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## A pulmonary trunk banding model of pressure overload induced right ventricular hypertrophy and failure

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**TITLE:**

A Pulmonary Trunk Banding Model of Pressure Overload Induced Right Ventricular Hypertrophy and Failure

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**KEYWORDS:**

Animal Model; Rats; Right Ventricular Hypertrophy; Right Ventricular Failure; Pulmonary Trunk Banding; Pulmonary Hypertension; Congenital Heart Disease

**SHORT ABSTRACT:**

We present a surgical method to induce right ventricular hypertrophy and failure in rats.

**LONG ABSTRACT:**

Right ventricular (RV) failure induced by sustained pressure overload is a major contributor to morbidity and mortality in several cardiopulmonary disorders. Reliable and reproducible animal models of RV failure are therefore warranted in order to investigate disease mechanisms and effects of potential therapeutic strategies. Banding of the pulmonary trunk is a common method to induce isolated RV hypertrophy but in general, previously described models have not succeeded in creating a stable model of RV hypertrophy and failure.

We present a rat model of pressure overload induced RV hypertrophy caused by pulmonary trunk banding (PTB) that enables different phenotypes of RV hypertrophy with and without RV failure. We use a modified ligating clip applier to compress a titanium clip around the pulmonary trunk to a pre-set inner diameter. We use different clip diameters to induce different stages of disease progression from mild RV hypertrophy to decompensated RV failure.

RV hypertrophy develops consistently in rats subjected to the PTB procedure and depending on the diameter of the applied banding clip, we can accurately reproduce different disease severities ranging from compensated hypertrophy to severe decompensated RV failure with extra-cardiac manifestations.

The presented PTB model is a valid and robust model of pressure overload induced RV hypertrophy and failure that has several advantages to other banding models including high reproducibility and the possibility of inducing severe and decompensated RV failure.

## **INTRODUCTION:**

The right ventricle (RV) can adapt to a persistent pressure overload. In time, however, adaptive mechanisms fail to sustain cardiac output, the RV dilates and eventually the RV fails. RV function is the main prognostic factor of several cardiopulmonary disorders including pulmonary arterial hypertension (PAH), thromboembolic pulmonary hypertension (CTEPH), and various forms of congenital heart disease with a pressure (or volume) overload of the RV. Despite intense treatment, RV failure remains a predominant cause of death in these conditions.

As a consequence of the unique properties<sup>1,2</sup> and embryological development<sup>3</sup> of the RV, knowledge derived from left heart failure cannot simply be extrapolated to right heart failure. Animal models of right heart failure are therefore needed in order to investigate the mechanisms of RV failure and potential pharmacological treatment strategies.

There are experimental models of pulmonary hypertension induced by SU5416 combined with hypoxia (SuHx)<sup>4</sup> or monocrotaline (MCT)<sup>5</sup>, which induce RV failure secondary to disease in the pulmonary vasculature. These models are used to evaluate therapeutic effects of drugs that target the pulmonary vasculature. Both the SuHx and the MCT model are non-fixed afterload models of RV failure. Consequently, it is not possible to conclude if an improvement in RV function after an intervention is secondary to afterload reducing pulmonary vascular effects or if it is caused by direct effects on the RV. In addition, the MCT model has several extra-cardiac effects.

In experimental pulmonary trunk banding models, the afterload of the RV is fixed due to a mechanical constriction of the pulmonary trunk. This allows for the investigation of direct cardiac effects of an intervention on the RV independent from any pulmonary vascular effects<sup>6-9</sup>. Usually, the banding is performed by placing a needle along the pulmonary trunk. Then a ligature is placed around the needle and the pulmonary trunk and tied with a knot, and the needle is removed leaving the suture around the pulmonary trunk. Depending on the gauge of the needle, different degrees of constrictions can be applied, but despite this approach being widely used, it has some disadvantages. First, the diameter of the banding is not exactly the same as the outer diameter of the needle as the ligature is tied around both the needle and the pulmonary trunk. Second, there may be significant variation to how tightly the knot is tied making it difficult to reproduce a certain degree of banding. This will lead to a variation in banding diameter and thereby a larger dispersion. Finally, the knot may come loose over time.

One study applies a half-closed tantalum clip around the pulmonary trunk<sup>10</sup>. They compressed the clip around the pulmonary trunk to an inner area of 1.10 mm<sup>2</sup> and compared it to rats subjected to banding with a suture using an 18G needle. Overall, banding with the clip was associated with less peri-surgical complications and data variance.

Based on the principles described by Schou *et al.*<sup>11</sup>, we further developed and characterized the pulmonary trunk banding (PTB) model of RV hypertrophy and failure. Here, we present our experience using this model based on results from previous studies<sup>12,13</sup>. For this model, a titanium clip is compressed around the pulmonary trunk to an exact preset inner diameter, which may be adjusted in order to induce distinct RV failure phenotypes.

## **PROTOCOL:**

All rats were treated according to Danish national guidelines, and all experiments were approved by the Institutional Ethics Review Board and conducted in accordance with the Danish law for animal research (authorization number 2012-15-2934-00384, Danish Ministry of Justice).

### **1. Adjustment of the Ligating Clip Applier**

Note: The banding of the pulmonary trunk is performed with a modified open ligating clip applier with an angled jaw. The applier is modified with an adjustable stop mechanism to stop the compression when the jaws reach an exact distance from each other. When a small titanium ligating clip is compressed with the modified applier, a lumen persists between the legs of the clip with a specific diameter according to the adjustment of the stop mechanism (**Figure 1**).

1.1. Choose the diameter of the desired banding, *e.g.*, 0.6 mm.

1.2. Adjust the ligating clip applier until the distance between the jaws is 1.0 mm when fully compressed. This leaves a lumen of 0.6 mm as the two clip legs have a thickness of 0.2 mm each.

[Place **Figure 1** here]

### **2. Preparation of the rat**

Note: Other regimens of analgesics may be applied.

2.1. Use Wistar rat weanlings weighing approximately 100-120 g. In order to maintain body temperature during the surgery, use a covered heating pad.

2.2. For surgery, use a mechanical ventilator set to a tidal volume of approximately 1.75 mL and respiratory rate of 75 per min.

2.3. Anaesthetize the rat with sevoflurane (7% mix in 1.5 L of O<sub>2</sub>) in an induction chamber for 5 minutes. Intubate the rat using a 17G IV cannula, where the distal 2 mm of the needle have been cut off in order for the soft catheter to cover the tip. Remove the needle and connect the cannula to the ventilator.

2.4. Place the rat on its back on the heating pad. Make sure that the intubation is correct by observing the movements of the thorax. These should be without side differences and in pace with the ventilator.

Note: Absence of movements of the thorax, abdominal contractions and inflation of the stomach in the upper left abdomen are signs of a misplaced tube. Remove the cannula, put the rat back in the induction chamber and re-intubate.

2.5. After correct intubation, reduce the sevoflurane to maintenance concentration (3.5% mix in O<sub>2</sub>, 1.5L/min) and fix the paws of the rat to the heating pad.

2.6. Confirm proper anesthetization by checking withdrawal reflexes of the extremities using a forceps to squeeze the paws of the rat.

2.7. Inject the rats with buprenorphine (0.1 mg/kg subcutaneously (s.c.)) and carprofene (5 mg/kg s.c.) to relieve post-operative pain.

2.8. Shave the chest and disinfect with chlorhexidine.

### **3. Isolation of the pulmonary trunk**

3.1. With a pair of scissors, make a 1 cm incision in the skin along the middle part of the sternum. Identify the major pectoral muscle and cut its sternal attachment. Identify the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> costa below.

Note: This can be useful if bleeding from the anterior thoracic artery is a recurrent issue.

3.2. Optionally, grab the 2<sup>nd</sup> costa with a fixation forceps, put a suture (4-0, multifilament, absorbable) around the 2<sup>nd</sup> costa from the 1<sup>st</sup> intercostal space to the lower medial part of the 2<sup>nd</sup> intercostal space. Tie a firm knot in order to ligate the anterior thoracic artery.

3.3. Cut the 4<sup>th</sup>, 3<sup>rd</sup>, and 2<sup>nd</sup> costa close to the sternum with a pair of scissors and carefully dissect the intercostal muscles until a complete left thoracotomy has been performed. If any bleeding from the anterior thoracic artery occurs, compress with a pean and ligate the artery.

3.4. Insert a retractor between the sternum and the costae and open it to get a good operating field. At the top of the field is the thymus covering the aorta and the pulmonary trunk. Carefully lift the thymus using a pean and flip it upwards in order to expose the aorta and the pulmonary trunk below.

3.5. Guide the tip of a small surgical hooklet with a 85° angle through the transverse pericardial sinus located behind the left atrial appendage. Pull it halfway back through the sinus and guide the tip of the ear hook upwards until it appears between the ascending aorta and the pulmonary trunk.

3.5.1. Remove any connective tissue covering the tip with an iris scissor in order to separate the pulmonary trunk from the ascending aorta.

3.5.2. Repeat the step with a larger hook (optional).

3.6. Grab the end of an approximately 10 cm ligature (4-0, multifilament) with an angled muscle forceps and guide it around the pulmonary trunk through the passage made with the hooks. Seize the end of the ligature with another forceps and withdraw the first one. Now the pulmonary trunk is separated from the ascending aorta and can be controlled by the ligature around it.

#### **4. Application of the Clip**

4.1. Load the adjusted ligating clip applier with a clip. Carefully guide one of the jaws and one leg of the clip through the passage around the pulmonary trunk. Use the ligature to gently pull the pulmonary trunk upwards and into the fork of the clip.

4.2. When the pulmonary trunk is in the fork of the clip and the two tips of the clip legs are free of any connective tissue, compress the clip with the applier to apply the banding.

4.3. Observe how the RV immediately dilates in response to the banding and remove the ligature.

#### **5. Closing of the Thorax**

5.1. Remove the pean from the thymus and reposition it to its natural position. Remove the retractor.

5.2. Close the thorax in three layers: the intercostal layer, the major pectoral muscle, and the skin with suture (4-0, multifilament, absorbable). Inject 2 mL of saline s.c. to replace fluid lost during surgery.

5.3. Turn the sevoflurane off and extubate the rat when it starts breathing spontaneously.

5.4. Treat the rats with buprenorphine (7.4 µg/mL) in the drinking water for the following three days or apply a similar analgesic protocol. After three days, the rats have recovered and are without discomfort.

## 6. Sham Surgery

6.1. Perform a sham surgery by following all of the steps above except for the application of the clip (step 4).

### REPRESENTATIVE RESULTS:

Using the described PTB procedure in previous studies from our group<sup>12,13</sup>, we induced RV hypertrophy (PTB mild) by banding with a 1.0 mm clip, a moderate degree of RV failure (PTB moderate) by banding with a 0.6 mm clip and a severe degree of RV failure (PTB severe) by banding with a 0.5 mm clip. The rats subjected to the severe banding developed extra-cardiac manifestations of RV failure including liver failure and ascites (**Figure 5E**). All rats were evaluated seven weeks after the PTB and sham rats underwent the same procedure only without the application of the clip. Perioperative mortality was less than 1 in 6. Seven weeks survival rate was 80% for rats subjected to severe banding and close to 100% in rats subjected to mild or moderate banding or sham surgery.

For evaluation of the effects of the PTB procedure, we used echocardiography together with cardiac magnetic resonance imaging (MRI) to assess RV volumes and cardiac output. Tricuspid annular plane systolic excursion (TAPSE) was measured as the distance of the tricuspid annular plane with the RV contraction in the apical four-chamber view. An average of three cycles outside respiration was used as a representative value. RV end-diastolic volume (EDV) and end-systolic volume (ESV) was assessed by drawing of the endocardium in a series of short axis images through the RV obtained by MRI for each rat and RV ejection fraction (EF) calculated as  $EF = (EDV - ESV) / EDV$ . Cardiac output was measured between the pulmonary valves and the clip using a phase-contrast MRI sequence. Digital recordings of RV pressures were obtained by a micro tip catheter installed in the RV before euthanasia. Further details of the methods have been described previously<sup>12</sup>. RV hypertrophy was evaluated as the ratio of the RV weight divided by the weight of the left ventricle (LV) plus septum and as the weight of the RV divided by the length of the tibia to correct for the size of the rat. All methods were applied as described previously<sup>12</sup>.

In one week, elevated RV pressures and RV dysfunction evident by a decrease in cardiac output and TAPSE had developed in the PTB rats compared to sham operated rats. Accordingly, interventions or pharmacological treatments can be initiated already at this time point if one aims to investigate effects on established RV failure. After additional six weeks, RV pressure had further increased. The differences of the moderate vs the severe RV failure phenotype were even more pronounced shown by a stepwise decrease in both cardiac output and TAPSE with increased severity of the banding (**Figures 2 and 3**). Detailed hemodynamic differences between PTB mild rats and PTB severe rats 4 weeks after surgery have been published by our group previously<sup>14</sup>.

The PTB procedure also caused RV dilatation evident by an increase in both RV EDV and RV ESV in the moderate PTB rats compared to sham operated rats and in the severe PTB rats compared to both moderate PTB and sham. A stepwise decrease in RV EF was also seen (**Figure 4**).

The development of RV hypertrophy was associated to the magnitude of the pressure overload applied by the clip. The ratio of the RV over the LV plus septum weight increased stepwise from rats with a mild banding with a clip of 1.0 mm over rats banded with a moderate 0.6 mm clip to rats banded with a severe 0.5 mm clip. Similar results were seen for the RV weight corrected for the size of the rat by dividing with the length of the tibia. The hypertrophy was also seen as an increase in cardiomyocyte cross sectional area in the PTB rats compared to sham operated rats. Apart from hypertrophy of the cardiomyocytes, the pressure overload also induced other morphological changes of the RV associated with RV failure including RV fibrosis. In rats subjected to severe banding, decompensated RV failure was induced. This phenotype was characterized by signs of backward failure including hepatic congestion seen as a dark discoloration of the liver. Hepatic congestion was usually accompanied by ascites (**Figure 5**).

#### **FIGURE AND TABLE LEGENDS:**

##### **Figure 1: The PTB procedure.**

(A) The surgical instruments used for the PTB procedure including the ligating clip applier (blue arrow). (B) The adjustable stop mechanism of the ligating clip applier. Turning the cogwheel (blue arrow) will adjust the position of the pin (yellow arrow), which stops the closing of the applier when the jaws reach a certain distance from each other. The distance corresponds to twice the thickness of the legs of the clip plus the inner diameter of the clip, when the clip is compressed, and can be calibrated by using for example a needle with a known outer diameter. (C) The applier compresses a titanium clip to an exact inner diameter pre-specified by adjustment of the applier. (D) The inner diameter of the compressed clip can be adjusted in order to induce different severities of RV hypertrophy and failure. For the data presented, an inner diameter of 1.0 mm was used to induce mild RV hypertrophy, an inner diameter of 0.6 mm was used to induce moderate RV failure, and an inner diameter of 0.5 mm was used to induce severe RV failure. (E) The clip after application around the pulmonary trunk.

##### **Figure 2: Effects of PTB one week and seven weeks after the procedure.**

(A) Right ventricular (RV) systolic pressure (B) cardiac output and (C) tricuspid annular plane systolic excursion (TAPSE) measured one week after sham or PTB operation with a moderate or a severe banding respectively. (C, D and E) The same measures seven weeks after the procedures and further development of RV failure<sup>12</sup>. Data presented as mean  $\pm$  SEM. One-way ANOVA with *post hoc* Bonferroni analysis. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$  PTB vs sham and PTB severe vs PTB moderate.

##### **Figure 3: Effects of the PTB procedure assessed by echocardiography.**

(A) A representative four chamber view and (B) measurements of velocity time integral (VTI) in the pulmonary trunk (upper panel) and tricuspid annular plane systolic excursion (TAPSE)



(lower panel) in a sham operated rat. (C and D) Similar images for a PTB rat subjected to moderate banding. All images are seven weeks after sham operation or banding.

#### **Figure 4: Magnetic resonance imaging of PTB rats.**

Cardiac magnetic resonance imaging (MRI) of PTB and sham operated rats. (A) Representative four chamber images (upper panel) and short axis views (lower panel) of sham rats (left) and PTB rats with a moderate degree of RV failure (right). In the PTB rat, the high RV pressures caused septum bulging (blue asterisk). (B) The PTB procedure induced RV dilatation evident by an increase in both RV end diastolic volume (EDV) and RV end systolic volume (ESV). (C) RV ejection fraction (EF) decreased<sup>12</sup>. Data presented as mean  $\pm$  SEM. n = 11-13 per group. One-way ANOVA with *post hoc* Bonferroni analysis. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\*\* $p < 0.0001$  PTB vs sham and PTB severe vs PTB moderate.

#### **Figure 5: Anatomical data and histology.**

RV hypertrophy measured as (A) RV divided by the LV plus septum and (B) RV weight divided by tibia length from rats with mild RV hypertrophy, moderate RV failure, and severe RV failure seven weeks after the PTB procedure. (C) Representative images of histological sections stained with hematoxyline eosine for measurement of cardiomyocyte cross sectional area and (D) picosirius red analyzed under polarized light for fibrosis from sham rats (left) and PTB rats with moderate RV failure (right). (E) A healthy liver (left) and a discolored liver with congestion (right) from a PTB rat with severe RV failure<sup>12,13</sup>. Data presented as mean  $\pm$  SEM. One-way ANOVA with *post hoc* Bonferroni analysis. \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  PTB vs sham, PTB moderate vs PTB mild and PTB severe vs PTB moderate.

#### **DISCUSSION:**

We describe an accessible and highly reproducible method of pulmonary trunk banding using a modified ligating clip applier to compress a titanium clip around the pulmonary trunk. By adjusting the applier to compress the clip to different inner diameters, distinct phenotypes of RV hypertrophy and failure can be induced including severe RV failure with extra-cardiac manifestation of decompensation.

Although simple, the protocol contains a few critical steps. Importantly, the rats cannot be too big when they undergo the PTB procedure. In our experience, Wistar rat weanlings weighing 100-120 g are suitable for the procedure. In larger rats, application of a severe banding may lead to acute RV failure and death. Other studies<sup>6-10,15</sup> have mainly used larger rats (160-260 g) but also larger diameters of their respective bandings (1.27-1.65 mm).

The application of banding of less severity may also explain the relative modest increases in RV pressures reported by other groups. Banding with an 18G needle (1.27 mm) leads to RV systolic pressures in the range of 70-90 mmHg<sup>6-9</sup>. In one study<sup>6</sup>, this was not sufficient to cause RV fibrosis or reduce cardiac output. Here, we report RV pressures of approximately 90 mmHg for a moderate banding and 110 mmHg for a severe banding. With a severe banding, we have also been able to create a phenotype of decompensated RV failure with extra-cardiac manifestations including hepatic congestion and ascites<sup>12</sup>. Pulmonary trunk banding by ligation

using a 20G needle (0.902 mm) caused liver fibrosis, nutmeg-like liver congestion and ascites in Sprague Dawley rats<sup>15</sup> despite the relatively mild constriction compared to our study. This may be explained by different rat strains responding differently to the banding. There are significant differences with regards to metabolism<sup>16</sup>, adrenergic tone and heart rate<sup>17</sup> between rat strains. Even within the same rat strain, various characteristics including growth rate may vary with different vendors<sup>18</sup>. This should always be taken into account. For the specific rat strain used, it is therefore critical that well-designed pilot studies are performed in order to determine the banding diameter and the follow-up time needed for the desired RV failure phenotype to develop. The clip model can potentially be used in rat neonates as opposed to the ligating technique previously used<sup>19</sup>, but we have no experience with this and the same considerations as mentioned above applies before initiating a study.

The PTB model has a few limitations. Firstly, the very proximal occlusion by the clip around the pulmonary trunk represents the conditions of pulmonic stenosis or CTEPH more than the distal narrowing of the smaller pulmonary arteries seen in PAH. The adaptation of the RV to the increased afterload may vary depending on the location of the obstruction(s)<sup>20</sup>. Secondly, the application of the clip during surgery causes a very sudden increase in RV afterload different from the gradual increase in pulmonary vascular resistance in PAH. The procedure is, however, performed in rat weanlings (100-120 g) giving rise to a progressively increased RV afterload relative to body weight with growth of the animals. During the seven weeks period after surgery, the body weight of the rats increases approximately fourfold and hence relative RV afterload increases proportionally inducing a progressive disease development<sup>6</sup>.

Using a modified ligating clip applier and a titanium clip for banding of the pulmonary trunk, we were able to induce RV failure. The method has several advantages including high reproducibility and the possibility of creating different disease severities from mild RV hypertrophy to decompensated RV failure by adjusting the diameter of the banding clip. Changing the diameter by 0.1 mm resulted in distinct RV failure phenotypes ranging from moderate and compensated RV failure to severe and decompensated RV failure demonstrating accuracy of this pulmonary trunk banding method.

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#### **DISCLOSURES:**

The authors have nothing to disclose

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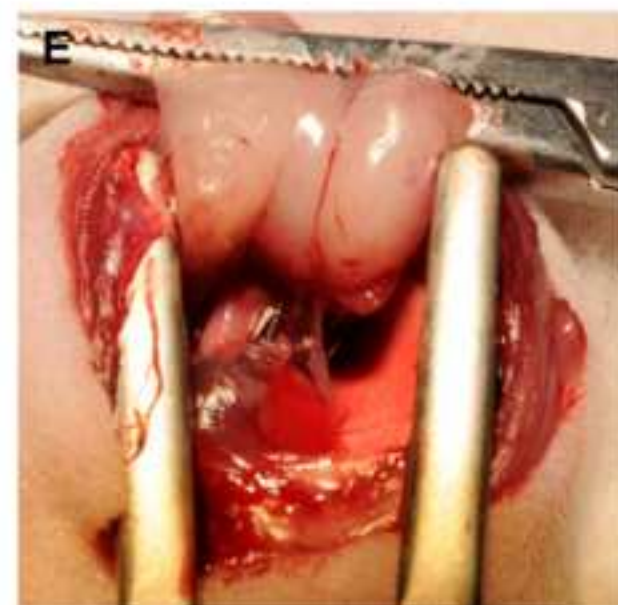
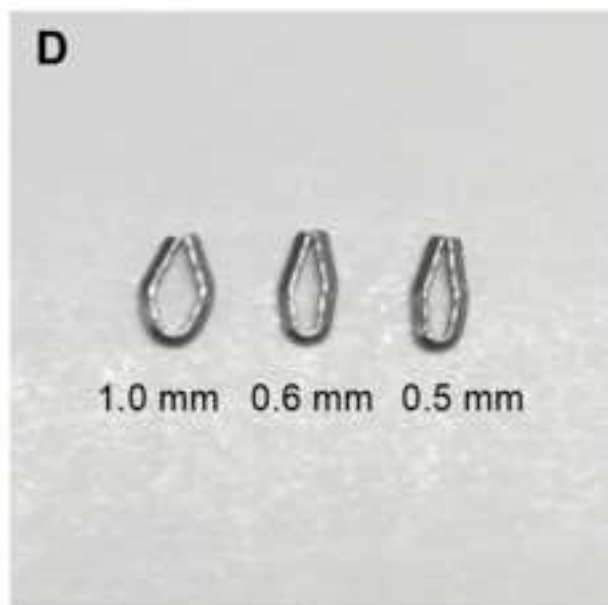
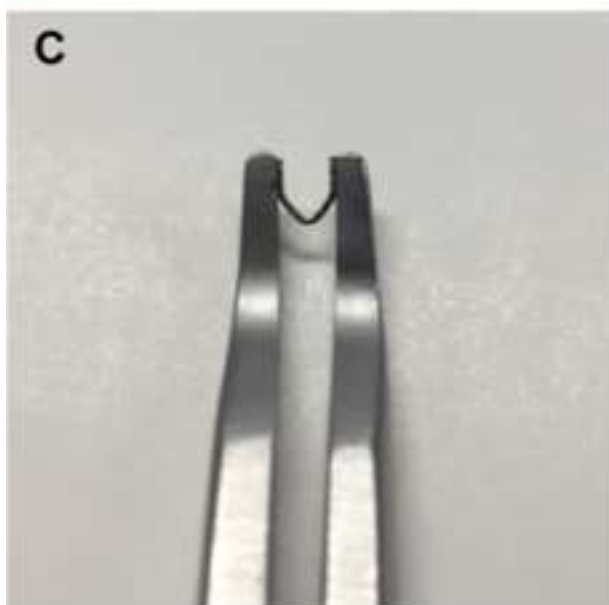
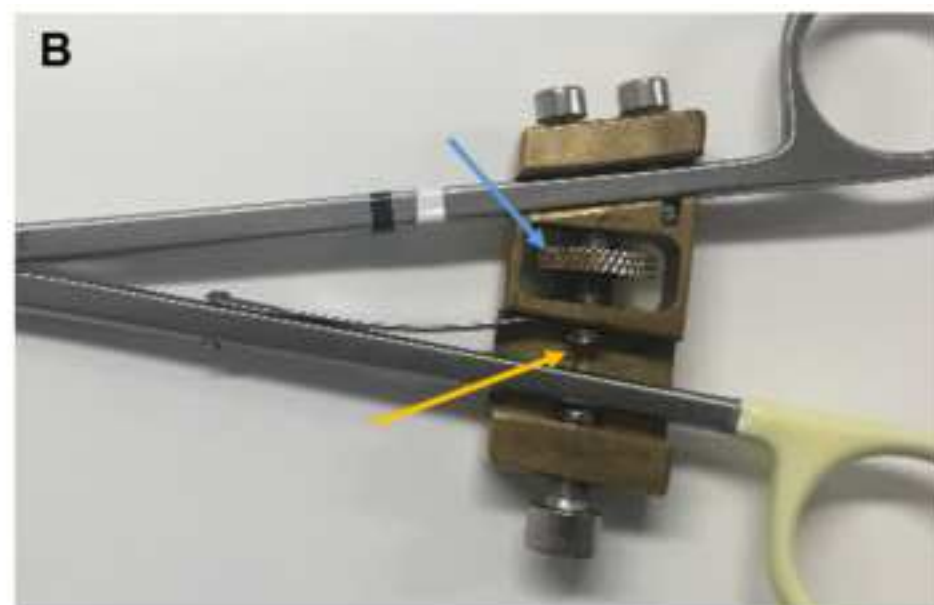
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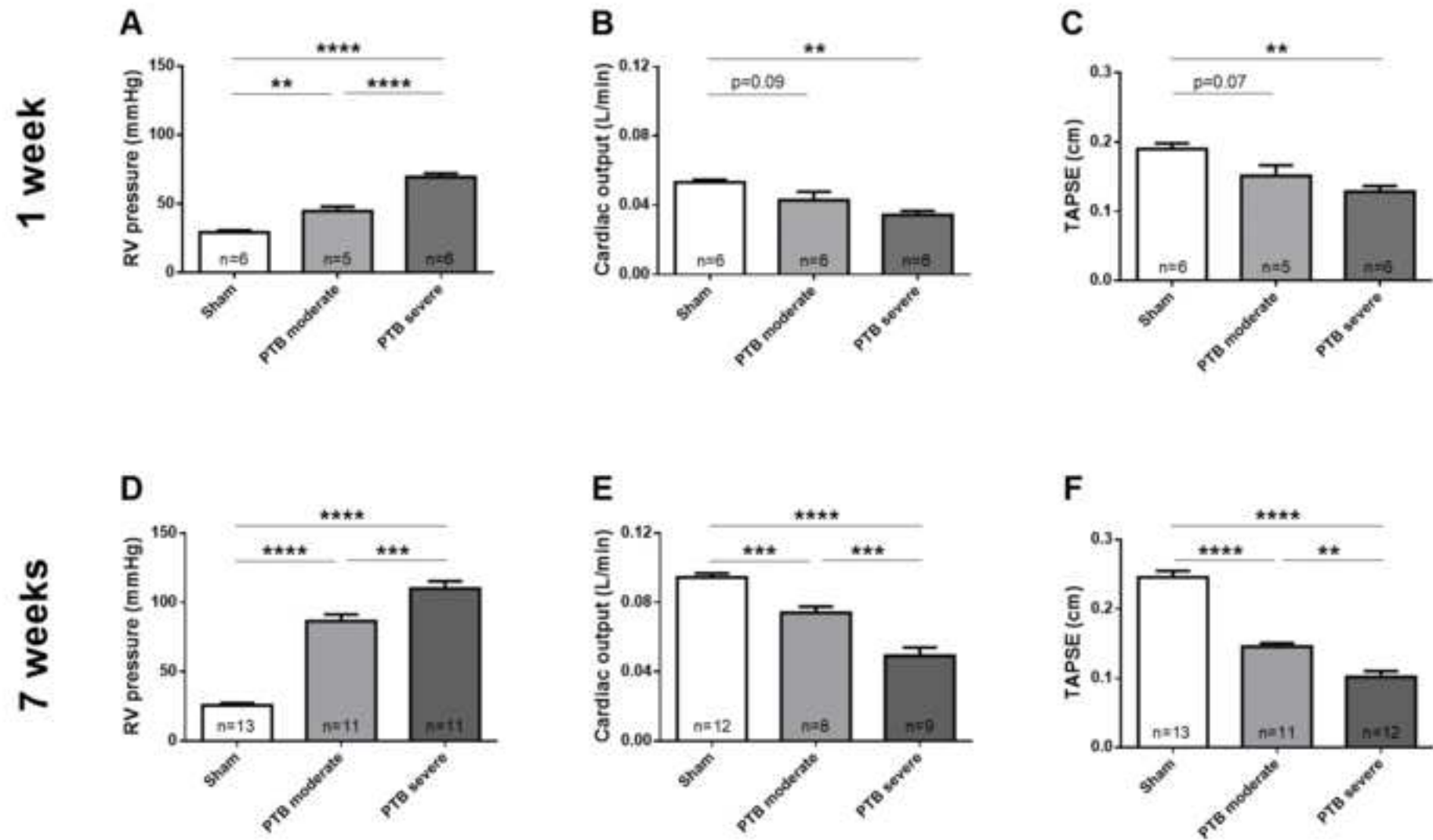
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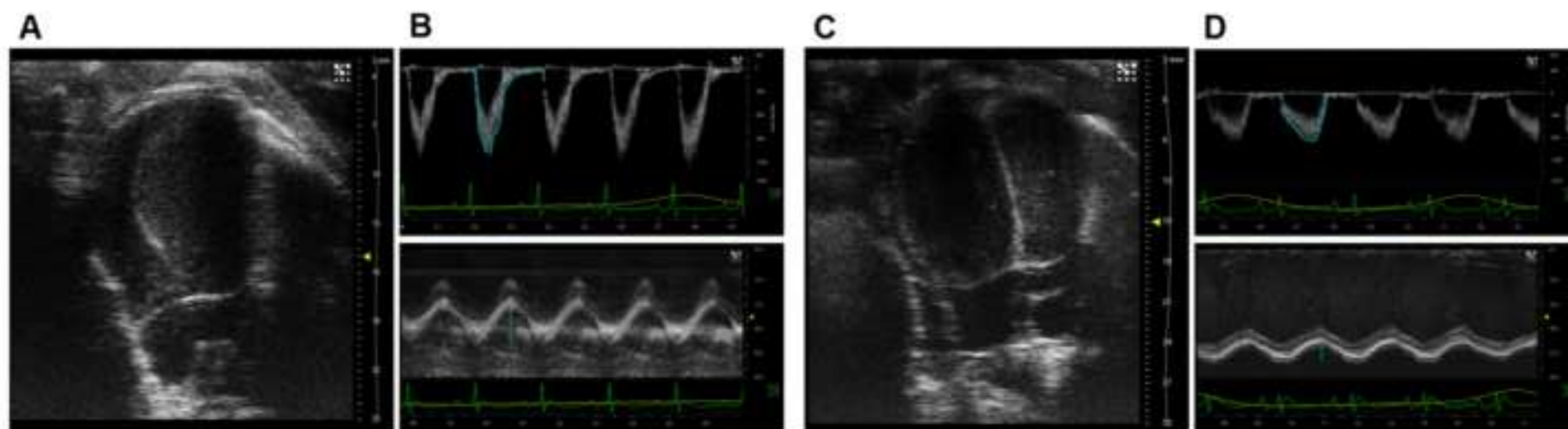
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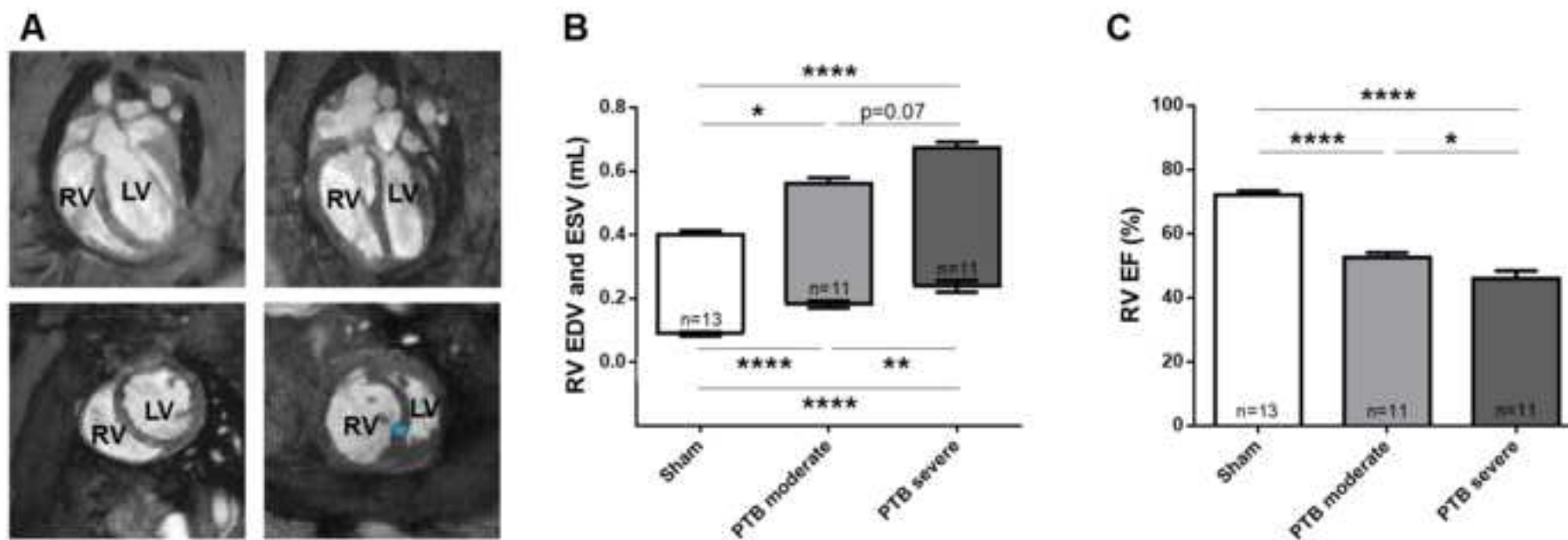
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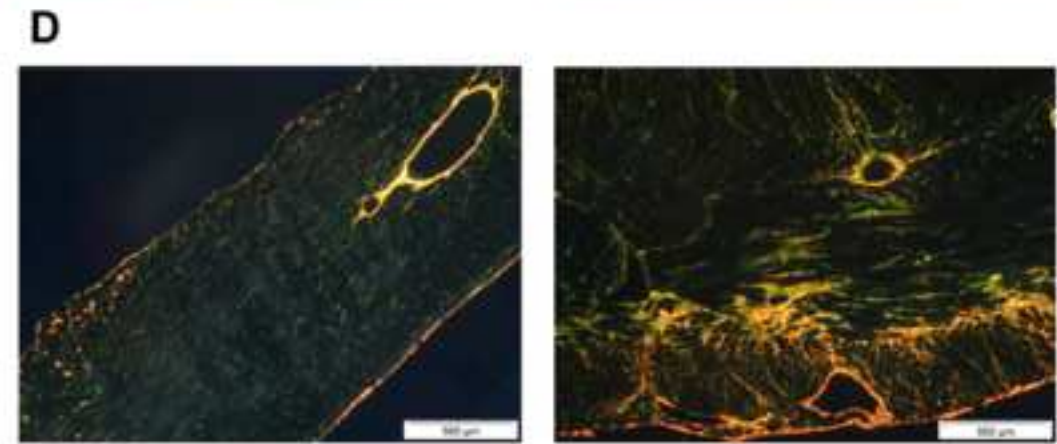
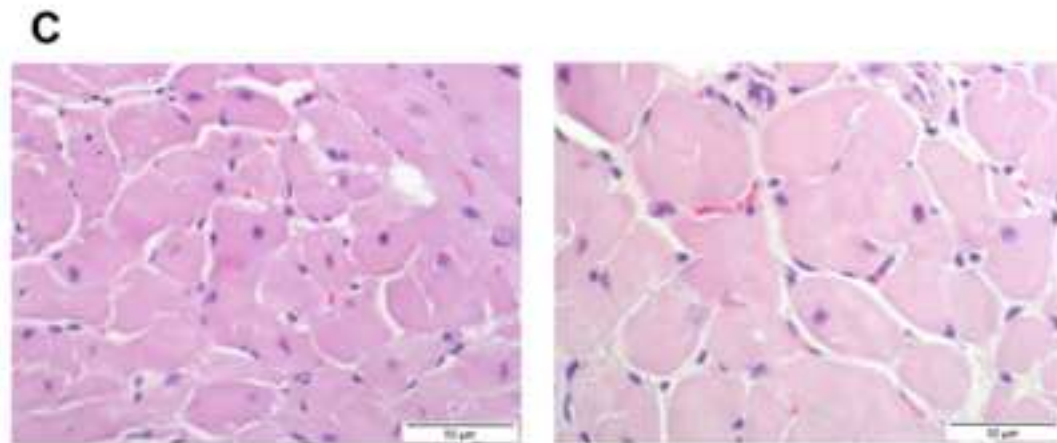
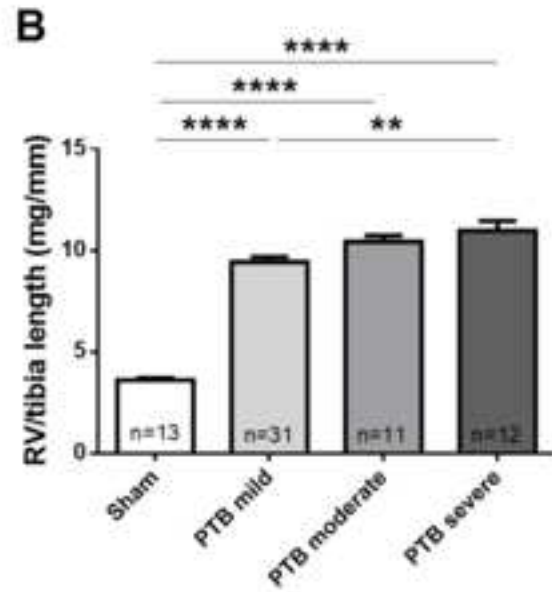
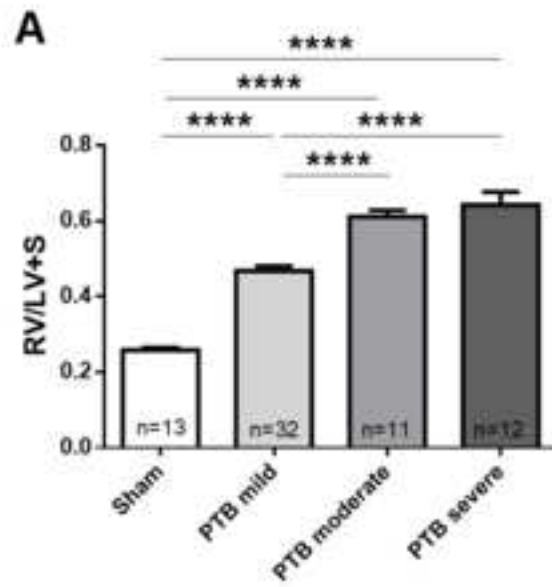












Name of Material/ Equipment	Company	Catalog Number
17G IV Venflon Cannula	Becton Dickinson, US	393228
1 mL syringe + 26G needle	Becton Dickinson, US	303172 & 303800
4-0 absorbable multifilament suture	Covidien, US	GL-46-MG
4-0 multifilament ligature	Covidien, US	LL-221
Buprenorphine	Indivior UK Limited	
Carprofene	ScanVet, DK	27693
Chlorhexidine	Faaborg Pharma, DK	
Contractor	Aesculap, Germany	BV010R
Ear Hooklet	Lawton, Germany	66-0261
Eye gel	Decra, UK	
Forceps, Delicate Tissue	Lawton, Germany	09-0020
Forceps, Dissecting	Lawton, Germany	09-0013
Gas Anesthesia System	Penlon Limited, UK	SD0217SL
Hair trimmer	Oster	76998-320-051
Horizon Open Ligating Clip Applier	Teleflex, US	137085
Horizon Titanium Clips	Teleflex, US	001200
Induction chamber	N/A	
Iris Scissor	Lawton, Germany	05-1450
Iris Scissor	Aesculap, Germany	BC060R
Mechanical ventilator	Ugo Basile, Italy	7025
Microscissor	Lawton, Germany	63-1406

Microscope	Carl Zeiss, Germany	303294-9903
Needle Holder	Lawton, Germany	08-0011
Pean	Lawton, Germany	06-0100
Pro-Optha	Lohmann & Rauscher, Germany	16515
Saline 9 mg/mL	Fresenius Kabi, DK	209319
Sevoflurane	AbbVie, US	
Surgical hook	Lawton, Germany	51-0665
Surgical Tape	3M, US	1530-0
Temperature Controller	CMA Microdialysis; Sweden	8003760
Weighing machine	VWR, US	
Wistar rat weanlings	Janvier Labs, France	

## Comments/Description

Distal 2 mm of the needle have been cut off

Polysorb, violet, 5x18"

Polysorb, violet, 98"

Local procurement, Temgesic 0.3 mg/mL

Norodyl 50 mg/mL

Local procurement

Blunt, self retaining, 70 mm

Small, 14 cm, tip modified to an angle of 85°

Lubrithal, Local procurement

1 regular, 1 with tip modified to an angle of 100°

Sigma Delta Vaporizer

Modified with adjustable stop mechanism

Small

TITEGRIP

Halsted-Mosquito, straight

Tampon

Sevorane, Local procurement

Cushing, 19 cm, tip modified to an angle of 90°

Micropore

CMA 450

RjHan:WI, 100-120 g



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A pulmonary trunk banding model of pressure overload induced right ventricular hypertrophy and failure

Stine Andersen<sup>1</sup>, Jacob Schultz<sup>1</sup>, Sarah Holmboe<sup>1</sup>, Julie Birkmose Axelsen<sup>1</sup>, Mona Sahlholdt Hansen<sup>1</sup>, Mads Dam Lyhne<sup>1</sup>, Jens Erik Nielsen-Kudsk<sup>1</sup>, Asger Andersen<sup>1</sup>  
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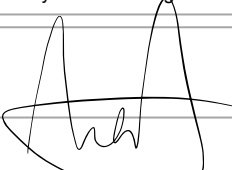
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Department:	Department of Cardiology	
Institution:	Aarhus University Hospital	
Article Title:	A pulmonary trunk banding model of pressure overload induced right ventricular hypertrophy and failure	
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## **Reply to reviewers regarding the manuscript JoVE58050 entitled: A pulmonary trunk banding model of pressure overload induced right ventricular hypertrophy and failure**

The authors gratefully appreciate the thoughtful comments from the editor and reviewers. As requested, we have attached a “marked up” version of the revised manuscript with changes indicated in red. Page and line numbers corresponding to the changes in the revised manuscript are mentioned in the point-by-point responses to editorial and reviewer comments below.

### **Editorial comments:**

*Changes to be made by the Author(s):*

*E1: 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.*

Answer E1: The paper has been carefully proofread.

*E2: Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please revise lines: first paragraph of the introduction,*

Answer E2: The first paragraph of the introduction has been replaced by:

“The right ventricle (RV) can adapt to a persistent pressure overload. In time, however, adaptive mechanisms fail to sustain cardiac output, the RV dilates and eventually the RV fails. RV function is the main prognostic factor of several cardiopulmonary disorders including pulmonary arterial hypertension (PAH), thromboembolic pulmonary hypertension (CTEPH), and various forms of congenital heart disease with a pressure (or volume) overload of the RV. Despite intense treatment, RV failure remains a predominant cause of death in these conditions.” (p. 1 ll. 46-51).

*E3: Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

Answer E3: Trademark (™) and registered (®) symbols have been removed from the table of equipment and materials.

*E4: Please provide an email address for each author.*

Answer E4: An e-mail address for each author has been provided in the online submission system. E-mail address for corresponding author is provided in the Author and Affiliations section.

*E5: 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. Please remove the purchasing links as well.*

Answer E5: Commercial names have been removed from the manuscript (long abstract ll. 32-33, p. 2 ll. 96-98, 99-101, and 103-104, p. 3 l. 157, p. 5 ll. 237-238, p. 6 ll. 281-282, and p. 7 ll. 323-324). Links have been removed from the manuscript (p. 2 ll. 97-98 and 99-101). The table of equipment and materials has been updated according to journal guidelines.

*E6: Please mention how proper anesthetization is confirmed.*

Answer E6: The following passage has been added to the manuscript:

"2.6 Confirm proper anesthetization by checking withdrawal reflexes of the extremities using a forceps to squeeze the paws of the rat." (p. 2 ll. 125-126).

*E7: Please do not abbreviate journal titles.*

Answer E7: Abbreviations of journal titles have been replaced by full titles in the reference list.

---

#### **Reviewers' comments:**

##### **Reviewer #1:**

###### *Manuscript Summary:*

*In this manuscript, the authors described a new experimental surgical device and various stages of right heart failure for pulmonary artery banding in order to create a model of right ventricle failure in rats. They compared their results (at 1 and 7 weeks after surgery) of this procedure with those of a sham operated group.*

###### *Major Concerns:*

*C1: The authors should report the number of animals in each group and how they calculated the "sample size". Please describe the number of each section, and the survival data.*

Answer C1:

We appreciate the comment and agree that power calculation should be performed when designing studies using the PTB model. The calculated power is dependent on the expected efficacy of the planned intervention. We did not perform power calculation for these data, as they illustrate a model that can be used for future studies and no intervention was introduced in animals subjected to PTB. The data we published can be used for power calculation when planning future studies that involve an intervention.

The number of rats in each group has been specified on the graphs of figure 2, 4, and 5 and indications of group sizes have been deleted from figure legends.

The following passage has been added to the manuscript:

“Perioperative mortality was less than 1 in 6. Seven weeks survival rate was 80% for rats subjected to severe banding and close to 100% in rats subjected to mild or moderate banding or sham surgery.” (p. 4 ll. 186-188).

*C2: In the Methods, the paragraph "Echocardiographic measurements" should be made more concise, schematic, and, as a consequence, easily readable. How did the authors calculate the RV EF and RV pressure? Please explain the calculate method.*

Answer C2: We appreciate the comment. The methods used for evaluation of hemodynamics (echocardiography, cardiac magnetic resonance imaging and invasive pressure measurements) have been described in detail previously<sup>1</sup>, but we have elaborated the section and added the following passage to the manuscript:

“Tricuspid annular plane systolic excursion (TAPSE) was measured as the distance of the tricuspid annular plane with the RV contraction in the apical four-chamber view. An average of three cycles outside respiration was used as a representative value. RV end-diastolic volume (EDV) and end-systolic volume (ESV) was assessed by drawing of the endocardium in a series of short axis images through the RV obtained by MRI for each rat and RV ejection fraction (EF) calculated as  $EF = (EDV - ESV) / EDV$ . Cardiac output was measured between the pulmonary valves and the clip using a phase-contrast MRI sequence. Digital recordings of RV pressures was obtained by a micro tip catheter installed in the RV before euthanasia. Further details of the methods have been described previously<sup>12</sup>.” (p. 4 ll. 191-200).

*Minor Concerns:*

*C3: In figure 5, there was no description of D.*

Answer C3: Figure legend for figure 5 has been updated with the following description of D:

“(D) picrosirius red analyzed under polarized light for fibrosis from sham rats (left) and PTB rats with moderate RV failure (right).” (p. 6 ll. 173-275).

## **Reviewer #2:**

*Manuscript Summary:*

*Andersen S. et al. describe a protocol of pulmonary trunk banding (PTB) operation in rats. Mechanisms of right ventricular hypertrophy and failure remain understudied area and PTB is a technique of choice for its investigations. Detailed description of the technique is of high interest for investigators. The manuscript is well written. The protocol is described in sufficient details.*

*Major Concerns:*

*No major concerns*

*Minor Concerns:*

*C1: How was sufficient analgesia controlled. Please, provide short description.*

Answer C1: Please refer to answer E6.

*C2: Fig. 5 D must be picosirius red staining under polarized light. please, specify this point. Additionally, provide correct labeling of sub-figures in the figure legend.*

Answer C2: Text and labelling of subfigures have been corrected. Please refer to answer to comment 3 from reviewer #1.

*C3: Throughout the manuscript, the groups of mild, moderate and severe constriction are described. However, data for mild constriction group are shown only of Fig. 5. Please, show data for this group on Figures 2-4.*

Answer C3: We appreciate the comment, but unfortunately we do not have data from MRI and invasive in-vivo pressures from our mild PTB operated animals at 1 and 7 weeks. We do however have data from a study published previously illustrating the difference 4 weeks after PTB surgery<sup>2</sup>. We have chosen not to include these data in this study, as we believe it to be confusing having both mild, moderate, and severe and 1, 4 and 7 weeks after surgery included in the paper. We have clarified this in the manuscript accordingly and added the mentioned paper to the reference list:

“Detailed hemodynamic differences between PTB mild rats and PTB severe rats 4 weeks after surgery have been published by our group previously<sup>13</sup>.” (p. 4 ll. 210-212).

*C4: In figure legends, stars for statistical significances are explained as in comparison to sham group only. On the figures, bars indicate significances between banding groups as well. Please, modify correspondingly.*

Answer C4: Figure legends of figure 2, 4, and 5 have been updated according to the figures (p. 5 ll. 250-251, and p. 6 ll. 266-267 and ll. 277-278).

### **Reviewer #3:**

#### *Manuscript Summary:*

*A reliable RV overload model will be valuable for studying RV failure mechanism. Authors improved pulmonary artery banding operations with precise clipping. These improvements established a highly efficient and reliable banding method. With precise constriction, authors demonstrated that different severities of banding will introduce different consequences in RV function. It explains some controversies in the field. The followings are a few questions about results and procedure details.*

#### *Major Concerns:*

*C1: What's the death rate for each banding severity at different time points?*

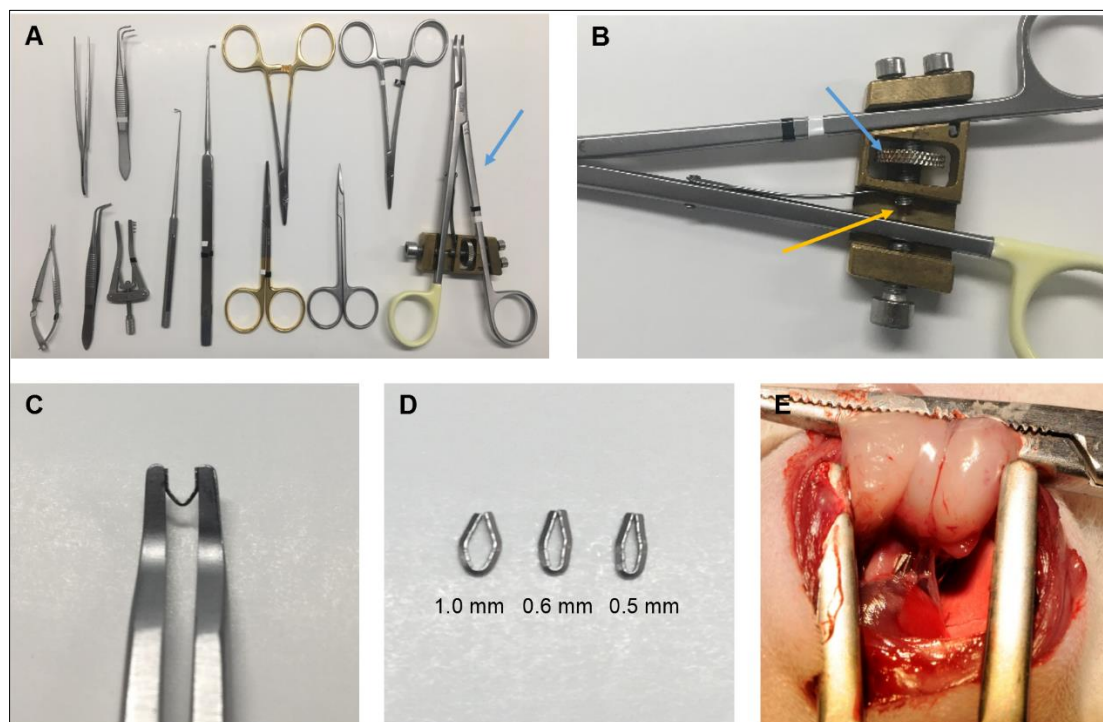
Answer C1: Please refer to answer to comment 1 from reviewer #1.

*C2: It will be easier for others to follow if authors could describe the stop mechanism of clip applicator in more details.*

Answer C2: We appreciate the comments and have sought to clarify how the applicator works to make the protocol easier to follow. Figure 1 has been updated to include an enlarged image of the stop mechanism of the ligating clip applicator (B) and the figure legend has been changed to provide a more detailed description of the mechanism:

“(A) The surgical instruments used for the PTB procedure including the ligating clip applicator (blue arrow). (B) The adjustable stop mechanism of the ligating clip applicator. Turning the cogwheel (blue arrow) will adjust the position of the pin (yellow arrow), which stops the closing of the applicator when the jaws reach a certain distance from each other. The distance correspond to 2 x the thickness of the legs of the clip plus the inner diameter of the clip, when the clip is compressed, and can be calibrated by using for example a needle with a known outer diameter.” (p. 5 ll. 232-237).

Updated figure 1:



C3: Authors used the diameter of the banding loop to represent the banding severity. However, the area of banding loop will change if a different length of clip is to be used. So, it will be desirable to use the area of banding loop as an indicator of banding severity. It will be easier to compare different studies later.

Answer C3: We appreciate the comment, but as seen in figure 1, E, the pulmonary artery does not completely fill the area of the banding clip. Hence, reporting the area of the banding clip will not be correct either. The correct way would be to measure the area of the pulmonary artery in the banding clip post mortem. Unfortunately, these data are not available.

Minor Concerns:

C4: In the following sentence, "This leaves a lumen of 0.6 mm as the two clip legs have a diameter of 0.2 mm each", the word "diameter" should be replaced by "thickness" to avoid confusion.

Answer C4: The sentence have been updated accordingly (p. 2 l. 104).

C5: All the abbreviations should be explained when it first appears in the main text.

The manuscript has been updated accordingly (p. 4 ll. 191-192 and ll. 194-196).

C6. A neonatal PTB model has recently been published (*J Thorac Cardiovasc Surg* 2017;154:1734-9). It used a conventional method to perform banding. It should be commented whether the new method can be applicable in neonatal rats.

Answer C1: Thank you for pointing this out. This method could potentially be applied to rat neonates and also mice, but we have no experience in doing so. We have added this to the manuscript:

"The clip model can potentially be used in rat neonates as opposed to the ligating technique previously used<sup>19</sup>, but we have no experience with this and the same considerations as mentioned above applies before initiating a study." (p. 6 ll. 308-310).

#### **Other comments/changes:**

This method study is based on data and experiences from previous studies from our group. This have been clarified in the manuscript:

"Here we present our experience using this model based on results from previous studies<sup>12,13</sup>." (p. 1 l. 86-87).

"...in previous studies from our group<sup>12,13</sup>, ..." (p. 4 l. 180)

Additionally, the histological images in figure 5 have been updated and minor adjustments have been made to the sentences: "In addition, the MCT model has several extra-cardiac effects" (p. 1 l. 64-65)" and "In experimental pulmonary trunk banding models, the afterload of the RV is fixed due to a mechanical constriction of the pulmonary trunk" (p. 1 l. 67-68).

#### **References**

- 1 Andersen, S. *et al.* Effects of bisoprolol and losartan treatment in the hypertrophic and failing right heart. *Journal of cardiac failure*. **20** (11), 864-873, (2014).
- 2 Andersen, A., Povlsen, J. A., Botker, H. E. & Nielsen-Kudsk, J. E. Right ventricular hypertrophy and failure abolish cardioprotection by ischaemic pre-conditioning. *Eur J Heart Fail*. **15** (11), 1208-1214, (2013).