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Beneficial effect of combined therapy with macitentan and sildenafil in a rat model of pulmonary artery hypertension

폐동맥 고혈압 백서 모델에서 macitentan 과 sildenafil 병합 요법의 우심실 재형성에 미치는 영향

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A doctoral degree in Clinical Medical Sciences

Beneficial effect of combined therapy with macitentan and sildenafil in a rat model of pulmonary artery hypertension

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ABSTRACT

Abstract

Background: Recent reports have noted that a new dual endothelin-receptor antagonist, macitentan reduced morbidity and mortality among patients with pulmonary arterial hypertension. We investigated the efficacy of macitentan in combination with sildenafil on hemodynamic and morphological parameters in rats with monocrotaline-induced pulmonary artery hypertension (PAH).

Method: We proceeded an experiment by planning two different protocol to find out the effect of drugs on pulmonary hypertension and right ventricle. Anti-remodeling protocol: One week after monocrotaline (MCT) injection, the rats were randomly assigned to macitentan (MAC) treatment (30mg/kg p.o, qd, N=16), macitentan (MAC) combined with sildenafil (SIL) treatment (MAC+SIL group, N=16, MAC 30mg/kg p.o qd, SIL 50mg/kg p.o, bid), or normal saline only group (MCT group, N=16). Reverse remodeling protocol: Three weeks after monocrotaline injection, elevated RV-RA pressure gradient was confirmed by echocardiography. The rats were randomly assigned to MAC treatment (N=14), sildenafil combination treatment (MAC+SIL group, N=14). RV afterload is assessed by measurement of PASP from TR velocity and right atrial pressure. For quantification of
RV performance, TAPSE, FAC were measured. A pressure-volume analysis performed at the 7th week after MCT.

**Results:** Serial echocardiograms revealed that significant pulmonary hypertension was developed three weeks after MCT injection and it was getting severe with time. The increases in PASP and ratio of right ventricular weight to body weight were significantly attenuated in the macitentan and combination with sildenafil groups (20.7±1.5 for sham vs 57.3±5.7 for MCT vs 36.8±1.6 for MAC vs 34.7±2.3 mmHg for MAC+SIL). When given at a delayed stage (with established RV hypertrophy and failure), combination therapy with macitentan and sildenafil improved survival rate and exercise capacity but not RV hypertrophy and decreased inflammation cytokines.

**Conclusion:** Macitentan administered at an early stage prevented the development of PAH and RV hypertrophy and failure and exercise intolerance caused by chronic experimental PAH. Combination therapy of macitentan and sildenafil is more effective than macitentan treatment alone significantly and prevent exercise intolerance after administration of RV failure without any cardiovascular toxic effects in the monocrotaline-induced PAH model.
Keywords: Pulmonary artery hypertension, Macitentan, Sildenafil, RV remodeling

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*p<0.05 for difference from sham
LIST OF ABBREVIATIONS

PH= pulmonary hypertension
PAH= pulmonary arterial hypertension
MCT= monocrotaline
MAC=macitentan
SIL=sildenafil
PASP= pulmonary artery systolic pressure
RV= right ventricle
RA= right atrium
RVSP=right ventricle systolic pressure
BP=blood pressure
IHC=immunohistochemistry
TAPSE; tricuspid annular plane systolic excursion
INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive, life-threatening disease that affects the pulmonary vasculature\(^1\). The histopathology is marked by vascular proliferation/fibrosis, remodeling, and vessel obstruction. Development of PAH involves the complex interaction of multiple vascular effectors at all anatomic levels of the arterial wall. The hallmarks of disease include vasoconstriction, thrombosis, and inflammation with subsequent vessel wall remodeling and cellular hyperproliferation\(^2, 3\). Current therapies for pulmonary arterial hypertension target abnormalities in one of three intracellular pathways with signaling dysfunction\(^1\): the endothelin, prostacyclin or nitric oxide pathway. However, no single class of drug has been found to be consistently effective in treating all patients, which suggests that no single dominant pathogenic role\(^4\). It has been hypothesized that combination therapy with agents that target several different pathways may potentially increase the overall therapeutic effect and provide additional clinical benefits\(^5, 6\).

A novel, dual endothelin-receptor antagonist, macitentan developed by modifying the structure of bosentan with an aim to increase efficacy and safety, has been reported to significantly reduce morbidity and mortality among patients with pulmonary arterial hypertension\(^7\). Sildenafil citrate, a selective inhibitor of phosphodiesterase type 5, also
improves exercise capacity and hemodynamics in patients with symptomatic pulmonary hypertension\textsuperscript{8}. Additionally, growing evidence has proven that sildenafil attenuated pulmonary inflammation, exhibits anti-hypertrophic, and anti-apoptotic effects on myocytes that may limit myocardial remodeling in response to stress and attenuate the substrate for heart failure development\textsuperscript{9-11}.

Accordingly, we hypothesized that combination therapy with macitentan and sildenafil may have beneficial effect in PAH because the process of right ventricular remodeling shares few of aforementioned molecular mechanisms. In the present study, we evaluated long-term combination effect of macitentan and sildenafil on survival, right myocardial function, remodeling and exercise capacity in the monocrotaline induced pulmonary hypertension (PH) rat model. Additionally, we investigated potential mechanisms behind the beneficial effects of combination therapy using histology and molecular analysis.

MATERIALS AND METHODS

\textit{Method}

Animal Handling, Monocrotaline Induced PAH, Study Protocol
Male Sprague Dawley (SD) rats (n=103) with an initial weight of 350-400g were employed in the present study. The rats were housed under controlled temperature (21°C), humidity (55%) and lighting (12:12-h light-dark cycle) with free access to tap water and standard rat chow. The experimental protocols were approved by Institutional Animal Care and Use Committee of Seoul National University Hospital [IACUC number; 13-0040-CIA0].

Currently, the monocrotaline (MCT) induced PAH rat model, chronic hypoxia with or without Sugen 5416 and pulmonary artery banding are used to study experimental PAH\textsuperscript{12}. However, none of the animal models fulfills the pathophysiology of human PAH. Concerns have been raised about the MCT rat model since many therapies were successful in MCT rats, but not in humans with PAH\textsuperscript{13}. Additionally, we observed 10% failure rate in making MCT induced PAH. We, therefore, investigated long-term progression with large numbers and reversibility of MCT induced PAH with medication in rats for over 4 weeks, using a dose of 60 mg·kg\textsuperscript{-1} in a randomized placebo-controlled study design, since it is known that a high dose of MCT (80 mg·kg\textsuperscript{-1} ) is fatal within 3 weeks of administration\textsuperscript{14}. MCT (Sigma/C2401, Sigma-Aldrich) was dissolved in 0.5 N of HCl, and the pH was adjusted to 7.4 with 0.5 N NaOH. The solution was given as a single subcutaneous injection (60
mg/kg) to rats. Sham rats received an equal volume of isotonic saline.

Experiments were carried out by planning two different protocols to find out the effect of drugs on pulmonary hypertension and right ventricle. Model 1 was planned to study the effect of drugs on right ventricular remodeling with reference to post-administration exercise capacity, right ventricular function, and histological changes before progression of right ventricular imperfection and hypertrophy (on echocardiography). One week after monocrotaline (MCT) injection, the rats were randomly divided into three groups as macitentan (MAC) treatment (MAC group, 30mg/kg p.o, qd, N=16), macitentan (MAC) combined with sildenafil (SIL) treatment (MAC+SIL group, N=16, MAC 30mg/kg p.o qd, SIL 50mg/kg p.o, bid), or normal saline only group (MCT group, N=16). Twelve sham rats were compared to MCT induced PH rats. Model 2 was planned to study the effect of drugs on ‘reverse remodeling’ after re-modeling of right ventricle in which the randomized drugs administration was conducted after identifying right ventricular hypertrophy and pulmonary hypertension. Three weeks subsequent to monocrotaline (MCT) administration, we confirmed the development of PAH with RV dilatation, elevation in PASP by transthoracic echocardiography (TTE) and the rats were randomly assigned to macitentan (MAC) treatment (MAC group, 30mg/kg, p.o. qd, N=14), macitentan (MAC) combined with sildenafil
(SIL) treatment (MAC+SIL group, N=14, MAC 30mg/kg p.o qd, SIL 50mg/kg p.o, bid), or normal saline only group (MCT group, N=12). Three rats without formation of PH for three-week period were excluded in Model 2. We started the treatment 3 weeks after monocrotaline injection and continued for 5 weeks according to the results of our pilot study; (1) pulmonary artery systolic pressure (PASP) by tricuspid regurgitation was observed to be more than 20 mmHg as compared with baseline echocardiography and (2) no additional death occurred during this period. Cardiac function, exercise capacity, and body weight were monitored every 2 weeks. At the end of 8-week after monocrotaline injection (19-week age, same Model 1 and 2), pressure-volume analysis was performed and the hearts, lungs, and livers were harvested for tissue determinations. The detailed experimental protocol is shown in Fig. 1.

Macitentan at a dose of 30mg/kg/day (Actelion, Switzerland) was administered orally by gavage once a day based on the previous finding that maximal effective dose of macitentan decreased mean pulmonary arterial pressure by 30 ± 5 mm Hg. Although this dose is high for humans, rats metabolizes MAC at a higher rate, and this dose yields a free plasma concentration of 1.0 to 1.5 pg/ml of ET-1 concentration, within the specific and therapeutic range for dual ET_A/ET_B receptor antagonist. An oral dose of 100mg/kg/day sildenafil citrate (Hanall,
Korea) was provided by gavage twice a day based on the previous studies showing the pleiotropic vascular effect in pulmonary hypertension and heart failure models\textsuperscript{10,11,17}. The dose administered is also high for humans; however, it yields a free plasma concentration of 10 to 15 nM, within the specific and therapeutic range for PDE5A among rats with high metabolites.
Survival Analysis

We examined the effects of macitentan or combination therapy of macitentan with sildenafil on the survival of PH rats. The day of oral administration of MCT was defined as day 0. This survival analysis covered the entire experimental period of 56 days.
그림 1 experimental protocol, a schemiatic illustration

Blue narrow indicates the time at which the body weight, echocardiography and exercise capacity were measured. The asterisk indicates the time at which we obtained pressure-volume loop after harvesting experimental rats.

wk; week, MCT; monocrotaline, N.S; normal saline
Physiologic study

Echocardiography

Transthoracic echocardiography was performed 1 week before and 1, 3, 5 and 7 weeks after MCT injection on spontaneously breathing rats placed on their dorsal recumbent position. Rats were lightly sedated with inhalation of the lowest possible dose of isoflurane (initially 4%, then approximately 2-3%) mixed with oxygen. Images were acquired with a 9 MHz transducer connected to a Toshiba echocardiography machine (Nemio, Toshiba Co., Tokyo, Japan). Right ventricular (RV) free-wall thickness and end-diastolic cavity dimension were measured using M-mode echocardiography at the papillary muscle level. Fractional area change (FAC) and tricuspid annular plane systolic excursion (TAPSE) were evaluated in the apical four-chamber view so that the tricuspid and mitral valves could be clearly visualized. Even though all rat hearts were horizontally displaced in the diaphragm so that apical four-chamber view is relatively for shortening of left ventricular apex, RV FAC and TAPSE were offered consistent image in this animal model. Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. The sample volume was placed proximal (1-2 mm) to the pulmonary valve leaflets and the acceleration time as well as velocity-time integral were measured. Acceleration time was measured from the time of onset of
systolic flow to peak pulmonary outflow velocity. The systolic right ventricular (RV)-right atrial (RA) pressure gradient was calculated using the peak tricuspid regurgitation (TR) velocity (v) in the modified Bernoulli equation (\( \Delta P=4v^2 \)). True pulmonary artery systolic pressure (PASP) could not be measured since inferior vena cava was not visualized with relatively large liver volume in rats.

All parameters were evaluated on an average of five consecutive beats. All studies were performed by an experienced sonographer with experience in performing rat echocardiography and data acquisition for more than 1000 cases and who was blinded to the treatment group. Two observers, also blinded to treatment assignment, analyzed the images.

*Exercise Test and BP Monitoring*

Maximal exercise capacity was evaluated with Rota Rod Treadmill (Ugo Basile, Comerio, Italy). Typically, during the test, rats run on a knurled drum as the drum rotates in order to avoid falling off. Animals were trained twice before the test to adjust and get familiar with the treadmill. Treadmill speed was gradually increased from 3 rpm (revolutions per minute) to 15 rpm every 1 minute. Exercise time was recorded. The observer blinded to the study group recorded episodes of
the immobility response due to exhaustion.

Systolic and diastolic blood pressures were measured in conscious rats via the tail-cuff method (Biopac System Inc) at 1 week before and 1, 3, 5, 7 weeks after injection of MCT. At least 1-day interval was given for the BP measurements after echocardiographic examination or exercise test to minimize the stress on the animals.

**Invasive Hemodynamic Measurements**

At 7 weeks (56 days) after MCT injection, invasive hemodynamic measurements were obtained using a Millar catheter (SPR-869; Millar Instruments, Houston, TX) inserted into the right ventricle pressure catheter. The rats were sedated by inhalation of a mixture of isoflurane (4%) and oxygen. An endotracheal intubation was performed, and the animals were mechanically ventilated using a pressure-controlled respirator and a mixture of air and oxygen. The left jugular vein for infusion of hypertonic saline (10%) was prepared to determine parallel conductance. A midsternal thoracotomy was performed and a conductance catheter was inserted via the RV apex into the RV. After allowing for stabilization from surgical preparation, data were continuously recorded on a personal computer using a pressure-volume unit (model MPVS-300; ADInstruments, Colorado Springs, CO).

Heart rate, RV end-diastolic volume, RV end-systolic volume, RV
end-diastolic pressure, RV peak systolic pressure, and RV-end systolic pressure were assessed from pressure-volume loops. RV pressure upstroke and fall (dP / dt_max and dP / dt_min, respectively) were calculated. The slopes, end-systolic and end-diastolic elastance (E_{ES} and E_{ED}) were determined by linear regression.

To obtain Vo, additional loops were acquired during injection of 100µl of 10% hypertonic saline via the cannula in the jugular vein. True volume was determined by measurement of the aortic flow using an ultrasonic flow probe (Transonic systems, Maastricht, The Netherlands) around the descending thoracic aorta.

**Histopathological analysis**

After hemodynamic measurements were performed, the rats were euthanized and the hearts and lungs were harvested and weighed.

**Biochemical analysis of serum**

Plasma biochemical analysis was performed within 24 hours of blood collection. Blood was collected from the rats sacrificed under isoflurane anesthesia after hemodynamic study. B-type natriuretic peptide (BNP) (AbCam Inc., Cambridge, MA) protein analysis in plasma was performed according to the manufacturer’s instructions. We
collected blood in a plastic EDTA (lavender-top) tube. The tube was centrifuged and separate plasma was collected into a plastic specimen transport tube within one hour and frozen immediately. In brief, a 1:4-fold dilution of plasma for BNP was incubated for 2 hours at room temperature using the manufacturer’s assay reagents. BNP concentrations were calculated from their assay standard curve optical density value. For estimation of liver enzymes (AST and Total bilirubin) and creatinine level, the blood samples were centrifuged after allowing for clotting (1-3 hours) at 2000 revolution per minute for 15 minutes. The sera were assayed for serum aspartate aminotransferase (AST) and total bilirubin and creatinine.

**RNA isolation**

Fresh RV apex was immediately stored in RNAlater (Ambion/Applied Biosciences, Streetsville, ON, Canada) at -80°C until use. The hearts were homogenized in TRIzol Reagent (Invitrogen, Burlington, ON, Canada), and total RNA was isolated according to the manufacturer’s instructions. RNA was further purified to remove contamination in genomic DNA and concentrated using an RNeasy Plus Mini kit (Qiagen, Mississauga, ON, Canada). Samples with optical density ratio 260/280 >1.8, 28S/18S >1.6 measured using a Bioanalyzer 2100 (Agilent, Santa Clara, CA) were selected for RT-PCR.
Total RNA was isolated using RNeasyPlus Mini kit (Qiagen) and cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara) with total RNA sample (1 µg) according to the manufacturer’s instruction. cDNA (1 µg) was amplified by PCR using TaKaRa Ex Taq™ kit (Takara) with specific primers (Table 1). GAPDH was chosen as an endogenous control. Quantification of band intensity was analyzed using TINA 2.0 (RayTest) and normalized to the intensity of GAPDH.
Table 1. Primer Sequences for Real Time PCR

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**Immunohistochemical Analysis**

Mid ventricles were removed for histopathology, preserved in 4% paraformaldehyde and embedded in paraffin. The tissue was sectioned into four µm sections, stained with Masson’s trichrome for evaluation of the degree of fibrosis. No correction was made for tissue shrinkage by the fixation procedure, under an assumption that this factor is equal in all the samples.

**Statistical analysis**

Data are presented as mean±S.E.M. The normality of all parameters was tested using the Kolmogorov-Smirnov test. The differences among groups were compared by the unpaired t-test or one-way analysis of variance, followed by the Bonferroni test. In cases in which normality was excluded, the non-parametric Kruskal-Wallis H test was performed, and the Mann-Whitney U test was used for post-hoc analysis. Repeated measures ANOVA was performed to analyze the changes in several variables over time such as RV-RA pressure gradient, RV wall thickness, body weight and exercise capacity. Survival data was evaluated by the Kaplan-Meier method with pair wise comparison conducted using the log-rank test. All statistical analyses were performed using SPSS 17.0 version (SPSS Inc., Chicago, US) and p-values of < 0.05 were
considered statistically significant.
RESULTS

Model 1: anti-remodeling effect of macitentan

Survival Analysis

A total of 62 rats were employed in this study. Upon echocardiography, one rat died during anesthesia and one rat with poor echo window was excluded from the study. Half of 16 rats in MCT group died during the study period \([P=0.009,\text{ compared with sham}].\) However, no deaths were noted in Sham group and survival rates between MAC and MAC+ SIL were comparable \([3\text{ rats died in each group}].\) Kaplan-Meier survival curve showed a difference between MCT rats and medicated rats \([\text{MAC or MAC+ SIL}]\) \((P=0.04)\) (Figure 2).

The time curves of body weights of the experimental groups over a 7 weeks period after MCT injection indicate a reduced weight gain in MCT rats. The MCT rats showed pronounced growth retardation with resultant significantly lower body weight after 7 weeks than Sham rats \([593.6±9.3\text{ for sham vs 491.0±9.0 for MCT rats, } p < 0.05]\). The MCT animals started to lose weight at around 3 weeks, which is a sign of heart failure as comparable to cachexia in patients with chronic heart failure. In contrast to Sham, MCT rats showed an obvious lack of physical activity. However, weight gain tended to lag behind the sham rats in MAC and MAC+SIL rats without any statistical significance.
[557.5±7.8 for MAC and 550.7±7.8 for MAC+SIL] (Figure 3).
Figure 2. Survival analysis.

Kaplan-Meier survival curve showed a difference between MCT rats and medication rats [MAC or MAC+ SIL] (P=0.04).
Figure 3. Serial change in body weight
Hemodynamic study

**RV Remodeling after MCT injection by echocardiography**

Baseline examinations showed no differences in RV diameters and RV function among the four groups. Initially, in most of the rats, the TR jet was not observed at baseline echocardiography. However, development of TR was prominent, although late and various time points, feature of rats injected with MCT. The TR velocity is used in the modified Bernoulli equation \( 4v^2 \) to establish the RV-RA pressure gradient, which is an estimate of the PASP. Seven weeks after MCT injection, untreated rats exhibited higher PASP as compared with sham (20.7±1.5 for sham vs 57.3±5.7 for MCT, P<0.05). As expected, administration of 30mg/kg/day MAC decreased PASP by 35% (36.8±1.6mmHg; P < 0.05). However, combination treatment with sildenafil did not cause a further decrease statistically and reduced PASP by 39% (34.7±2.3 mmHg; P < 0.05, compared with MCT). LV end diastolic dimension and systolic dimension revealed that MCT rats have smaller D-shaped LV on the LV short axis view. However, treatment with MAC and combination therapy with sildenafil showed preservation of LV dimension (Table 2, Figure 4). Conventional RV variables revealed that MCT rats have dilated and hypertrophied RV with decreased systolic
function at 7 weeks. RV functions evaluated by FAC, TAPSE and PAAT were significantly decreased as compared to those of sham rats. Macitentan treatment attenuated decreased RV function (RV FAC 25±10 for MCT vs 43±7% for MAC, P < 0.05). Combination therapy with sildenafil led to no further attenuation in RV dysfunction as compared with MAC only treated rats.
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<th>MAC+SIL</th>
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<td><strong>RV FAC</strong></td>
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<td><strong>PASP, mmHg</strong></td>
<td>20.7±1.5</td>
<td>57.3±5.7*</td>
<td>36.8±1.6†</td>
<td>34.7±2.3†</td>
</tr>
</tbody>
</table>

Table 2. Echocardiographic data at 7th weeks after monocrotaline injection. HR; heart rate, SBP; systolic blood pressure, LV; left ventricle, RV; right ventricle; TAPSE; tricuspid annular plane systolic excursion, PASP; pulmonary artery systolic pressure

* P<0.05 compared with sham. † P< 0.05 compared with MCT
Figure 4 – Representative figures of echocardiography
MAC; macitentan, MCT; monocrotaline, MAC+SIL; combination therapy
macitentan and sildenafil, TAPSE; tricuspid annular plane systolic excursion,
PASP; pulmonary artery systolic pressure
Heart rate and systemic blood pressure

We observed no difference in heart rate or systemic blood pressure between the sham and MCT induced PH rats during the study. The heart rate and systemic blood pressure were measured by tail cuff method. Even though a trend towards SBP lowering was present in rats treated with MAC+SIL as compared with Sham, there were no significant changes in blood pressure and heart rate among the groups (Figure 5).
Figure 5- Serial change in blood pressure and heart rate

* P= 0.05 compared with sham
**Exercise Capacity**

There was no difference in exercise duration among the groups until the first week after monocrotaline injection. Thereafter, exercise capacity was impaired progressively in PAH group as compared with sham group (at 7th week, 601±19 for sham vs 342±17 seconds for MCT, p < 0.05). However, exercise duration was maintained in the MAC treated PAH rats (501±18 seconds for MAC, p < 0.05 for difference from MCT). The exercise capacity of MAC+ SIL was comparable with MAC only group (501±18 for MAC vs 483±18 seconds for MAC+SIL, figure 6).
Figure 6 – Comparison of exercise duration among the four groups.

* P < 0.05 compared with sham, + P < 0.05 compared with MCT
Invasive hemodynamic measurement

At the 7th week after monocrotaline injection, rats underwent invasive hemodynamic assessment and were sacrificed for the pathological analysis. Heart rate, systemic blood pressure, dP/dtMax (mmHg/s) were not different among the group. However, RV end systolic volume (ESV, µl) and end diastolic volume (EDV, µl), pulmonary peak pressure (mmHg), Ea (mmHg/µl) Lung weight (g) and Ratio RV weight/ (LV+septum) weight were greater in MCT group compared with sham group. Macitentan treatment prevented right ventricular hypertrophy. Maxitentan or combination therapy with sildenafil for 7 weeks significantly reduced RV/ (LV+septum) by 21% and 20% respectively (p<0.05). The lung weight significantly decreased in macitentan treated rats by 30% for MAC only and by 30% combination with SIL, respectively (p < 0.05).
<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MCT</th>
<th>MAC</th>
<th>MAC+SIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>310±15</td>
<td>305±10</td>
<td>307±15</td>
<td>302±17</td>
</tr>
<tr>
<td>Ratio RV/(LV+IVS)</td>
<td>0.33±0.19</td>
<td><strong>0.5±0.31</strong></td>
<td><strong>0.41±0.20</strong></td>
<td>0.39±0.21</td>
</tr>
<tr>
<td>Lung wt (g)</td>
<td>1.62±0.21</td>
<td><strong>3.01±0.49</strong></td>
<td>2.01±0.77</td>
<td>2.03±0.89</td>
</tr>
<tr>
<td>RVESV, µl</td>
<td>245±74</td>
<td><strong>634±280</strong></td>
<td>380±100</td>
<td>634±280</td>
</tr>
<tr>
<td>RVEDV, µl</td>
<td>438±108</td>
<td><strong>804±386</strong></td>
<td>604±205</td>
<td>804±386</td>
</tr>
<tr>
<td>P peak, mmHg</td>
<td>30.5±6.5</td>
<td><strong>65±16.2</strong></td>
<td>35±10.5</td>
<td><strong>65±16.2</strong></td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;Max&lt;/sub&gt;, mmHg/s</td>
<td>2705±780</td>
<td>2340±900</td>
<td>2550±670</td>
<td>2340±900</td>
</tr>
<tr>
<td>Ea, mmHg/µl</td>
<td>0.15±0.07</td>
<td><strong>0.24±0.19</strong></td>
<td>0.15±0.06</td>
<td>0.24±0.19</td>
</tr>
</tbody>
</table>

Table 3. Hemodynamic parameters 7<sup>th</sup> weeks after monocrotaline injection. Data are means±S.E.M.; MCT; monocrotaline only group, MAC; macitentan, MAC+SIL; combination therapy macitentan with sildenafil, HR; heart rate, RV; right ventricle, wt; weight, ESV, end-systolic volume; EDV, end-diastolic volume *p<0.05 for difference from sham.
Histopathological analysis with cardiac fibrosis

Gross pathological examination showed that significant enlargement of heart with hypertrophy in MCT group at 7th week (Figure 7). Cardiac fibrosis and lung fibrosis were significantly increased in MCT group as compared with sham. However, hypertrophy and fibrosis were reduced in macitentan treated PH rats. We found no difference in interstitial fibrosis or perivascular fibrosis between MCT only group and sildenafil combination group (MCT+SIL).

**Figure 7**- Comparison of the pathologic results. The extent of perivascular fibrosis and interstitial fibrosis of heart were significantly greater in MCT group compared with MAC or MAC+SIL group (P<0.05)
Biochemical analysis of serum for drug safety measurement

The levels of aspartate aminotransferase, total bilirubin and creatinine were two or three times more than the upper limits of the normal range in MCT group as compared with sham or medication treated rats. Serum BNP level was higher in MCT group but significantly decreased in the macitentan or combination treatment groups (Figure 8).
Figure 8. Quantitative measurement of BNP, Creatinine, Total bilirubin and aspartate aminotransferase levels. * P < 0.05 compared with sham, + P < 0.05 compared with MCT
Model 2: reverse-remodeling effect of combination therapy with macitentan and sildenafil

Survival Analysis

On the contrary to Model 1 (anti-reverse remodeling), the survival analysis of ‘Model 2’, which was the macitentan and sildenafil co-administration group revealed higher survival rate than macitentan single group (57% survival rate for macitentan only group vs 78% survival rate for combination therapy with sildenafil group, p=0.04). Advancement to pressure-volume loop study, echocardiography (echocardiography data could be obtained for only 4 weeks after monocrotalin injection) and histological study was not possible due to the unpredictable large number of experimental rat’s death before reaching 7th week after the injection of monocrotaline. There were no significant changes in blood pressure and heart rate among the groups.
Figure 9. Survival analysis. Kaplan-Meier survival showed that maxitentan and sildenafil co-administration group was higher survival rate than maxitentan single group (survival rate 57% for macitentan only vs 78% for combination therapy with sildenafil, p=0.04).
Exercise test

There was no difference in exercise duration among the groups until the 3rd week after monocrotaline injection when RV failure occurred. Thereafter, progressive impairment in exercise capacity was observed in MCT group as compared with medication group (at 5 weeks after monocrotalin injection, 384±24 for MCT vs 466±20 for MAC vs 483±17 for MAC+SIL). Exercise duration was maintained in the group receiving combination therapy with sildenafil as compared with macitentan only group (421±20 for MAC vs 483±17 for MAC+SIL, P=0.01 between MCT and MAC+SIL).
Figure 10. Comparison of exercise duration among the three groups. * P=0.01 compared with MCT. Wk; week, MCT; monocrotaline, MAC; macitentan, MAC+SIL; combination therapy with macitentan and sildenafil.
RT-PCR for inflammatory markers

Large and unpredictable mortality rate was observed during model 2 scheme; hence, we could not evaluate histological analysis. Since most of the rats were found dead, it was impossible to assess ??? by measuring weight of the heart and lung tissue and therefore statistical outcomes could not be obtained. After considering several possible theoretical mechanisms about improvement in exercise ability in the combination therapy group, RT-PCR was implemented for genes that are associated with inflammatory response.

Pro-inflammatory cytokines like, iNOS, IL 1β and IL-6 were elevated in monocrotaline only group whereas treatment with macitentan (we used sham RNA in model 1)
Figure 11. Quantitative measurement of pro-inflammatory cytokines.

* P < 0.05 compared with MCT.  + P < 0.05 compared with MAC+SIL. MCT; monocrotalin, MAC; maxitentan, MAC+SIL; combination therapy with macitentan and sildenafil
Discussion

Pulmonary circulation is intimately coupled with RV function in health and disease. The importance of the right ventricle in PAH has been confirmed in major survival studies demonstrating direct relation of haemodynamic factors with right ventricle function. have been identified as significant predictors of mortality, including mean pulmonary pressure, right atrial pressure, cardiac output and cardiac index\textsuperscript{4, 19, 20}. Despite the significant advances in our understanding and clinical practice in PAH over the past decades, RV failure remains the common fatal pathway and consequence of PAH. However, our understanding about RV failure is limited because of limited research in this area.

In this study, we used an established, MCT-induced PAH model to study the structure-function relationship of RV remodeling and effectiveness and safety of combination therapy with macitentan and sildenafil. The major findings from the study are as follows: (1) macitentan administered at an early stage prevented the development of PAH and RV hypertrophy and failure and exercise intolerance caused by chronic experimental PAH, (2) combination therapy with macitentan and sildenafil at an early stage did not led to prevention of development of RV failure as compared with macitentan only treatment, (3) when given at a delayed stage (with established RV hypertrophy and failure),
combination therapy with macitentan and sildenafil improved survival rate and exercise capacity but not RV hypertrophy and partially involved inflammation pathway, and (4) macitentan treatment corrected MCT-induced impairment of LV filling. Our data provides evidence that combination therapy with macitentan and sildenafil is safe and effective treatment of PAH detected after RV remodeling and additionally chronic inflammation is reversible on therapeutic treatment in a manner independent of cardiac hypertrophy remodeling.

**Macitentan use for pulmonary artery hypertension**

Current clinical research in pulmonary arterial hypertension (PAH) focuses on the development of more potent and less toxic drugs that target pathophysiologic pathways, which are important in PAH with special emphasis on endothelin, nitric oxide and prostacyclin pathways. Endothelin is one of the most potent vasoconstrictor ever identified with additional proliferative and profibrotic activities. Endothelin exerts its effects by binding to two distinct receptor isoforms in the pulmonary vascular smooth muscle cells, endothelin-A and -B receptors. Recently, the US Food and Drug Administration has approved a new ERA macitentan to treat PAH in adults. Support for approval of macitentan comes from the recently published SERAPHIN trial. Macitentan is a dual ERA that was developed by modifying the
structure of bosentan with an aim to increase efficacy and safety. The characteristics of macitentan include slow receptor dissociation kinetics and enhanced tissue penetration\(^{16}\). In contrast to other ERAs, macitentan has a low propensity for drug-drug interactions\(^{21}\). However, until date, little is known about RV reverse remodeling. Iglarz et al. demonstrated that macitentan (100 mg·kg\(^{-1}\)·d\(^{-1}\)) significantly prevented pulmonary vascular remodeling, RV hypertrophy, and increase in cardiomyocyte diameter when compared to bosentan (300 mg·kg\(^{-1}\)·d\(^{-1}\)). Cardiac protection by macitentan was associated with a significant attenuation of genes related to cell hypertrophy and extracellular matrix remodeling. Microautoradiography and high performance liquid chromatography analysis showed greater distribution of macitentan than bosentan in the RV and pulmonary tissues\(^{22}\). The superior efficacy of macitentan on RV remodeling, a known predictive marker of mortality\(^{23}\), associated with a greater distribution in the RV suggests that macitentan may possess potential for imparting superior long-term benefit on clinical outcome as compared to bosentan. We showed similar results in model 1- anti remodeling. Macitentan administered at an early stage prevented the development of PAH and RV hypertrophy and failure and exercise intolerance caused by chronic experimental PAH. Additionally, we demonstrated that combination therapy with macitentan and sildenafil
at an early stage led to no further prevention in development of RV failure as compared with macitentan only treatment. These results suggest that monotherapy by macitentan is enough for the patients with early detected PAH. However, in case of patients detected with PAH after RV remodeling\textsuperscript{24}, early combination therapy should be considered for improvement of survival benefit.

**Combination therapy - macitentan with sildenafil**

In this study, combination of macitentan and sildenafil was found to be more effective in preventing MCT-induced pulmonary hypertension than treatment with macitentan alone after RV remodeling. While combination therapy was not that better than the single macitentan treatment in preventing RV remodeling at early stage of treatment, combination therapy did led to significant reduction in mortality and exercise intolerance in course of treatment after RV remodeling. We presume that some anti-inflammatory mechanism of sildenafil could be the cause for observed results. In recent years, there has been considerable interest in studying the effect of sildenafil on endothelial cell protection that may trigger a signaling cascade and generation of nitric oxide (NO) by phosphorylation of endothelial NO synthase (eNOS). An important property of sildenafil is its ability to increase eNOS generation and inducible NOS proteins in the heart and this has a direct cause and effect relationship in protection against inflammation
as well as apoptosis in cardiomyocytes\textsuperscript{25}. Sildenafil is effective in improving PAH-related RV dysfunction; however, it is still debatable whether sildenafil exerts its therapeutic effects via its action on the pulmonary vasculature (like after load unloading) or by direct antihypertrophic effect on RV remodeling. Kass and Kim\textsuperscript{10, 11, 26} have proposed that sildenafil prevents, arrests, and even reverses LV hypertrophy, fibrosis, and dilatation in mice subjected to LV pressure overload induced by thoracic-aortic constriction or volume overload in their studies. Recently, two independent research groups examined whether sildenafil provides similar, direct protection against RV remodeling, as shown in LV by Kass and colleagues\textsuperscript{27, 28}. Surprisingly, the studies provided evidence that sildenafil does not prevent but even exacerbates RV hypertrophy in a pre-established RV hypertrophy model induced by pulmonary trunk artery banding, indicating that sildenafil prevents myocardial remodeling in PAH mainly through an indirect action via RV unloading. The discrepant effects of sildenafil on pressure-overload–induced LV versus RV hypertrophy could possibly be due to different mechanisms involved in the development of hypertrophy between the RV and LV. It is being increasingly recognized that, in addition to important differences in gene expression, embryology, and physiology, the RV and LV may have divergent responses to stress, including activation of different signaling
Our data also showed that early combination therapy with macitentan and sildenafil did not lead to further prevention of RV remodeling in PAH model by monocrotaline. However, in the delayed phase treatment, combination therapy improved survival rate and exercise intolerance as compared with single macitentan therapy (57% survival rate for macitentan only group vs 78% survival rate for combination therapy with sildenafil group, p=0.04). Unfortunately, large extent of mortality rate failed to show the RV reverse remodeling by echocardiography or pressure-volume loop. We demonstrated that some pro-inflammatory cytokines were decreased in the group of combination therapy with sildenafil. Humbert and college studied the concentration of interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor-alpha (TNF alpha) in the serum of 29 patients with severe PAH referred to their center for lung transplantation. Results were compared by analyzing serum for similar parameters from 15 normal controls and nine patients with pulmonary hypertension secondary to chronic obstructive pulmonary disease (COPD-PH). TNF alpha serum levels were within the normal range in each group. The result was contrary to increased IL-1 beta serum levels in severe PAH (118 +/- 36 pg/ml, mean +/- SEM) as compared with controls (3 +/- 1 pg/ml, p < 0.001) or COPD-PH patients (3 +/- 1 pg/ml, p < 0.001). IL-6...
serum concentrations were also higher in severe PAH (66 +/- 20 pg/ml) than in controls (14 +/- 6 pg/ml, p < 0.01)\textsuperscript{31}. This study demonstrates increased serum levels of IL-1 beta and IL-6 in severe PAH, and suggests a role for proinflammatory cytokines in PAH. We showed similar results in model 2.

**Monocrotalin (MCT) induced pulmonary hypertension model and study limitation**

The causes of PAH are complex and yet not completely understood. Three rodent models have been recurrently considered fundamental for the investigation of human pulmonary hypertension (PH); the chronic hypoxia exposure model, monocrotaline (MCT) lung injury model and pulmonary artery banding model (PAB).\textsuperscript{12} In the model of PAB has technical variation according to researcher and severe PAH do not develop in hypoxia exposure model. MCT rat model was chosen for the present research. The MCT rat model continues to be a frequently investigated model of PAH, since it offers technical simplicity, reproducibility and low cost as compared with other models of PAH. MCT is known to cause damage to both alveolar lining cells and pulmonary vascular endothelial cells as early as 24 h after injection. It also causes lung, liver and myocardial direct toxicity and very different of human PAH. However, it is true that (almost) all animal models are
imperfect and that it matters which aspect, mechanism, or manifestation of disease a particular model can reproduce or investigate. In this regard, the beneficial effect of macitentan on early PAH and combination therapy on delay PAH might have clinical implication. Further research is needed to delineate the exact mechanisms involved to allow for translation into the clinical setting.

**Conclusion**

Macitentan attenuates RV remodeling and prevents exercise intolerance in a rat model of chronic PAH when treated in early phase. Combination therapy with macitentan and sildenafil improved survival benefit and exercise intolerance in RV failure rats induced by PAH. It is proposed that this benefit might be associated with anti-inflammatory synergistic effects of the medications, macitentan and sildenafil. Further research is needed to delineate the exact mechanisms involved and demonstrate the beneficial effects in patients with chronic PAH.

**Conflict of interest**

None declared
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국문 초록

서론: 최근의 연구들은 새로운 이중 내피세포 수용체 길항제인 막시텐탄이 폐동맥 고혈압 환자들에게 유병률과 사망률을 줄인다는 결과를 보여주었다. 이에 본 저자는 마시텐탄과 실데나필의 병용 요법이 모노크로탈린을 이용한 폐동맥 백서 모델에 적용하여 우심실의 혈역학적인 호전을 살펴 보고자 했다.

방법: 실험은 두 가지의 다른 설계로 진행되었다. 첫 번째 설계는 우심실의 재형성을 억제하는 효과에 대한 실험으로 모노크로탈린을 투여한 후 마시텐탄 그룹과 (macitentan, MAC 30mg/kg p.o, qd, N=16), 마시텐탄과 실데나필 병용 그룹 (MAC 30mg/kg p.o qd, SIL 50mg/kg p.o, bid, N=16), 혹은 아무 약제도 투약하지 않은 폐동맥 고혈압 그룹 (monocrotaline, MCT N=16)으로 나눈다. 두 번째 설계는 재형성된 우심실의 역-재형성 효과에 대한 실험으로 모노크로탈린을 투여한 후 3 주가 지난 시점에서 우심실의 압력이 커지고 우심실 기능이 감소함을 확인하고 백서를 마시텐탄 투여 그룹 (MAC,
N=14), 실험군 실험군 (SIL, N=14)으로 나눈다. 우심실의 기능을 확인하기 위해 심초음파와 운동 부하 검사를 시행하고 각군당모노크로탈린 투약 후 7 주까지 약물을 투약하였다. 7 주 이후 압력-용적곡선을 구하고 심장을 적출하여 조직학적 분석을 시행하였다.

결과: 심초음파에서 유의한 폐 고혈압이 모노크로탈린 투약군에서 형성되었으며 우심실 기능의 저하가 일어났다. 그러나 마시텐탄과 실험군 실험군 투여한 그룹에서는 폐 동맥의 압력의 상승은 크게 나타나지 않았다. (20.7±1.5 for sham vs 57.3±5.7 for MCT vs 36.8±1.6 for MAC vs 34.7±2.3 mmHg for MAC+SIL, P= 0.05). 또한 우심실 부전이 나타난 이후 투약을 한 역-재형성 설계에서는 마시텐탄과 실험군의 병용 요법이 생존률을 증가시키고 운동능력을 향상 시켰으며 염증 반응을 일으키는 물질들을 감소시켰다.

결론: 마시텐탄을 폐동맥 고혈압 초기에 투약시 우심실의 재형성과 부전을 막으며 운동능력을 향상 시킨다. 마시텐탄과 실험군의 병용 요법은 마시텐탄 단독에 비해 우심실 기능 부전과 재형성이 일
어난 상태에서는 효과가 더 우수하며 운동능력이 유지시키는 효과가 있다.

주요어: 폐동맥 고혈압 백서 모델, 마시텐탄, 실데나필, 병용요법
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