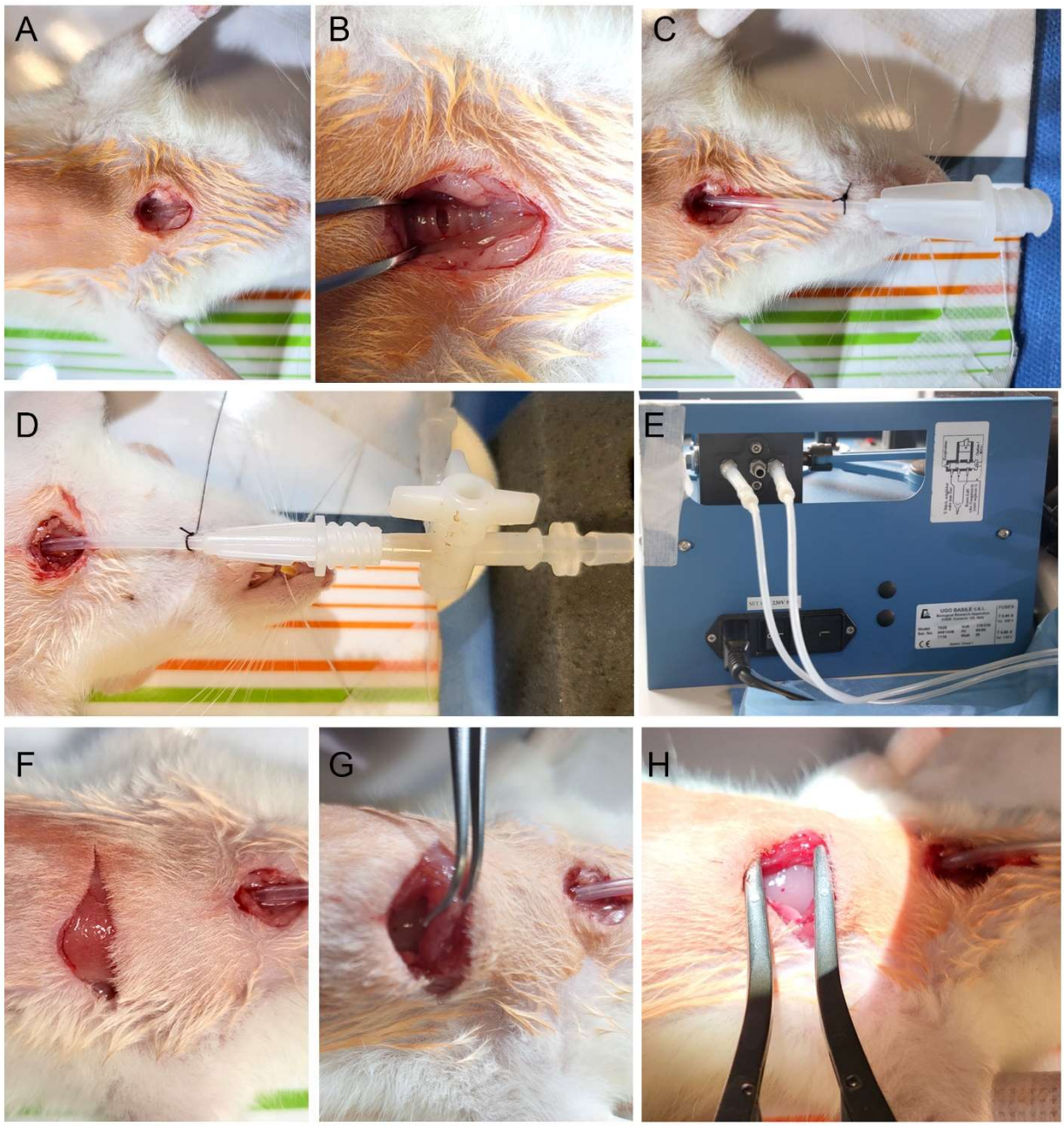

Figure 3. Tracheotomy and thoracotomy
Imagess illustrate the surgical steps for the tracheotomy: **A**, cervical midline incision; **B**, incision of the trachea between two cartilaginous rings; **C**, tracheal cannula inserted into the trachea and secured with a suture; **D**, tracheal cannula connected to a mechanical ventilator (shown in **E**). Images illustrate the surgical steps for the thoracotomy: **F**, skin incision between the second and third ribs; **G**, cutting of muscles; **H**, creation of a thoracic surgical window by spreading the second and the third ribs.

Figure 5

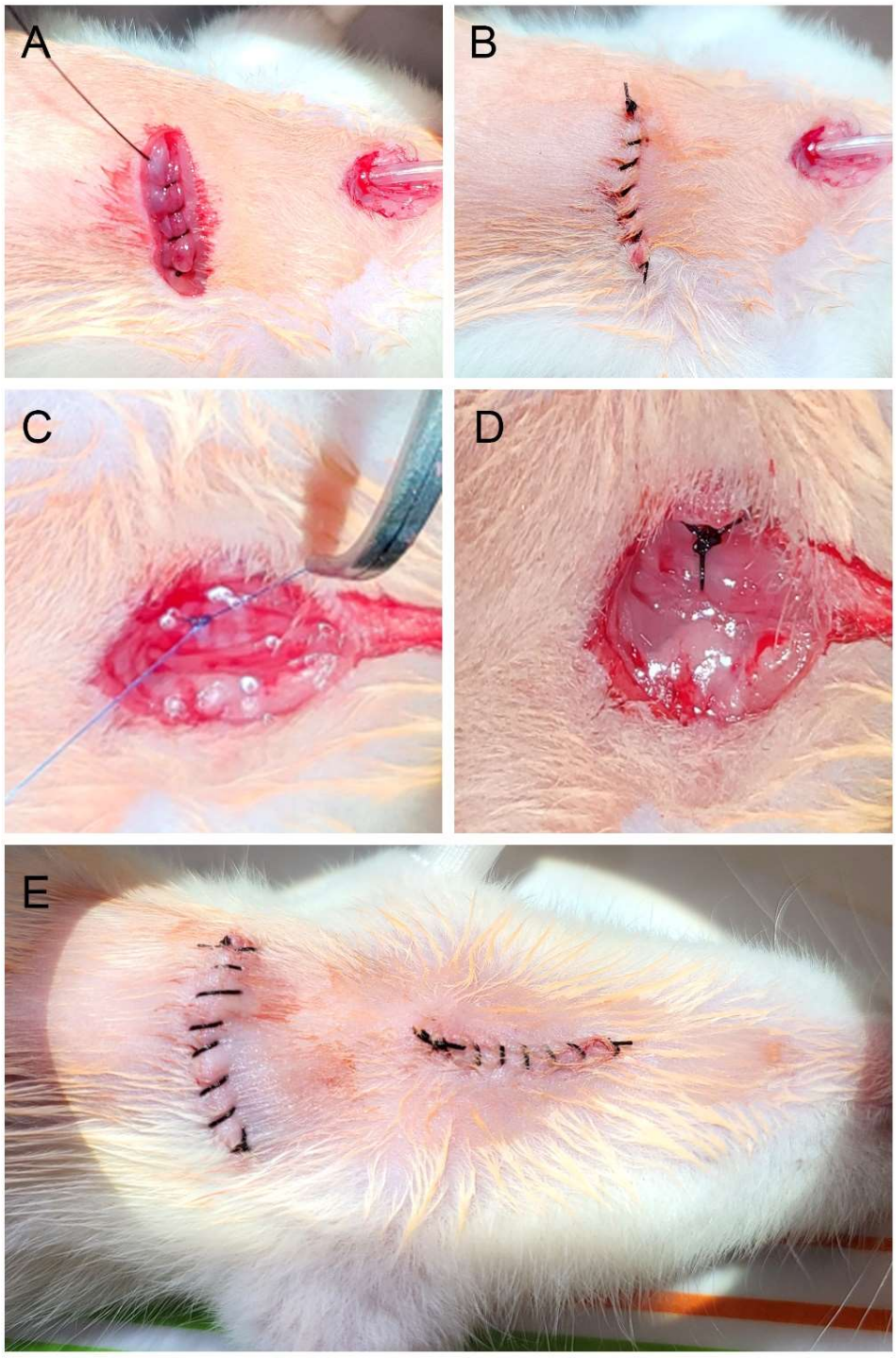

Figure 5. Wound closure
Images illustrate closing of the thoracic upper muscles (**A**) and the skin (**B**) with a simple continuous suture. The trachea (**C**) and the infrahyoid muscles (**D**) are closed by a simple suture and the skin on the neck (**D**) by a simple continuous suture.

Figure 7

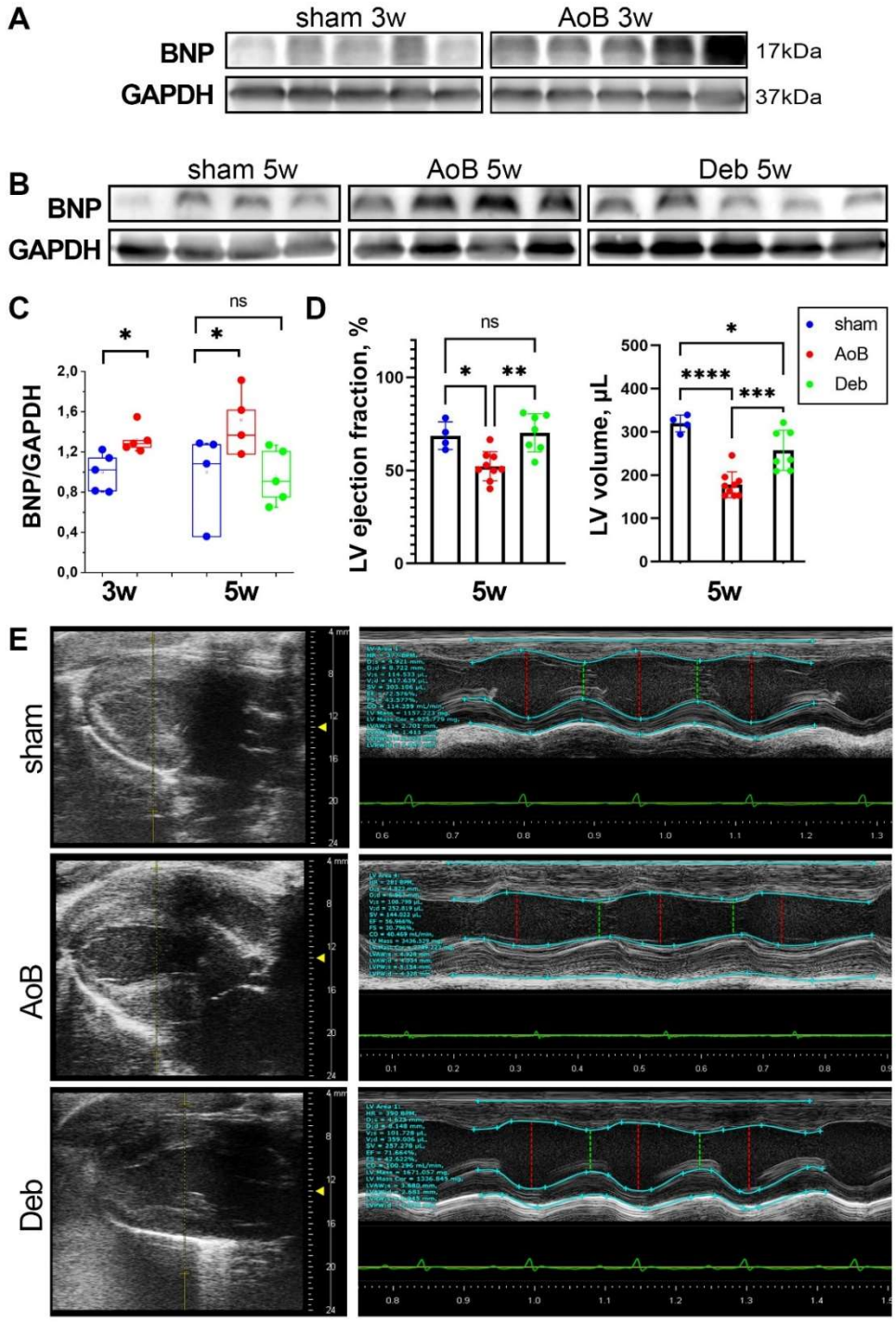

Figure 7. Normalization of left ventricular function by aortic debanding
A, Representative Western blots show protein levels of BNP and with GAPDH as loading control in left ventricles (LV) of AoB rats at week 3 after aortic banding (n=5) and in corresponding sham controls (n=5). **B**, Representative Western blots show BNP and GAPDH in left ventricles (LV) of AoB rats at week 5 after aortic banding (n=4), in Deb rats at week 5 (n=5),

and in sham controls at the corresponding time after primary surgery (n=4). **C**, Box and whisker plots show quantification of BNP expression normalized to GAPDH and sham control at the corresponding time after primary surgery. Boxes show median, 25 and 75 percentiles, respectively; whiskers indicate minimum and maximum values. For statistical analyses Student's t-test was used *, *p*-value < 0.05. **D**, Bar graphs (mean±standard deviation) show LV ejection fraction and volume in sham (n=4), AoB (n=9) and Deb (n=7) animals at week 5 as measured by echocardiography from M- and B-mode images. **E**, Representative M-mode echocardiographic images show changes in LV fractional shortening in sham, AoB and Deb animals at week 5. For statistical analyses Mann–Whitney U test was used *, *p*-value < 0.05.