

Supplementary Material

Effects of chronic treprostinil treatment on right heart hypertrophy and failure

Sofie Axelgaard¹; Sarah Holmboe¹; Steffen Ringgaard²; Thomas Andersen¹; Stine Andersen¹; Mona S. Hansen¹; Asger Andersen¹; Jens E. Nielsen-Kudsk¹

¹Department of Cardiology, Institute of Clinical Medicine, Aarhus University Hospital, Denmark.

²The MR Centre, Institute of Clinical Medicine, Aarhus University Hospital, Denmark.

Dose Study

Methods

A total number of 28 Male Wistar rats were included in the dose finding study. Fifteen 150 g 0.5PTB rats and 12 250 g healthy rats were randomized to 14 days of treatment with treprostinil in low, moderate or high dose administered by Alzet osmotic mini pumps. (Fig. 2) Plasma concentration of treprostinil was analysed in blood samples drawn from the tail vein every third day. Evaluation by echocardiography, MRI and pressure volume measurements was performed and systemic blood pressure measured before euthanization of the animals.

Results

- Plasma concentrations

Administration of treprostinil by continuous subcutaneous infusion using Alzet osmotic pumps ensured fairly stable plasma concentrations during the entire study after a stabilisation period of 4-7 days. As expected, plasma concentrations were up to two fold higher in 150 g rats than in 250 g rats.

The mean plasma concentration of treprostinil in the low dose rats during the entire study was 2.22 ng/kg corresponding to a free treprostinil concentration of 0.20 ng/mL because of a 91 % plasma protein binding, 3.80 ng/kg (0.35 ng/kg free treprostinil) in the moderate dose rats and 10.59 (0.96 ng/kg free treprostinil) in high dose rats. (Fig. 3)

- Blood pressure

Mean arterial pressure (MAP) was 4 % higher in animals treated with moderate dose treprostinil than animals treated with low dose treprostinil. Animals treated with high dose treprostinil had an 11 % decrease in blood pressure compared to low dose animals. (Fig. 4)

Discussion

Investigation of differential effects of treprostinil administered by Alzet osmotic pumps in previous studies has focused on doses between 45 and 100 ng/kg/min.^{i ii iii} Human with pulmonary arterial hypertension are given an initial

dose of 1.25 ng/kg/min which is increased over time and can reach values up to 100 ng/kg/min corresponding to a plasma concentration of 1.2 ng/kg/min when administered subcutaneous or intravenous.

Recently, we have investigated the acute in vitro effects of increasing doses of treprostinil in healthy animals in our Langendorff Model. The most effective dose on RV developed pressure, systolic pressure, rate pressure product and dP/dt max was 1.5 ng/mL. (Holmboe et al; Paper under review)

Thus, our highest dose corresponded to plasma concentrations closest to the most effective one in vitro and still corresponded to clinical relevant plasma concentrations. However, the 11 % blood pressure drop should be taken into consideration, as the magnitude of the drop can cause systemic effects blunting the response. For this reason, we found it relevant to also investigate the effects of a dose not influencing the systemic blood pressure.

Therefore, the conclusion of the dose study was to investigate the effects of moderate dose treprostinil (300 ng/kg/min) and high dose treprostinil (900 ng/kg/min) in our disease model.

Expanded Methods

Histology

The right ventricle was immersion fixated in 4% formaldehyde buffer (pH 7). The tissue was dehydrated in graded ethanol. After tissue embedding in paraffin, 2 µM sections were cut and stained to estimate the degree of fibrosis (Sirius Red), cardiomyocyte diameter and quantity per area (Haematoxylin-Eosin) and right ventricular capillary density (CD-31 immune histochemistry).

The sections were stained with Collagen specific Sirius Red to visualizise fibrosis in a polarised light microscope. The mean area of fibrosis was evaluated by capturing five digital RGB color images of the right ventricle in each animal (ColorViewII, Soft Imaging System, Germany) using the 4×objective (BX50F4, Olympus, Japan).

Data were analysed in ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2007). Images were converted to 8bit gray scale and threshold adjusted

manually. The fibrosis fraction was calculated in a semi quantitative manner as an average percentage of area from on section.

Another set of sections was stained with haematoxylin and eosin in order to estimate RV cardiomyocyte cross-sectional area (CSA). CSA was expressed as an average of twenty-five cardiomyocytes cut transversally at the level of the nucleus from five locations randomly distributed over the right ventricle.

Finally, immunohistochemical staining for CD31 was performed to visualize capillaries. Three images were obtained per animal at random locations with cross-sectioned cardiomyocytes. The capillaries and cardiomyocytes were counted manually in each image, and capillary density was expressed as the number of capillaries per mm² and per cardiomyocyte.

All the histological analyses were performed by an operator blinded to the clinical source of the samples.

Gene expression studies

RNA from snap-frozen right ventricular tissue (-80°C) was isolated by using a commercial RNA purification kit (NucleoSpin® RNA II, Macherey-Nagel).

30 mg of right ventricular tissue was homogenised (TissueLyser) for 2 x 90 seconds. Lysis buffer and mercaptoethanol was added and vortexed. The lysate was filtered using the NucleoSpin® Filter. Ethanol was added to the lysate and loaded to the NucleoSpin® RNA II Column. The column was centrifuged for 30 seconds at 11000 x g. 350 uL desalting buffer was added before another 1 minute of centrifugation. The column was incubated for 15 minutes with 95 uL DNase reaction mixture to digest DNA and centrifuged three times before the RNA was isolated in 27 uL RNase-free H₂O.

The RNA concentration in the samples was determined by spectrophotometry (Eppendorf® BioPhotometer). Total RNA was reverse transcribed into complimentary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following a standard protocol.

First strand cDNA was diluted and RT-qPCR performed using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific) along with specific primers for the following genes: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and β myosin heavy chain (β -MHC). All measured transcript expression

levels were normalised to the housekeeping gene GAPDH and made relative to the normalised mRNA level of SHAM rats.

Primer sequences:

GAPDH: TTAAGGGCATCCTGGGCTACACT (forward)
TTACTCCTTGGAGGCCATGTAGG (reverse)

ANP: TAGATCTGCCCTCTTGAAAA (forward)
TCCAATCCTGTCAATCCTAC (reverse)

BNP: GCTGGAGCTGATAAGAGAAA (forward)
TTGAACTATGTGCCATCTTG (reverse)

β -MHC: GAGCTTCTAGACATGCTGCT (forward)
TGGAGTTCTTCTTCTGGA (reverse)

Supplemental figures

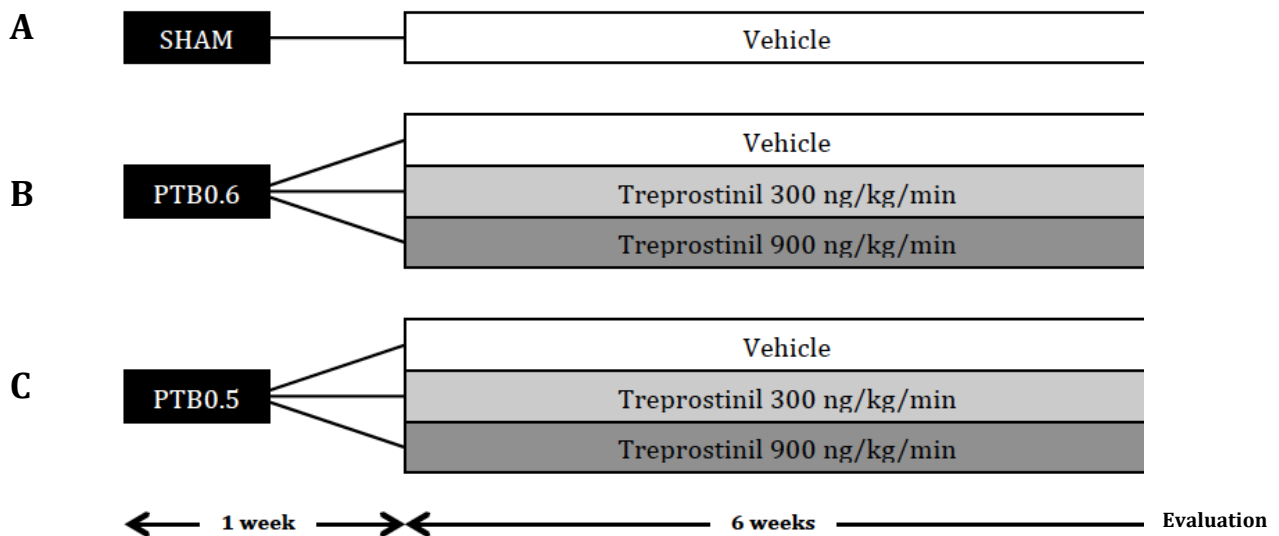


Figure 1) Schematic illustration of the study design.

A) Sham. B) Pulmonary Trunk Banding with 0.6 mm-banding clip. C) Pulmonary Trunk Banding with 0.5 mm-banding clip. 8 days after surgery, animals were randomized to a moderate dose treprostinil (300 ng/kg/min), high dose treprostinil (900 ng/kg/min) or vehicle (Saline). After 6 weeks of treatment, cardiac function was evaluated by echocardiography, MRI and invasive pressure-volume measurements.

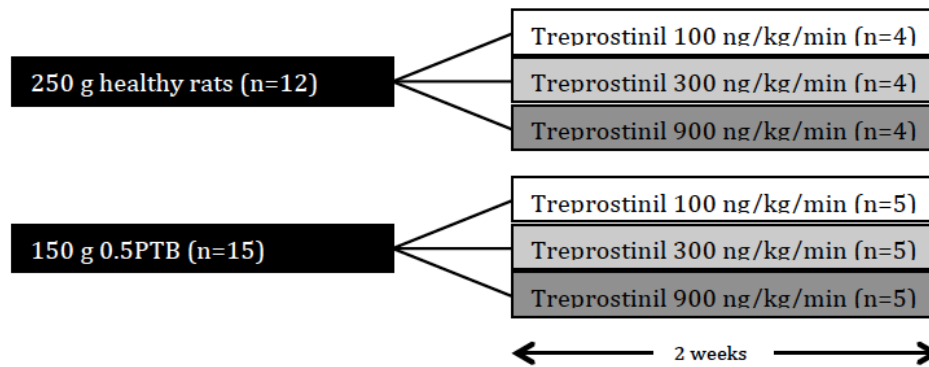


Fig. 2) Study design of the dose study. 0.5PTB: PTB banded animals with a clip size of 0.5mm. 8 days after surgery PTB rats weighing 150 g were randomized to two weeks of treatment with low dose, moderate dose or high dose treprostnil. Healthy 250 g rats were randomized to the same three doses. On day 13 of treatment, echocardiography was performed. MRI was performed on day 14. On day 15, systemic blood pressure was measured, PV relations were obtained and blood samples were drawn before euthanization of the animals.

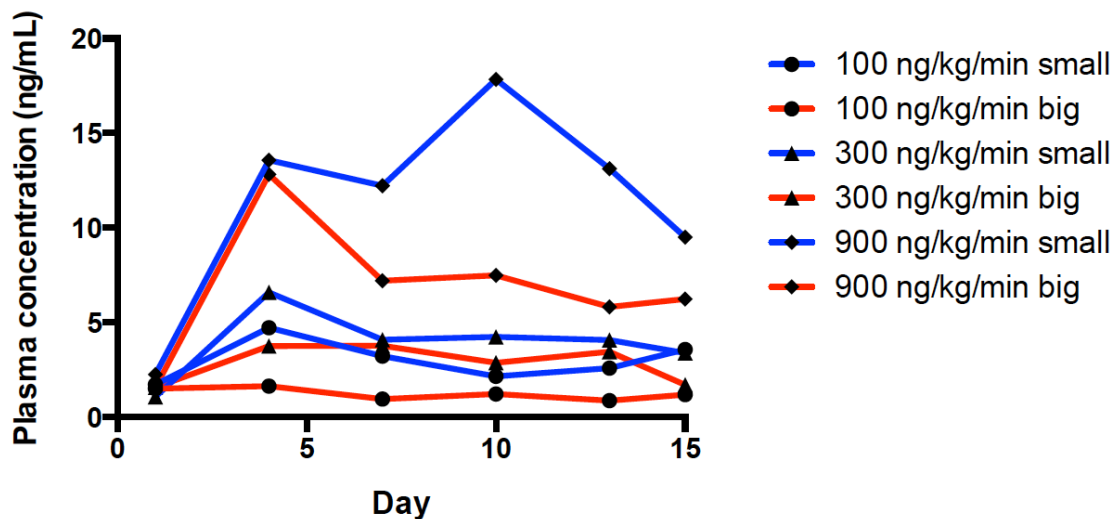


Fig. 3) Plasma concentrations of treprostnil during the study period in different groups.

100 ng/kg/min small: PTB animals weighing 150g at time of pump insertion and treated with treprostnil (100 ng/kg/min). 100 ng/kg/min big: Healthy animal weighing 250g at time of pump insertion and treated with treprostnil (100 ng/kg/min). 300 ng/kg/min small: PTB animals weighing 150g at time of pump insertion and treated with treprostnil (300 ng/kg/min). 300 ng/kg/min big: Healthy animal weighing 250g at time of pump insertion and treated with treprostnil (300 ng/kg/min). 900 ng/kg/min small: PTB animals weighing 150g at time of pump insertion and treated with treprostnil (900 ng/kg/min). 900 ng/kg/min big: Healthy animal weighing 250g at time of pump insertion and treated with treprostnil (900 ng/kg/min).

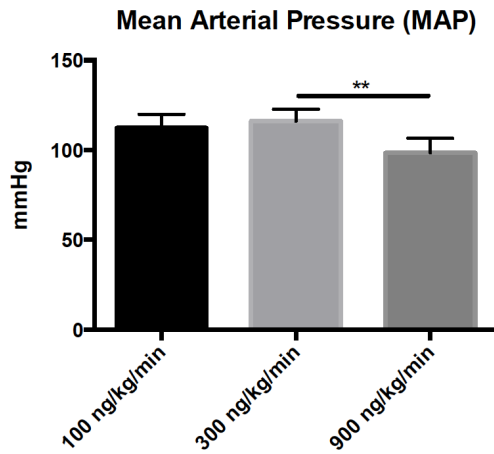


Fig. 4) Mean arterial pressure (MAP). 100 ng/kg/min: PTB and healthy animals treated with treprostinil (100 ng/kg/min) (n=7); 300 ng/kg/min: PTB and healthy animals treated with treprostinil (300 n/kg/min) (n=7); 900 ng/kg/min: PTB and healthy animals treated with treprostinil (900 ng/kg/min) (n=6). **P<0.005

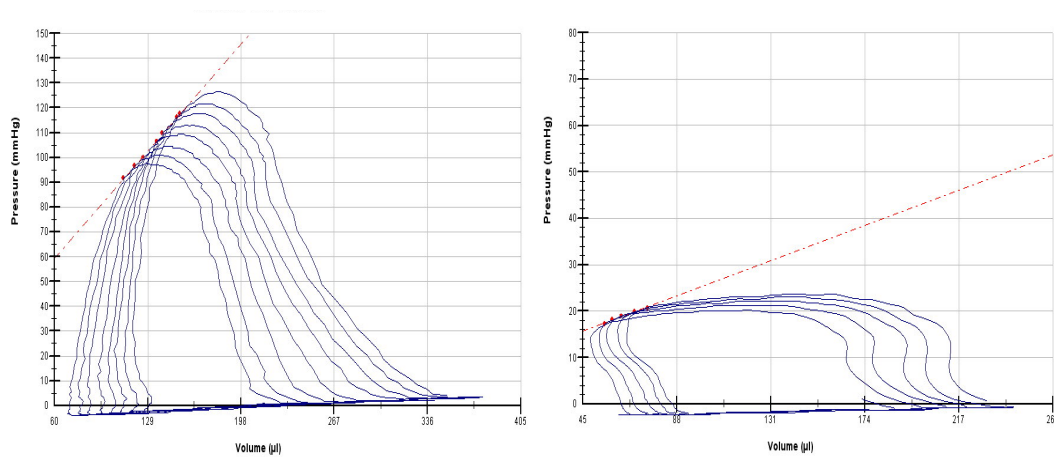


Fig. 5) Pressure-volume relations in a PTB rat (left) and a SHAM rat (right).

The slope of the end-systolic pressure-volume relationship (systolic elastance), E_{es} , is increased in the PTB rat either by increased wall thickness, muscle contractility or both. PTB: Pulmonary Trunk Banding.

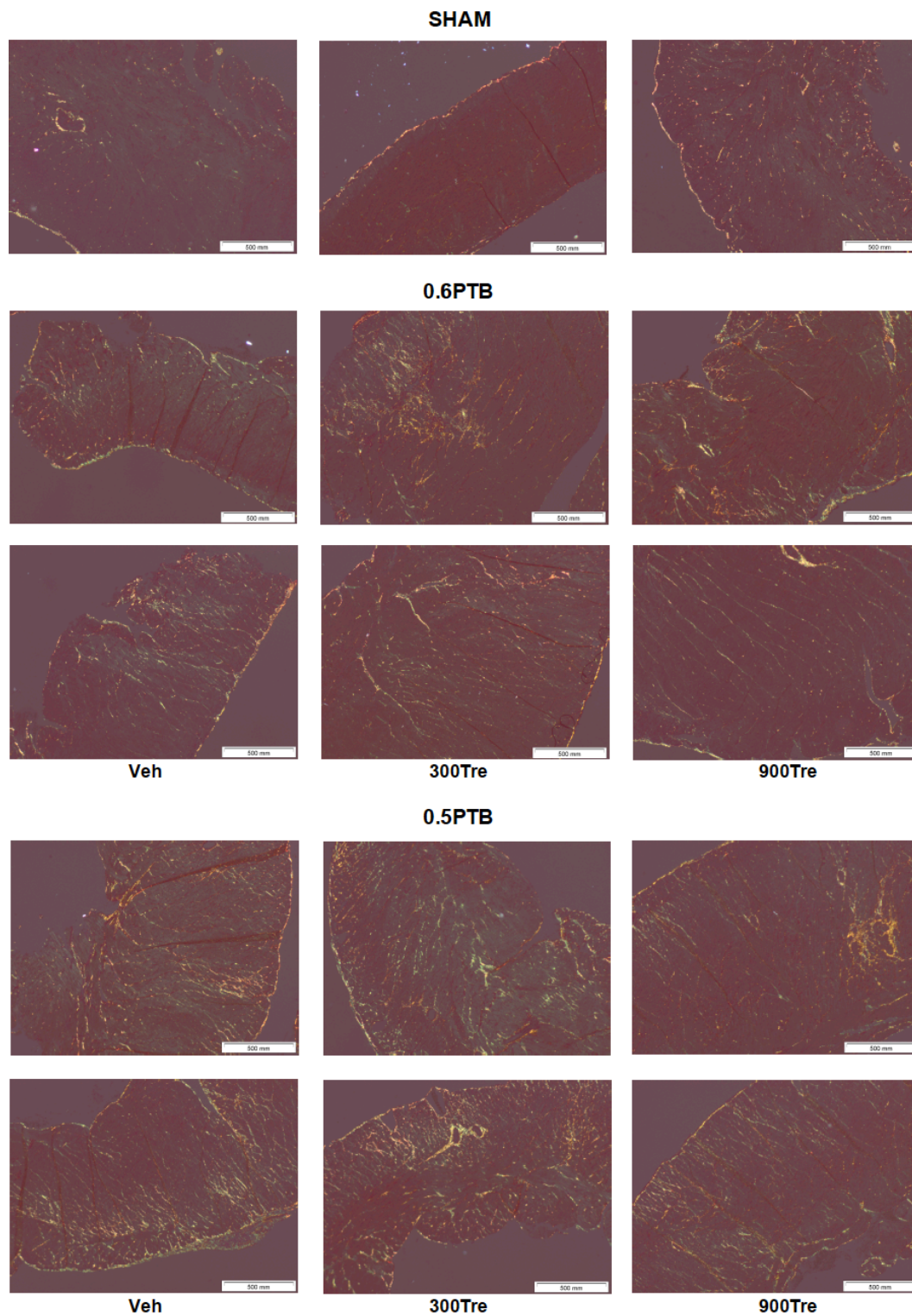


Fig. 6) Histological sections stained with Sirius Red for visualization of right ventricular fibrosis fraction.

Sham: Sham-operated animals treated with vehicle (saline); 0.6PTB: Pulmonary Trunk Banding with 0.6 mm-banding clip. 0.5PTB: Pulmonary Trunk Banding with 0.5 mm-banding clip. Veh: Treatment with saline. 300Tre: Treatment with 300 ng/kg/min treprostinil; 900Tre: Treatment with 900 ng/kg/min treprostinil.

Supplemental references

ⁱ van Albada ME, van Veghel R, Cromme-Dijkhuis AH, Schoemaker RG, Berger RM. Treprostinil in advanced experimental pulmonary hypertension: beneficial outcome without reversed pulmonary vascular remodeling. *J Cardiovasc Pharmacol.* 2006 Nov;48(5):249-54.

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