Dexamethasone reverses monocrotaline-induced pulmonary arterial hypertension in rats

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**Abbreviations**

BMPR2: Bone morphogenetic protein receptor 2

IL-6: Interleukin 6

MCT: Monocrotaline

mRNA: messenger ribonucleic acid

PAH: Pulmonary arterial hypertension

PASMC: Pulmonary arterial smooth muscle cells

PVR: Pulmonary vascular resistance

RVH: Right ventricular hypertrophy

RV/LV+S: Ratio of the right ventricular weight to left ventricular plus septal weight (Fulton’s index)

VSD: Ventricular septal defect

**Keywords:**

Pulmonary arterial hypertension

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ABSTRACT

Pulmonary arterial hypertension (PAH) is associated with dysregulated bone morphogenetic protein receptor type 2 (BMPRII) signaling and pulmonary vascular inflammation. We evaluated the effects of dexamethasone on monocrotaline (MCT)-induced PAH in rats for potential reversal of PAH at late time-points.

Saline-treated controls, MCT-exposed, MCT-exposed and dexamethasone-treated rats (5mg/kg/day, 1.25mg/kg and 2.5mg/kg/48-hourly) were evaluated at day 28 and day 35 following MCT for haemodynamic parameters, right ventricular hypertrophy, morphometry, immunohistochemistry, IL-6 and BMPR2 expression.

Dexamethasone improved haemodynamics and pulmonary vascular remodeling, preventing PAH development at early (day1-14, day1-28) and reversing PAH at late time points (day14-28, day21-35) following monocrotaline, as well as improving survival in MCT-exposed rats compared to controls. Both MCT-induced pulmonary IL-6 overexpression and IL-6-expressing adventitial inflammatory cell infiltration were reduced with dexamethasone. This was associated with pulmonary BMPR2 down-regulation following monocrotaline which was increased with dexamethasone, in whole lung, and in control pulmonary artery smooth muscle cells. Dexamethasone also reduced proliferation of rat pulmonary artery smooth muscle cells in vitro.

Experimental PAH can be prevented and reversed by dexamethasone, and survival is improved. In this model, mechanisms may involve reduction of IL-6-expressing inflammatory cells, restoration of pulmonary BMPR2 and reduced proliferation of vascular smooth muscle cells.
INTRODUCTION

Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance, ultimately leading to right ventricular failure and death. The principal pathological finding is remodelling of small pulmonary arteries with marked proliferation of pulmonary artery smooth muscle cells (PASMC), resulting in obstruction of these resistance pulmonary arteries. Inflammatory mechanisms are believed to play a key role in both human and experimental PAH. In idiopathic PAH, infiltrates of macrophages and lymphocytes are found in the range of plexiform lesions with local expression of chemokines CCL2 (MCP-1), CCL5 (RANTES) and CX3CL1 (fractalkine). Histopathological specimens from patients displaying severe PAH in the context of connective tissue diseases suggests that inflammation and remodelling are key contributors to pulmonary vascular disease complicating inflammatory diseases. Pro-inflammatory cytokines including interleukin (IL)-1 and IL-6 are elevated in both human idiopathic PAH and MCT PAH. Auto-immunity is also demonstrated to contribute to PAH with patients characterized by circulating auto-antibodies. Pathogenic auto-antibodies target endothelial cells and may induce vascular endothelial apoptosis, promoting PAH development.

The suggestion that treatment with corticosteroids and/or immunosuppressants may dramatically improve PAH stems from the improvement seen in associated PAH following treatment for co-existing systemic inflammatory conditions, including POEMS (Polyneuropathy, Organomegaly, Endocrinopathy, Monoclonal immunoglobulin, Skin changes) syndrome, Castleman’s disease, systemic lupus erythematosus and mixed connective tissue disease. Immunosuppressive therapies including rapamycin and cyclosporin have been shown to attenuate the development of PAH in rats exposed to MCT including established PAH.
Earlier studies have shown that steroids prevent the development of MCT-PAH\textsuperscript{9,19-21}, although no studies have yet shown that steroids reverse established MCT-PAH.

Mutations in the BMPRII gene (\textit{BMPR2}) have been identified in more than 50% of FPAH patients and in 10% to 25% of IPAH patients\textsuperscript{22,23}. Reduced levels of BMPR1\textalpha\textsuperscript{24} and BMPR2\textsuperscript{25,26} mRNA expression are seen in the lungs of patients with heritable PAH and IPAH, and in other subtypes of PAH. This reduction in pulmonary \textit{BMPR2} is mirrored in MCT-PAH\textsuperscript{27,28}. These mutations disrupt BMP/Smad-mediated signalling\textsuperscript{29}, potentiate BMP/MAP kinase signalling\textsuperscript{30}, and could underlie the abnormal vascular cell proliferation observed in familial PAH\textsuperscript{31}. Interestingly, several studies suggest that dysregulation of the BMP pathway leads to vulnerability to an inflammatory second hit\textsuperscript{32-34}.

The aims of this study were to test the effects of dexamethasone on pulmonary hemodynamics, IL-6 and \textit{BMPR2} expression in asymptomatic and symptomatic phases of development of MCT-induced PAH in rats. We hypothesized that dexamethasone treatment could reverse hemodynamics in established MCT-PAH, and that hemodynamic improvements would correlate with normalization of IL-6 and BMPR2 mRNA levels.

\textbf{METHODS}

\textbf{Study design}

Male Wistar rats (100 g body weight) were maintained in a temperature-controlled room with a 12:12-h light-dark cycle and randomly divided into 1) saline-treated control group (n=20); 2)
monocrotaline-exposed group (n=20); 3) monocrotaline-exposed and 5mg/kg/day dexamethasone-treated group from day 1 to day 14 (n=20); and 4) monocrotaline-exposed and 5mg/kg/day (by intraperitoneal injection) dexamethasone-treated group from day 1 to day 28 (n=10); 5) monocrotaline-exposed and dexamethasone-treated from day 14 to day 28 group (3 dose ranges: 5mg/kg/day, 2.5mg/kg/48-hourly and 1.25mg/kg/48-hourly) and 6) monocrotaline-exposed and 5mg/kg/day dexamethasone-treated from day 21 to day 35 group. All rats had access to standard rat chow and water ad libitum. For monocrotaline (MCT) administration, rats received a single subcutaneous injection of 60 mg/kg MCT (Sigma-Aldrich, Lyon, France), which was dissolved in 1 N HCl, and the pH was adjusted to 7.4 with 1 N NaOH. Ten rats from groups 1) to 3) were killed to perform experiments at 14 days after the MCT exposure, remaining 10 rats of all groups were then killed at 28 or 35 days after MCT exposure.

Hemodynamics

As described by Stinger et al35, a 3.5 French umbilical vessel catheter (Tyco, Plaisir, France), angled to 90° over the distal 1 cm and curved slightly at the tip, was introduced into the right external jugular vein of rats anesthetized with 35mg/kg ketamine, 4mg/kg xylasine and 0.5mg/kg acepromazine. Following deaths during anaesthesia in preliminary experiments, presumed relative adrenal suppression was managed using a 5mg/kg dose of intraperitoneal dexamethasone prior to induction of anaesthesia. With the angle directed anteriorly, the catheter was inserted 2.5cm proximally, which placed the catheter in the right atrium. The catheter was rotated 90° counterclockwise and inserted 1.0cm further, which placed the catheter in the right ventricle, and when advanced an additional 1.5cm, in the pulmonary artery. Placement at each stage was confirmed by respective pressure contours. Hemodynamic values were automatically calculated
by the physiological data acquisition system cardiomax III (Phymep, Paris, France). Following exsanguination, the lungs were distended by infusion of OCT compound (Miles, Epernon, France) diluted in PBS (1:1) into the trachea, and quick-frozen in isopentane on dry ice and stored at -80°C. For Fulton’s index of right ventricular hypertrophy, the ratio of the right ventricular weight to left ventricular plus septal weight (RV/LV+S) was calculated.

Gene quantification by quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

RNA was extracted from rat lungs with the total RNA isolation minikit (Agilent technologies, France) and then eluted from silicate columns and reverse-transcribed using Omniscript Reverse Transcription kit (Qiagen, Courtaboeuf, France). Constitutively expressed \( \beta \)-actin was selected as an internal housekeeping gene control for the comparative \( C_T \) method for the relative quantification of BMPR2, and IL-6 mRNA expression. BMPR2, IL-6, and \( \beta \)-actin expressions were quantified by RT-PCR with TaqMan Gene Expression Assays (\( \beta \)-actin [Rn00667869_m1], BMPR2 [Rn01437210_m1], IL-6 [Rn00561420_m1], and TaqMan Universal PCR Master Mix followed in an ABI Prism7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France).

Immunohistochemistry

Immunohistochemistry was performed on 8 µm-thick sections of frozen tissue (-80°C). After routine preparation, slides were processed with the primary antibody anti-IL-6 1/600 (Abcam Rabbit polyclonal ab6672, Cambridge, England), then with the secondary antibody (Anti-rabbit,
kit En Vision+/HRP, Dako, Trappes, France). Control used for these antibodies included omission of the primary anti-body and incubation with irrelevant immunoglobulins of the same isotype.

**Pulmonary artery morphometry**

Sections of paraffin-embedded lungs were prepared and stained with hematoxylin and eosin. The slides were evaluated by light microscopy, and the extent of vascular remodeling assessed by a researcher blinded to the treatment groups. Three whole left lung sections from each rat were evaluated. The percentage medial wall thickness (media x1 / external diameter (E.D.) x100) and adventitial thickness (adventitia x1 / E.D. x100) of fully muscuarized pre-acinar pulmonary arteries was measured, using 10 randomly chosen vessels from each of the 3 sections for each rat. For arterioles (<80 µm E.D), the degree of muscularization was determined 1-3: (1 = no muscularization, not occluded; 2 = muscularization, not occluded; 3 = muscularization, fully occluded).

**Primary smooth muscle cell isolation and culture**

At baseline (control) and 21 days following exposure to monocrotaline, rats were killed by an overdose of pentobarbital. The lungs were immediately removed and proximal pulmonary arteries were isolated. PASMCs were isolated by enzymatic digestion, with purity and verification of PASMC using immunostaining for alpha-smooth muscle actin as previously described.

**Measurement of PASMC proliferation**
PASMCs were cultured to 80% confluence in passage 3-6. On day 0 of the proliferation assay, cells were detached with trypsin (0.05%)/EDTA (0.02%) and seeded in 48-well plates at a density of 5x10^4 cells/well (in DMEM, 10% FCS). After 48 hours of incubation (day 2), the cells were serum-starved (in DMEM, 0.1% FCS) for 24 hours. On day 3, cells were washed twice with PBS and re-cultured in DMEM (with 10% FCS), with dexamethasone (Sigma Aldrich, France). On the basis of preliminary experiments examining the antiproliferative effects of dexamethasone on 10% FCS–stimulated rat PASMCs, the final concentrations used were 10^{-7} to 10^{-8} mol/L. Controls were cultured in DMEM, 10% FCS. On day 4, 24 hours after the addition of dexamethasone, the cells were labelled with 3H-thymidine at 1 µCi/mL for 24h and frozen at -80c. After labelling was completed in all samples, the cells were washed in situ with 500 µL ice-cold uptake buffer, lysed with 500 µL of 0.1N NaOH and the radioactivity counted using liquid scintillation spectroscopy.

**Statistical analysis**

Data are expressed as mean (standard error of mean (SEM)), unless otherwise specified. Data were analysed using the non-parametric Kruskal–Wallis test followed by Dunn’s test for multiple comparisons, the Mann Whitney U test and the Spearman’s rank correlation. Differences were considered significant for p<0.05. Analyses were performed using Statview 5.0.

**RESULTS**
Dexamethasone treatment improves survival in established MCT-induced PAH

Treatment with dexamethasone 5mg/kg/day from day 14-28 significantly improved survival assessed at day 28, compared to MCT alone (log rank test, p<0.0001) (Figure 1). Survival was also significantly improved at day 35 (data not shown).

Dexamethasone treatment normalizes hemodynamics and right ventricular hypertrophy in established MCT-induced PAH

Twenty-eight days after MCT administration, hemodynamics showed a significant increase in mean pulmonary arterial pressure (mPAP) (40.8±6.4 mmHg vs. 16.4±1.5 mmHg in control animals, p<0.0001, Figure 2A), right ventricular systolic pressure (RVSP) (94.3±7.8 mmHg vs. 35.2±2.4 in control rats p<0.0001, Figure 2B) and RV/LV+S (0.6±0.1 vs. 0.26±0.06 in controls, p<0.0001, Figure 2C). Dexamethasone given at 3 dose ranges (5mg/kg/day; 1.25 and 2.5mg/kg/48 hourly) from day 14 to 28 (MCT+Dex D14-28) after MCT administration normalized the mPAP (p<0.05 for Dex5 and dex2.5, p=0.066 for Dex1.25), RVSP (p<0.0001 for dex5 and dex2.5, p<0.05 for dex1.25) and RV/LV+S (p<0.0001 for all doses) in a dose-dependent manner. Dexamethasone (5mg/kg) given from day 14 to 28 was no statistically different to control rats (p=0.11). Dexamethasone at 5mg/kg/day from day 21 to 35 (MCT+Dex 21-35) also normalized mPAP, RVSP and RV/LV+S (p<0.05 for all compared to MCT alone, Figures 3A-C). Hemodynamic indices were also normalized in all earlier ‘preventative’ earlier phase dexamethasone-treated groups (MCT+Dex D1-14 and MCT+Dex D1-28, p<0.001 for all) (data not shown).

Dexamethasone treatment reverses pulmonary vascular remodelling in established MCT-PAH
Dexamethasone treatment (Day 14-28) reduced the degree of muscularization of peripheral pulmonary arteries and arterioles as assessed by morphometric analysis. MCT significantly increased the percentage medial thickness of pre-acinar pulmonary arteries (expressed as media / external diameter x 100) at D28 compared to controls (31.4±10.1 vs. 10.8±4.93%, p<0.0001). This was reduced in a dose-dependent fashion in all the D14-28 dexamethasone-treated groups at D28 compared to MCT alone (20.7±9.47%, 18.8±7.7% and 14.7±7.46% for Dex1.25, Dex2.5 and Dex5 respectively vs. 31.4±10.1%, p<0.0001 for all dexamethasone groups). Pulmonary arterial adventitial thickness was also increased at D28 following MCT compared to control rats (36.5±34.3 vs. 8.83±4.95%, p<0.0001), which was reduced with all D14-28 dexamethasone doses (24.0±12.6%, 16.3±10.4% and 9.47±5.39 for Dex1.25, Dex2.5 and Dex5 respectively vs. 36.5±34.3%, p<0.0001 for Dex5 and Dex2.5, p<0.05 for Dex1.25) (Figure 4A, 4B). At D28 following MCT, there was a significant increase in pulmonary arteriolar (i.e. vessels <80 μm external diameter) muscularization, with an increase seen in the percentage of both non-occluded and occluded arterioles compared to control rats (p<0.0001). Following D14-28 dexamethasone treatment, the arteriolar muscularization score was reduced in a dose-dependent manner (p<0.05 for all groups) (Figure 4C).

**Dexamethasone reduces MCT-induced adventitial infiltration of IL-6-expressing inflammatory cells**

Twenty-eight days after MCT administration, immunohistochemistry showed only a weak staining of IL-6 in control lungs (Figure 5A) whereas adventitial infiltrating inflammatory cells displayed a strong IL-6 expression in MCT-exposed rats (Figure 5B). Lungs from 5mg/kg/day
Dexamethasone-treated rats (MCT+Dex 1-28) exhibited the same faint IL-6 staining as seen in control lungs (Figure 5C).

**Dexamethasone inhibits MCT-induced pulmonary IL-6 overexpression**

Twenty-eight days after MCT administration, pulmonary IL-6 mRNA expression measured by RT-qPCR was strongly upregulated (p<0.01). Dexamethasone (MCT+Dex 1-28), normalized whole lung IL-6 mRNA expression (p<0.05 vs. MCT-exposed lungs; no significant difference seen with control lungs). Furthermore, IL-6 was reduced by late (D14-28) dexamethasone treatment, p<0.05 vs. MCT-exposed lungs at D28) (Figure 6A & B).

**Dexamethasone increases pulmonary BMPR2 downregulation in MCT-induced PAH**

Twenty-eight days after MCT administration, pulmonary BMPR2 mRNA expression was strongly downregulated (p<0.01). MCT+Dex D1-28 (Figure 7A) and D14-28 (Figure 7B) increased whole lung BMPR2 expression (p<0.01 and p<0.05 for D1-28 and D14-28 respectively vs. MCT-exposed lungs, not significantly different to control lungs).

**Dexamethasone inhibits proliferation of cultured rat PASMC**

Proliferation of PASMC isolated from pulmonary hypertensive rats (at day 21 following MCT), as assessed by ³H-thymidine uptake, was inhibited following dexamethasone treatment in a dose-dependent manner compared to controls in complete medium without dexamethasone (38% reduction at dexamethasone x10⁻⁸M and 88% reduction at dexamethasone x10⁻⁷M, p<0.05 for both doses compared to controls). Similar results were obtained using manual cell counting techniques (data not shown). At dexamethasone x10⁻⁸M, cells isolated from control rats were
growth-inhibited more by dexamethasone compared with PASMCs from MCT-exposed rats (p<0.05) (Figure 8).

**Dexamethasone increases BMPR2 and reduces IL-6 in rat PASMC**

Treatment of rat PASMC with dexamethasone (x10^-8M) led to an increase in cellular BMPR2 as measured by qRT-PCR in control cells (p<0.05), compared to untreated cells, whereas there was no significant difference in BMPR2 following dexamethasone treatment in cells isolated from pulmonary hypertensive rats (Figure 9A). IL-6 was reduced following dexamethasone treatment (p<0.05 for both control and MCT-exposed cells) (Figure 9B).

**DISCUSSION**

The major findings of this study were the following: (1) dexamethasone improved survival in rats with established MCT-PAH, with normalization of hemodynamics, RV hypertrophy and pulmonary vascular remodelling at late time points after MCT; (2) in keeping with previous findings, MCT down-regulated BMPR2 expression and increased IL-6 activity: we showed that hemodynamic improvements with dexamethasone treatment were associated with a normalization of BMPR2, 3) reduced pulmonary IL-6 overexpression and a reduction in the adventitial infiltration of IL-6-expressing inflammatory cells. (4) Finally, dexamethasone inhibited rat PASMC proliferation which was associated with a reduction in IL-6 and an increase of BMPR2 in control PASMC and (5) cells isolated from pulmonary hypertensive rats appeared relatively resistant to the BMPR2 increase and the anti-proliferative effects of dexamethasone.
Monocrotaline is an ‘inflammatory’ model of PAH, comprising an initial asymptomatic inflammatory phase, followed by a less inflammatory symptomatic phase from day 14 with increased medial volume in both major and intra-acinar pulmonary arteries by 21 days exposure\(^{38, 39}\). Previous studies have similarly shown that preventive immunosuppressive therapy is effective in MCT-PAH when given before the onset of pulmonary vascular remodelling (i.e. prior to D14)\(^{9, 17, 18, 40-43}\). However, although there have been some studies showing reversal of PAH at later time-points with various anti-inflammatory therapies, none as yet have used glucocorticoids. The reversal of MCT-PAH beyond the onset of vascular remodelling is of clinical relevance. Although patients with idiopathic PAH are not believed to have a ‘steroid-responsive’ disease, clinical improvement of PAH is seen in associated ‘inflammatory’ conditions where immunosuppression (including glucocorticoids) was otherwise indicated, including PAH associated with connective tissue diseases\(^{44}\) and SLE\(^{45}\). Interestingly this improvement in PAH occurs especially in those with earlier, less severe PAH\(^{16}\), perhaps suggesting that immunosuppressive therapies may be more effective in proliferating ‘active’ lesions in early disease than in those with established ‘fixed’ pulmonary vascular lesions.

Among the wide spectrum of biological actions of glucocorticoids, dexamethasone has been shown to inhibit vascular cell proliferation\(^{46}\). The anti-proliferative findings in this study are in keeping with a study of prednisolone on PDGF-stimulated PASMC from PAH patients\(^{47}\), although this study used much higher doses (equating to dexamethasone \(3\times10\) \(\mu\)g and \(3\times10\) \(\mu\)M).

Mutations in the genes encoding the bone morphogenetic protein (BMP) receptor type II (\(BMPR2\)) have been shown to be important in familial idiopathic PAH\(^{22}\). BMPs are members of the TGF-\(\beta\) superfamily, which through type 1 and type 2 receptors contribute to regulation of cell
proliferation, differentiation and apoptosis. In humans, a variety of cell types, including PASMC and endothelial cells, synthesise and secrete BMPs. BMPRII-positive cells have also been shown to be closely associated with the inflammatory cell infiltrate in IPAH lesions. One of the normal roles of the BMP signalling pathway is believed to prevent runaway positive feedback loops in inflammatory cytokines. Reduced expression of BMPRII has been reported in most types of human PAH, and an attractive theory is that the dysregulated BMPRII signalling is followed by an inflammatory ‘second hit’ early in the pathogenesis of PAH. Our data are consistent with previous studies showing a reduction in BMPRII in MCT-induced PAH in rats, and as far as we are aware, this is the first study to show a glucocorticoid-induced increase in the low BMPRII levels in MCT-induced PAH. The importance of IL-6 has been shown in several studies of PAH. Patients with PAH have increased circulating IL-6 levels, and IL-6 is capable, on its own, of causing growth of vascular smooth muscle cells and PAH. In transgenic mice, IL-6 overexpression induces PAH associated with down-regulation of TGF-beta signalling. In keeping with previous studies, we found that MCT-induced PAH is also associated with increased IL-6 production. Kaposi’s sarcoma-associated herpes virus, which may cause PAH in human immunodeficiency virus (HIV)-negative Castleman’s disease, encodes a viral, constitutively active, form of IL-6. In addition, recent studies have suggested the involvement of viral infection or autoimmunity in the development of PAH. Thus there is substantial evidence that the unknown second hit is likely to be inflammatory in character. Hagen et al have identified a negative feedback loop between IL-6 and the BMP pathway, in which increased IL-6 induces BMP pathway activity, and increased BMP pathway activity suppresses IL-6. Furthermore, IL-6 enhances proliferation via activation of STAT3, persistent activation of which has been shown to reduce BMPRII expression, which may contribute to the loss of BMPRII during PAH development. Further work showing that although asymptomatic
BMPR2\(^{+/−}\) mice do not develop pulmonary hypertension spontaneously, under inflammatory stress, they are more susceptible than wild-type mice\(^{32}\), and that mice expressing a dominant negative BMPR2 in smooth muscle develop elevated right ventricular pressures, with an increase in cytokines and markers of immune response, when the transgene is activated \(^{33}\). BMPRII dysfunction and resulting loss of activity may therefore result in unopposed IL-6 production in the context of an as yet unknown inflammatory stimulus.

Our data suggest that dexamethasone interrupts the IL-6-BMPR2 negative feedback loop\(^{34}\) probably mainly through a dexamethasone-induced reduction in IL-6-expressing inflammatory cells, and possibly also a direct PASMC effect. The resulting increase in pulmonary BM\(PR2\) thus may restore the required dampening effects of BMPRII signalling on IL-6 function. The mechanisms through which glucocorticoids interact with BMPRII are unclear, but a gene expression profiling study of asthmatics receiving glucocorticoids suggests an important interaction between the sensitivity of the glucocorticoid receptor and BMPRII\(^{56}\).

Limitations of our study include the absence of BMPRII protein expression to confirm the findings in gene transcription; the lack of immunohistochemistry for BMPRII, and also the lack of IL-6 immunohistochemistry at later treatment time-points, although we replaced this with quantitative data using RT-PCR for IL-6 and BMPR2 at these time-points.

In conclusion, we have shown that treatment with dexamethasone reverses haemodynamics, reduces remodelling and improves survival in established MCT-induced PAH, with normalization of BM\(PR2\) expression and macrophage inflammatory responses. These findings provide new insight into the potential role of immunosuppressants in the treatment of human PAH via the regulation of the BMPRII/IL-6 pathways.
**FIGURES & LEGENDS**

**Figure 1: Survival: Dexamethasone treatment improves survival in MCT-induced PAH**

Treatment dexamethasone 5mg/kg/day from day 14-28 had a significant positive impact on survival at day 28 (log rank test, p=0.001) compared to MCT alone.

**Figures 2+3: Hemodynamics: Dexamethasone reverses hemodynamics and right ventricular hypertrophy in established MCT-induced PAH (at D28 and D35)**

Twenty-eight days after MCT administration, mean pulmonary arterial pressure (mPAP) (**Figure 2A**), right ventricular systolic pressure (RVSP) (**Figure 2B**) and right ventricular to-left ventricle+septal ratio (RV/LV+S) (**Figure 2C**) were significantly increased in MCT-exposed rats (* p<0.001 for all groups). Dexamethasone given at 3 doses (5mg/kg/day, 1.25 and 2.5mg/kg/48-hourly) from day 14 to 28 after MCT administration normalized the mPAP (p<0.05 for Dex5 and dex2.5, p=0.066 for Dex1.25), RVSP (p<0.0001 for dex5 and dex2.5, p<0.05 for dex1.25) and RV/LV+S (p<0.0001 for all doses). At the highest dose, rats treated with dexamethasone D14-28 were no different to control rats (NS). Dexamethasone at 5mg/kg/day from day 21 to 35 (MCT+Dex 21-35) also normalized mPAP, RVSP and RV/LV+S (p<0.05 for all compared to MCT alone) (**Figures 3A-C**).

**Figure 4: Morphometry: Dexamethasone treatment reverses pulmonary vascular remodelling**

Effect of dexamethasone treatment (Day 14-28) on the degree of muscularization of peripheral pulmonary arteries: A total of 30 intra-acinar vessels were analyzed in each of 3 lungs from each
rat exposed to MCT for 28 days and dexamethasone-treated group (from Days 14 to 28). **Figure 4A** shows increased medial thickness in pre-acinar pulmonary arteries at D28 following monocrotaline compared to controls (31.4±10.1 vs. 10.8±4.93%, p<0.0001), which was reduced with all doses of D14-28 dexamethasone at D28 (20.7±9.47%, 18.8±7.7% and 14.7±7.46% for Dex1.25, Dex2.5 and Dex5 respectively vs. 31.4±10.1%, p<0.0001 for all groups). **Figure 4B** shows increased pulmonary arterial adventitial thickness with MCT (36.5±34.3 vs. 8.83±4.95%, p<0.0001), also reduced following D14-28 MCT in a dose-dependent manner (24.0±12.6%, 16.3±10.4% and 9.47±5.39 for Dex1.25, Dex2.5 and Dex5 respectively vs. 36.5±34.3%, p<0.0001 for Dex5 and Dex2.5, p<0.05 for Dex1.25). Arteriolar muscularization and occlusion scores were increased at D28 following MCT (p<0.0001) and were reduced with D14-28 dexamethasone treatment at all doses (p<0.05 for all groups) (**Figure 4C**).

**Figure 5: Immunohistochemistry: effects of dexamethasone on infiltration of IL-6-expressing inflammatory cells:** Photographs of control, MCT D28 and D1-28 dexamethasone-treated lung

Haematoxylin and eosin elastic staining of paraffin embedded rat lung tissue. **Figure 5A:** Control lung showing pulmonary arteriole with single layer arteriolar wall and weak staining of IL-6. **Figure 5B:** Extensive vascular narrowing of small muscular pulmonary arteries and adventitial infiltrating inflammatory cells displaying a strong IL-6 expression in MCT exposed rats. **Figure 5C:** Lungs from dexamethasone-treated rats (MCT+Dex 1-28) showing a normal medial wall thickness of small pulmonary arteries and exhibiting a similar weak IL-6 staining as seen in control rats. **Figure 5D:** Negative control: omission of primary antibody.
Figure 6: Real time PCR: Impact of dexamethasone on pulmonary IL-6 mRNA expression in MCT-induced PAH.

Twenty-eight days after MCT administration, IL-6 mRNA expression was upregulated in lungs of MCT exposed rats (p<0.01). Dexamethasone 5mg/kg/day (MCT+Dex 1-28, Figure 6A; MCT+Dex 14-28, Figure 6B) normalized whole lung IL-6 mRNA expression (p<0.05). IL-6 mRNA is expressed normalized to the housekeeping gene beta actin.

Figure 7: Real time PCR: Impact of dexamethasone on pulmonary BMPR2 mRNA expression in MCT-induced PAH.

Twenty-eight days after MCT administration, BMPR2 mRNA expression was strongly downregulated in lungs of MCT exposed rats (p<0.001). Dexamethasone increased whole lung BMPR2 mRNA expression in MCT+Dex 1-28 rats compared to MCT exposed rats (p<0.01) (Figure 7A), and at day 28 following day 14-28 dexamethasone treatment (p<0.05) (Figure 7B).

Figure 8: Dexamethasone reduces proliferation of cultured rat PASMCs

Proliferation of PASMC, was assessed by ³H-thymidine uptake, was expressed as counts per minute (CPM), and confirmed using manual counting (data not shown). Proliferation of PASMC was inhibited following dexamethasone treatment in a dose-dependent manner compared to untreated controls, for both PASMC exposed to MCT (MCT+dex-8, MCT+dex-7) and non-MCT-exposed cells (Control+dex-8, Control+dex-7) (p<0.05 for dexamethasone x10⁻⁸M and p<0.001 for x10⁻⁷M, compared to controls). Cells isolated from control rats were growth-inhibited more by dexamethasone compared with PASMC from MCT-exposed rats at dexamethasone x10⁻⁸M (p<0.05).
**Figure 9: Dexamethasone increases BMPR2 and reduces IL-6 in cultured rat PASMCs**

PASMC were isolated from control and pulmonary hypertensive rats (day 21 following monocrotaline), treated with dexamethasone $10^{-8}$M, and RT-PCR was performed for cellular BMPR2 and IL-6 mRNA. Dexamethasone treatment increased BMPR2 in control cells (Control+dex, $p<0.05$ compared to untreated controls), but not in MCT-exposed PASMC (MCT+dex) compared to MCT-exposed untreated controls (MCT-dex) (**Figure 9A**). IL-6 was reduced in PASMC from both control and MCT-exposed PASMC following dexamethasone treatment compared to controls ($p<0.05$ for both groups) (**Figure 9B**).
REFERENCES


Figure 1
Figure 2

A

B

C

Figure 3
Figure 4
Figure 5


Figure 6
Figure 7
Figure 8
Figure 8: Effects of dexamethasone on PASMC $^3$H-thymidine uptake

Counts per minute (CPM)

Control  
Control+dex-8  
Control+dex-7  
MCT  
MCT+dex-8  
MCT+dex-7

# NS between groups
* p<0.05 between groups
** p<0.001 compared to control
A

**BMFR2 mRNA** normalised to beta actin mRNA

- Control-dex
- Control-dex
- MCT-dex
- MCT-dex

* p<0.05 compared to control
# NS between groups

B

**IL-6 mRNA expression** normalised to beta actin mRNA

- Control-dex
- Control-dex
- MCT-dex
- MCT-dex

p<0.05 compared to control
# NS between groups