Effects of Single Drug and Combined Short-term Administration of Sildenafil, Pimobendan, and Nicorandil on Right Ventricular Function in Rats With Monocrotaline-induced Pulmonary Hypertension

Telma M. Nakata, DVM, Ryou Tanaka, DVM, PhD, Rieko Yoshiyuki, DVM, Toshiharu Fukayama, DVM, Seijiro Goya, DVM, and Ryuji Fukushima, DVM, PhD

Abstract: This study was designed to assess the progression of pulmonary arterial hypertension (PAH) and the effectiveness of therapy using recently investigated echocardiographic parameters. PAH is characterized by the progressive elevation of pulmonary artery pressure and right ventricular hypertrophy and dysfunction, which ultimately results in right-sided heart failure and death. Echocardiography results and invasive measurements of right and left ventricular systolic pressures were compared after 3-week administrations of sildenafil (S group), pimobendan (P group), nicorandil (N group), and their combinations (SP and SPN groups) in male rats with monocrotaline (MCT)-induced pulmonary hypertension (M group) and without this condition (C group). The groups that received pimobendan alone and in combinations (SP and SPN groups) showed improvement in their echocardiographic parameters of systolic function. A significant improvement of diastolic function was achieved in the SPN group. Invasive measurements showed the most significant decreases of right ventricular systolic pressure in the N and SPN groups, and the use of pimobendan resulted in a comparatively low risk of adverse hemodynamic effects (left ventricular systolic pressure). Although our results suggested the attenuation of PAH severity in all treatment groups, PAH could not be reversed.

Key Words: phosphodiesterase inhibitors, ATP-sensitive potassium channel, cardiac function

(*J Cardiovasc Pharmacol*™ 2015;65:640–648)

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a multifactorial disease, characterized by the progressive elevation of pulmonary artery pressure (PAP) and pulmonary vascular resistance with subsequent right ventricle (RV) overload and hypertrophy, which ultimately leads to right ventricular dysfunction and right-sided heart failure.[1] The reference standard method for the hemodynamic assessment of the right heart chambers is the invasive approach, which is unfeasible for continuously monitoring drug therapy.[2–5] Transthoracic echocardiography is a noninvasive method that measures variables associated with the progression of PAH.[6,7] Previous studies have shown that RV function is an important predictor of longevity in human patients.[5,7,8] However, RV-pulmonary arterial coupling and the RV response to PAH therapy have not been widely investigated, although RV adaptation to the progressive increase in pulmonary vascular resistance and PAP has recently been shown to be related to functional capacity and survival.[9–11]

Previous investigations have suggested that phosphodiesterase-3 (PDE-3) and phosphodiesterase-5 (PDE-5) activities are increased in the smooth muscle cells of pulmonary arteries (with the exception of resistance arterioles) in rats with hypoxia-induced pulmonary hypertension (PH), resulting in decreased levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and increased vascular tone.[12–14] Additionally, studies have shown that treatment with cAMP-specific PDE-3 inhibitors (PDE-3i) and cGMP-specific PDE-5 inhibitors (PDE-5i) improve pulmonary hemodynamics in animals with induced increases in PAP, suggesting that a combination of both enzyme inhibitor types may provide additional effects.[15–17] Pimobendan is a PDE-3i and calcium sensitizer that reduces afterload and provides positive inotropy.[18–20] Sildenafil is a PDE-5i used in standard protocols of PAH therapy and has also been investigated for cardioprotective effects.[21–24] Parallel studies using adenosine 5′-triphosphate (ATP)-sensitive potassium (K_{ATP}) channel openers have shown that these compounds exert beneficial effects on pulmonary arterioles and myocardial hypertrophy attributable to vascular smooth muscle tone regulation.[25–27] Nicorandil, a K_{ATP} channel opener with nitrate-like action, was included in this study due to its vasodilatatory effects on the pulmonary resistance arterioles and main PA of rats.[28–31]

The purpose of this study was to compare the effects of sildenafil, pimobendan, and nicorandil in single and combined administration on PAP and RVD in rats with

Received for publication November 17, 2014; accepted January 25, 2015.
From the Department of Veterinary Surgery, Faculty of Veterinary Medicine, Tokyo University of Agriculture and Technology (TUAT), Tokyo, Japan.
T. M. Nakata was supported by grants from the Federative Republic of Brazil, Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior—CAPES.
The authors report no conflicts of interest.
Reprints: Telma M. Nakata, DVM, Tokyo University of Agriculture and Technology, 3-8-1 Harumi-cho, Fuchu, Tokyo, 183-8538 Japan (e-mail: nakatamary@gmail.com).

MCT-induced PH by assessing cardiac function and hemodynamics through echocardiography and invasive intraventricular pressure measurements.

MATERIALS AND METHODS

Experimental Design and Animal Model

This study was approved by the Institutional Animal Care and Use Committee of the Tokyo University of Agriculture and Technology and conformed to the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research, National Research Council, Washington DC, and National Academy Press, 1996.

Seven groups of adult male Wistar rats (12 weeks old, weighing 330–405 g) were housed at 22°C with a 12:12-hour light–dark cycle and access to food and water ad libitum. PAH was induced by a single intraperitoneal injection of MCT (60 mg/kg, Sigma-Aldrich, St. Louis, MO) previously dissolved in 1 N hydrochloric acid and pH adjusted to 7.4 using 1 N sodium hydroxide, diluted in distilled water to obtain a concentration of 30 mg/mL. For comparison purposes, a normal control group (C) received a 100-μL saline injection (n = 6). Four weeks after MCT injection, echocardiographic evaluation showed evidence of PAH, including the presence of tricuspid regurgitation (TR), a midsystolic notch on pulmonary flow profile with an increased preejection period (PEP) and decreased ejection time (ET), systolic/diastolic flattening of the interventricular septum, an enlarged right atrium and ventricle, increased free-wall thickness (>0.9 mm), reduced RV systolic function, and pericardial effusion. After examination, the rats were randomly divided into 5 treatment groups (n = 6 for each group); one group remained untreated as a PAH model group (M). Three of the 6 rats in the M group developed severe PH and died between 3 and 4 weeks after MCT injection. Another group of 6 rats was given the MCT injection to provide substitutes for the deceased rats in the M group; only 2 rats of this group survived, leaving 5 rats to be examined at the end of the study (7 weeks). Two rats of the N group died between the second and third week of drug administration (n = 4).

Experimental Protocols

Nicorandil (N) at a dose of 1.0 mg/kg, sildenafil (S) at 1.0 mg/kg, pimobendan (P) at 0.15 mg/kg, a combination of sildenafil at 1.0 mg/kg and pimobendan at 0.15 mg/kg (SP), and combination of sildenafil at 1.0 mg/kg, pimobendan at 0.15 mg/kg, and nicorandil at 1.0 mg/kg (SPN) were orally administered twice daily. At the end of 3 weeks of therapy, echocardiography and invasive hemodynamic measurements were performed within 24 hours for all rats under general anesthesia achieved using isoflurane 1.5% in oxygen at 1 L/min administered through a mask.

Echocardiography

The thoraxes of the rats were shaved, and the rats were positioned in right and left lateral recumbency to obtain short-axis and apical imaging of the heart, respectively. Doppler tracings were recorded at a sweep speed of 200 mm/s and sample gate of 1 mm using a ProSoundα7 (Hitachi-Aloka Medical, Tokyo, Japan) ultrasonographic system with a 7.5-MHz transducer and simultaneous electrocardiography (ECG) (Fig. 1). All measurements represented the mean of 5 cardiac cycles.

Left parasternal apical 4-chamber view was used to assess RV systolic function through RV fractional area change (FAC), tricuspid annular plane systolic excursion (TAPSE), and tricuspid annular systolic velocity (Sm-TV) (Fig. 1A–C). FAC was estimated as [(end-diastolic surface − end-systolic surface) × 100]/end-diastolic surface. TAPSE was determined using the M-mode of contraction on the longitudinal plane.[32–34] Pulsed-wave tissue Doppler (PW-TDI) velocities during systole (Sm-TV), early relaxation (E′), and atrial systole (A′) were processed from the middle of the basal segment of the RV-free wall. RV diastolic function was evaluated using the E/E′ peak velocity ratio, and RA area was measured at the end systole from the apical 4-chamber view (Fig. 1D, E).[19,20] A modified Bernoulli equation ($\Delta p = 4v^2$) was used to estimate RVSP using the peak velocity of the tricuspid regurgitant jet (v).[35] The gradient of the pressure RV-right atrium (Δp) plus RAP represents an echocardiography-derived estimation of PAP during the systolic phase (SPAP = RVSP).[33] RV global function was assessed through the myocardial performance index (MPI), which was obtained using the tricuspid valve closure–tricuspid valve opening time and PA ET measured from the PW-Doppler RV inflow and outflow signals of the tricuspid valve and pulmonic valve, respectively.

Progression of PAH was evaluated through measurements of RV wall thickness and RV end-diastolic diameter (RVEDD) using the M-mode of contraction on the short-axis plane. The PET/ET ratio was calculated using the PA PEP recorded from the onset of the Q wave on the ECG to the start of PA flow and the pulmonary valve flow period recorded using the pulsed-wave (PW) Doppler mode from the transverse short-axis view. Pericardial effusion was assessed using parasternal long- and short-axis views and graded according to the diastolic separation of the visceral and parietal pericardium as either small (<1.2 mm), moderate (1.2–2.4 mm), or large (>2.4 mm).

Hemodynamic Study

The rats were placed in the dorsal decubitus position to measure RVSP and LVSP. A 25-gauge needle was inserted into the RV and LV by the transthoracic approach.[36–38] Localization of the RV and LV was guided by simultaneous echocardiographic imaging. The needle was connected to a physiological pressure transducer and amplifier system (Life Scope BSM-5192; Nihon Kohden, Tokyo, Japan) to record pressure oscillation. Intraventricular pressures were recorded after the calibration and stabilization of cardiac rhythm as monitored with ECG leads and pulse oximetry.

Statistical Analysis

Continuous variables were expressed as mean ± SD. Categorical data were expressed as percentages and ordinals. Comparison of the means among the groups was performed by a one-way analysis of variance followed by Fisher's least

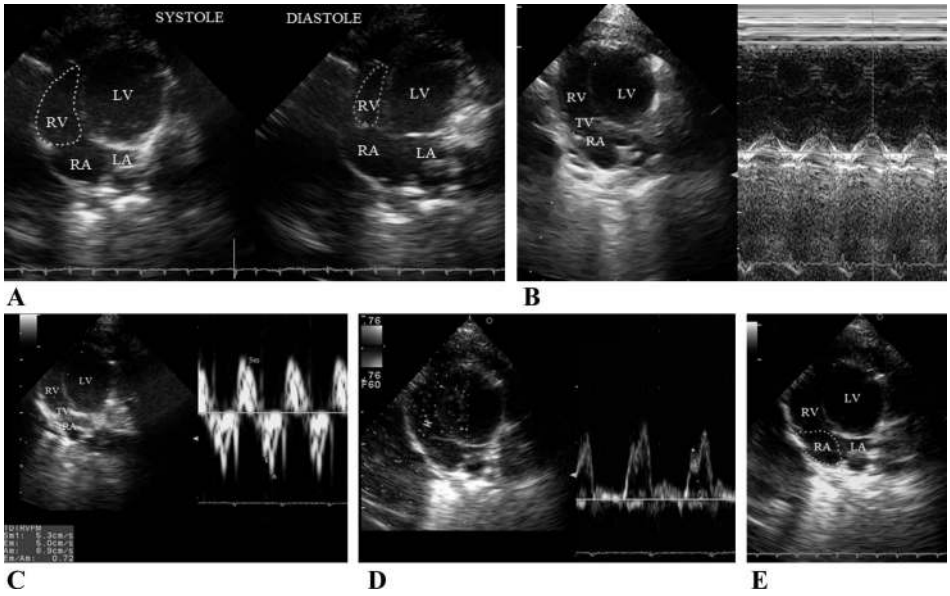

FIGURE 1. Normotensive male, 12 weeks -old, Wistar rat. Echocardiographic views are used to evaluate the right-side cardiac chambers. A, Apical 4-chamber view RV focus used to measure ventricular area and calculate FAC; (B) longitudinal movement of tricuspid annulus by M-mode imaging; (C) pulsed-wave tissue Doppler imaging of a single area of interest, targeted to the lateral tricuspid annulus, to measure systolic motion velocity (Sm), diastolic motion velocity (E′ wave), and calculate the E/E′ ratio using E wave velocity from (D) pulse-wave Doppler of RV inflow. E, RA focused used to measure right atrial area. LA: left atrium; LV: left ventricle; RA: right atrium; RV: right ventricle; TV: tricuspid valve.

significant difference post hoc multiple comparison tests. Significant differences between the groups were analyzed with paired Mann–Whitney rank-sum tests. A treatment was considered to ameliorate cardiac function if 2 or more of its parameters were significantly different from those of the M group and to improve cardiac function if also showed no statistically significant differences from the C group. The strengths of the correlations between the invasive and noninvasive measurements were assessed by Pearson's coefficients. Statistical significance was defined as $P < 0.05$.

RESULTS

Effects of Treatment on Measurements of Right Heart Structure and Function Evaluated by Echocardiography

The 2-dimensional, M-mode, and TDI data indicated improved cardiac morphology and function in all treatment groups compared with the M group. The results for each drug treatment and comparisons of parameters of systolic function between the groups are shown in Figure 2. Comparison between the groups showed that these values were not significantly different. The P, SP, and SPN groups showed significant improvements of RV-FAC and TAPSE (Fig. 2A, B), whereas only the P group showed improved Sm-TV (Fig. 2C). Although the PEP/ET ratios were ameliorated in all treatment groups, suggesting the attenuation of disease severity, all these values were significantly different from that of the normal C group (Fig. 3A). RVEDD and RV thickness showed reductions after treatment compared with those of the M group; however, these parameters also remained higher than those of the C group (Fig. 3B, C). Only the SPN group showed significant improvement of diastolic parameters (E/E′ ratio and RAD); however, SP treatment ameliorated diastolic dysfunction compared with the M group (Fig. 4A, B). The MPI indicated that all treatment groups, except the N group, exhibited improved global RV function when compared with the M group, but this value did not differ among the treatment groups (Fig. 4C). Pericardial effusion was observed more frequently in the P and SP groups than in the other treatment groups at 7 weeks after MCT injection and 3 weeks of therapy (Table 1).

Hemodynamic Effects

Invasive measurements and echocardiography-derived RVSP were significantly correlated in the groups that had lower RVSP, ie, in the *P* group (r = 0.98, $P = 0.001$), N group (r = 0.87, $P = 0.006$), and SPN group (r = 0.95, $P = 0.001$). Higher RVSP was associated with a modest correlation between invasive measurements and echocardiography-derived RVSP (r = 0.55, $P < 0.01$; data not shown). All observations of Doppler signal intensity and the velocity curve of TR flow showed fair or good quality for measuring RVSP. These results are similar to the findings described by Fisher et al[39] and may happen if measurements are not taken simultaneously. The high correlations observed may be partly due to the small number of rats with lower RVSP in these groups.

Despite its amelioration, RVSP was significantly higher in all treatment groups at the end of 3 weeks of therapy than in the C group (Fig. 5A). No significant differences were observed between the treatments.

LVSP was lower in the M group (49 ± 5.1 mm Hg) than in the C group (84.5 ± 10.3 mm Hg), as was heart rate (HR) (290 ± 36 vs. 450 ± 33 beats per minute). These reductions in LVSP (Fig. 5B) and HR (Fig. 5C) were partially reversed in all treatment groups. The groups that received pimobendan alone and in combinations (SP and SPN groups) did not significantly differ from the C group in terms of LVSP.

DISCUSSION

We hypothesized that cAMP-specific PDE-3 and cGMP-specific PDE-5 play important roles in PH pathogenesis and

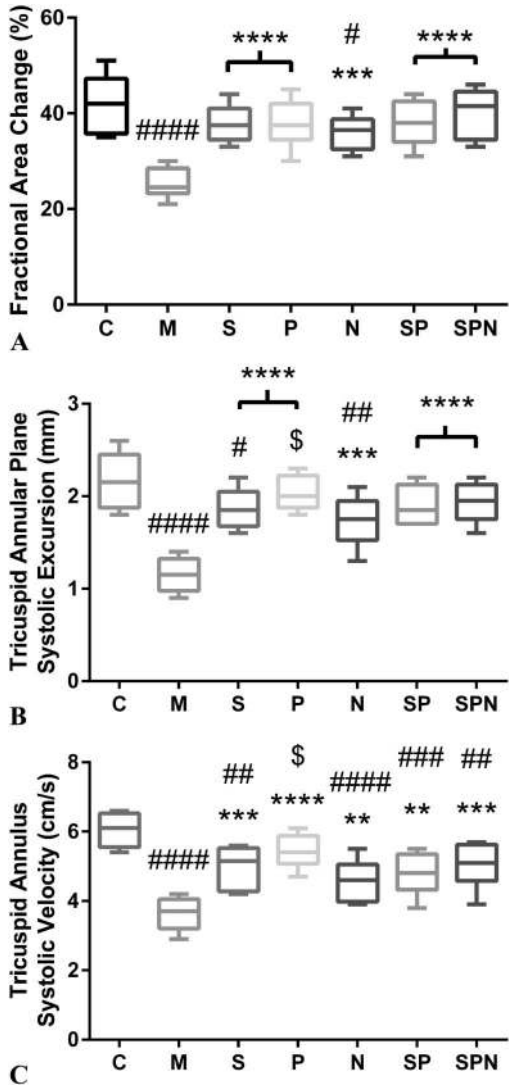

FIGURE 2. Echocardiographic assessment of right ventricular systolic function using (A) fractional area change, (B) tricuspid annular plane systolic excursion, and (C) tricuspid annulus systolic velocity after 3 weeks of therapy with sildenafil, pimobendan, nicorandil, and their combinations. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ versus control group; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus model group; \$$P < 0.05$ versus N group. Tick-up lines indicate all included groups. C, normal control (n = 6); M, MCT injection only (n = 5); N, nicorandil (n = 4); P, pimobendan (n = 6); S, sildenafil (n = 6); SP, sildenafil and pimobendan in combination (n = 6); SPN, sildenafil, pimobendan, and nicorandil in combination (n = 6).

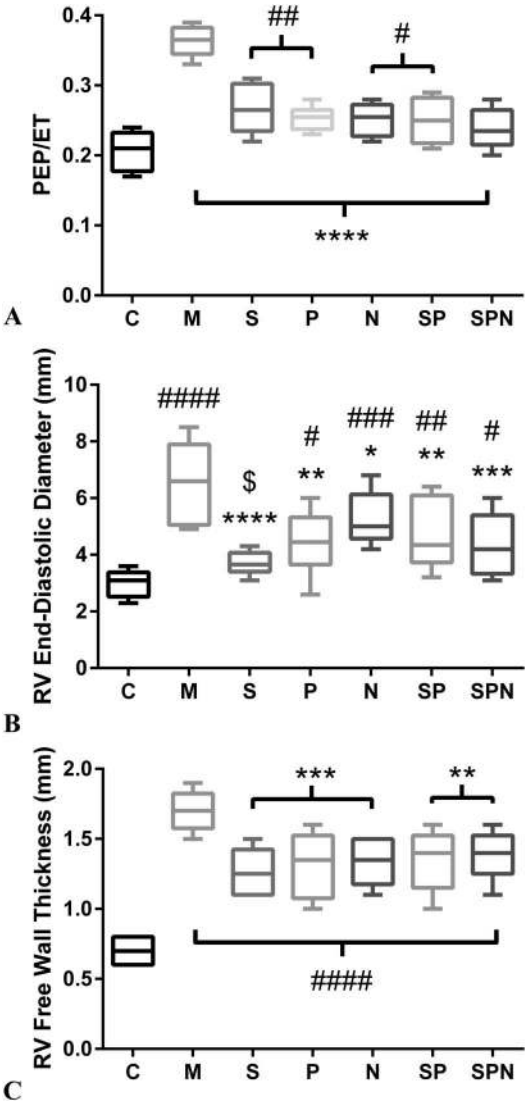

FIGURE 3. Echocardiographic assessment of pulmonary hypertension severity using (A) preejection period/ejection time ratio, (B) right ventricular end-diastolic diameter, and (C) right ventricular free wall thickness after 3 weeks of therapy with sildenafil, pimobendan, nicorandil, and their combinations. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ versus control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus model group; \$$P < 0.05$ versus N group. Tick-up lines indicate all included groups. C, normal control (n = 6); M, MCT injection only (n = 5); N, nicorandil (n = 4); P, pimobendan (n = 6); S, sildenafil (n = 6); SP, sildenafil and pimobendan in combination (n = 6); SPN, sildenafil, pimobendan, and nicorandil in combination (n = 6).

therefore that the combination of PDE-3 and PDE-5 inhibitors with an ATP-sensitive potassium channel opener would improve cardiopulmonary hemodynamics as measured by echocardiographic parameters. The pathological abnormalities caused by MCT injection in rats differ from those of PAH observed in humans due to presence of plexiform lesions in the latter; however, both sets of abnormalities may result in RV dysfunction.[40–42] Initial myocardial hypertrophy is related to adaptation to the pressure overload, and progressive increases in afterload result in ventricular remodeling associated with chamber dilation, eventually decompensation and development of right-sided heart failure.[7,8,43,44] RV hypertrophy is associated with decreased RV compliance, increased end-diastolic pressure, and subsequent RV diastolic dysfunction; furthermore, diastolic relaxation impairment is highly correlated with end-diastolic calcium levels, possibly due to the reduced production

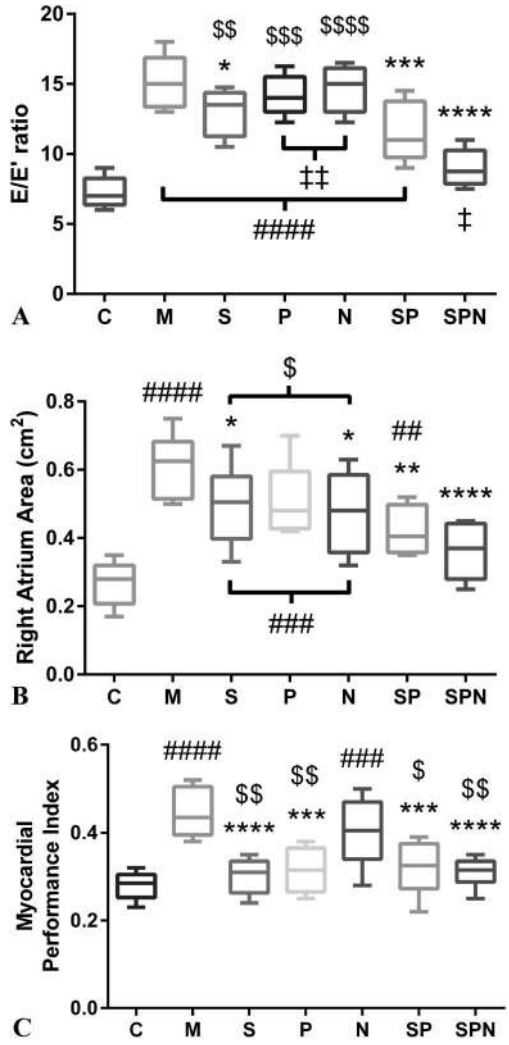

FIGURE 4. Echocardiographic assessment of right ventricular diastolic (A and B) and global function (C) after 3 weeks of therapy with sildenafil, pimobendan, nicorandil, and their combinations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus model group; ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ versus control group; \$$P < 0.05$, \$\$$P < 0.01$, \$\$\$$P < 0.001$, \$\$\$\$$P < 0.0001$ versus SPN group; ‡$P < 0.05$, ‡‡$P < 0.01$ versus SP group. Tick-up lines indicate all included groups. C, normal control (n = 6); M, MCT injection only (n = 5); S, sildenafil (n = 6); P, pimobendan (n = 6); N, nicorandil (n = 4); SP, sildenafil and pimobendan in combination (n = 6); SPN, sildenafil, pimobendan, and nicorandil in combination (n = 6).

of cAMP; nonetheless, systolic function may remain relatively normal for long periods of chronic afterload elevation before the development of overt systolic failure.[19,43,44] Echocardiography is a useful method for evaluating right-sided heart morphology and function in rats with MCT-induced PH and has been shown to be accurate for assessing cardiac function.[40,45–47] The echocardiographic parameters acquired from the M group were similar to those obtained by Boissiere et al and Hardziyenka et al in Wistar rats with MCT-induced PH.[45,46] The aims of PAH therapy include the reduction of RV afterload, improvement of RV contractility, and aversion of systemic arterial hypotension.[31,48]

Our results suggested that the administration of sildenafil alone may provide comparable hemodynamic effects to the administration of pimobendan, either alone or in combination with sildenafil, without statistically significant differences in the analysis of parameters of systolic function between treatment groups. However, Lobato et al[16] showed that the PDE-3 inhibitor milrinone, either alone or in combination with sildenafil, improved RV function to a greater extent than did sildenafil alone. Recent studies investigating the effects of PDE-5 inhibition, generally used for reducing PAP, on myocardium under overload have demonstrated significant reductions in necrosis and apoptosis.[49] Other studies have shown that sildenafil improves cardiac function and reverses myocardial hypertrophy in a rodent model of chronic cardiac pressure overload.[22,23] Additionally, increased PDE-5 expression has been demonstrated in the RV myocardium of patients with RV dysfunction.[50] Guazzi et al[51] suggested that sildenafil administration alone improves diastolic function. However, our results showed that sildenafil only generated statistically significant improvement in overall diastolic function when the treatment also included pimobendan and nicorandil (SPN group vs. single drug treatment groups); ie, the SPN group showed the most pronounced effects for the improvement of diastolic function. Nonetheless, a histopathological analysis was not performed, which could have confirmed a positive correlation between RV diastolic dysfunction improvement and the partial reversion of myocardial hypertrophy, as is suggested by echocardiographic parameters.[52] Although our results showed that pimobendan alone may significantly reduce RVSP with effects comparable with those of sildenafil, the combination of pimobendan and sildenafil did not result in an additional reduction of RVSP, which suggests that the association of a PDE-3i and a PDE-5i has a limited effect on the cGMP-PDE pathway. Previous studies have demonstrated that the administration of a PDE-3 inhibitor milrinone resulted in significantly inferior reduction of lobal arterial pressure compared with that of zaprinast, a PDE-5 inhibitor, when administered to a cat model of increased pulmonary vascular tone or patients with severe PH.[15,53] Discrepancies between the effects of milrinone and pimobendan may occur due to the calcium sensitizer property of pimobendan, which may improve cardiopulmonary hemodynamics.[19,54,55] However, our previous study in rats with MCT-induced PAH (30 mg/kg of MCT subcutaneously) did not find differences between rats given 6 weeks of pimobendan at 1.25 mg per rat and those in the model group; these results may differ from those of the present results due to the different disease stage induced by the lower dose of MCT and higher dose of pimobendan administered.[56] Administration of PDE-3 inhibitors for prolonged periods is related to development of myocardial hypertrophy.[57–59] Although only a fixed dose was used in our investigation, a larger study is necessary to demonstrate the lowest effective dose, as has been suggested by successful individual treatments with single or combined administrations of pimobendan at lower than recommended doses.[60–63] MPI is an important predictor of clinical status and survival in humans with PAH, and in this

TABLE 1. Number of Rats Presenting Pericardial Effusion According to Grade of Effusion

Effusion	M (n = 5)	S (n = 6)	*P* (n = 6)	N (n = 4)	SP (n = 6)	SPN (n = 6)
Small (<1.2 mm)	0	2	2	0	1	1
Moderate (1.2–2.4 mm)	2	1	1	2	1	1
Large (>2.4 mm)	3	0	1	0	1	0
Total	5	3	4	2	3	2

M, model group (MCT injection); S, treatment with sildenafil; P, treatment with pimobendan; N, treatment with nicorandil; SP, treatment with sildenafil and pimobendan in combination; SPN, treatment with sildenafil, pimobendan, and nicorandil in combination.

study, MPI improved in all treatment groups except the N group.[64] MPI assesses global cardiac function, and the lower performance of the N group reflects its systolic and diastolic function.[65] Nicorandil increases potassium channel conductance in the membranes of cardiomyocytes and smooth muscles cells, resulting in negative inotropy and vasodilation.[25] Therefore, the greater increase in RVEDD observed in the N group was associated with slightly improved RV function, which may explain the lower survival rate (67%) in this group. A study using nicorandil at 7.5 mg/kg, in an MCT-induced PAH rat model, showed an increased survival rate in the treatment group (73%) compared with that of the disease model group (39%) and the cessation of progress (but not the reversal) of PAH.[25] Sahara et al[29] have demonstrated the reduction of RVSP using 2.5 mg/kg of nicorandil in the same PAH model. Our results showed that 1.0 mg/kg of nicorandil could also reduce RVSP to the same degree obtained using 2.5 mg/kg, although the lower survival rate observed may have hampered the strength of these results. The N group showed less improvement of systolic function than did the other treatment groups, and an additional effect of nicorandil in the SPN group could not be demonstrated. The mechanisms of action of these drugs have shown partial interaction in their pathways for the regulation of smooth muscle tone through the inhibition of cGMP and cAMP or mitochondrial ATP-potassium channel opener activity; this association may potentiate the development of adverse effects.[31,66,67] However, even if sildenafil acts in the NO-cGMP pathway to release nitric oxide, the association between inhaled nitric oxide and sildenafil may have an additive beneficial effect in increasing and prolonging pulmonary vasodilation as has been suggested by previous studies.[68,69]

The neurohormonal activation induced by altered pulmonary arterial oxygen saturation aimed at overcoming severe hemodynamic dysfunction may hamper therapy effectiveness.[44,70–72] Right-sided heart failure results in reduced β-adrenoceptor density and is associated with induced abnormalities of the LV (morphology and function), subsequently reducing HR and LVSP, as has been observed in the present and previous studies.[44,73,74] Wang et al[75] also suggested that α-adrenoceptor stimulation may result in negative inotropy. The amelioration of RVSP induced by the vasodilator agents in this study may have decreased septal bowing to the left side and thus improved LVSP and HR compared with those of the M group; however, these parameters remained lower than those of C group. Previous investigation has suggested that a good correlation exists between RVEDD and decreased PAP.[47] Reductions in RVEDD were not accompanied by correlated reductions in RV wall thickness in this study, although both variables showed a certain degree of improvement. Indeed, although the short-term administration of pimobendan in patients with severe heart failure improves hemodynamics, its long-term administration is associated with loss of effectiveness or myocardium toxicity.[20,57–59] Although only a short-term administration of a lower than recommended dose of pimobendan was evaluated in this study, this therapy effectively reduced RV dysfunction and improved PAH. However, Walter et al[60] showed significant differences in PAP and RA pressure between patients with congestive heart failure who received 5 and 10 mg of pimobendan.

Echocardiography-derived RVSP was correlated with invasive measurements of RVSP under lower peak velocities of the TR jet. When higher flow velocities were present, the Doppler measurements overestimated RVSP and were poorly correlated with the invasive measurements. The discrepancies observed between the invasively measured and echocardiography-derived RVSP may occur due to the difficulty of aligning the Doppler beam with the regurgitant jet or impaired RV function, which reduces the accuracy of Doppler ultrasound measurements.[76–78]

An indirect assessment of the severity of PAP may be obtained by the PEP/ET ratio because PEP lengthens and ET shortens as PAP increases.[4] This ratio showed a good correlation with PAP and was significantly better in the SPN group than in the other groups.

The presence of pericardial effusion in PAH is associated with increased RA pressure and higher mortality rates; however, previous studies have reported that human patients with relatively small pericardial effusion have similar survival rates to those without effusion.[4,79] According to these results, we speculated that therapy with nicorandil alone or the SPN combination may reduce the formation of pericardial effusion associated with elevated RA pressure, which may result from the significant reduction of RVSP.[5,79]

The present results suggest that the combination of PDE-3 and PDE-5 inhibitors with nicorandil may improve PAP and RVD in the clinical therapy of patients with PAH. However, the results obtained from rats with MCT-induced PH may not be directly extrapolated to a clinical setting without taking the criteria specific to humans arising from variable responses to vasodilator therapy into consideration. Case reports have indicated that the combination of pimobendan and sildenafil, with or without nicorandil, results in clinical improvement (exercise tolerance, hemodynamic, and

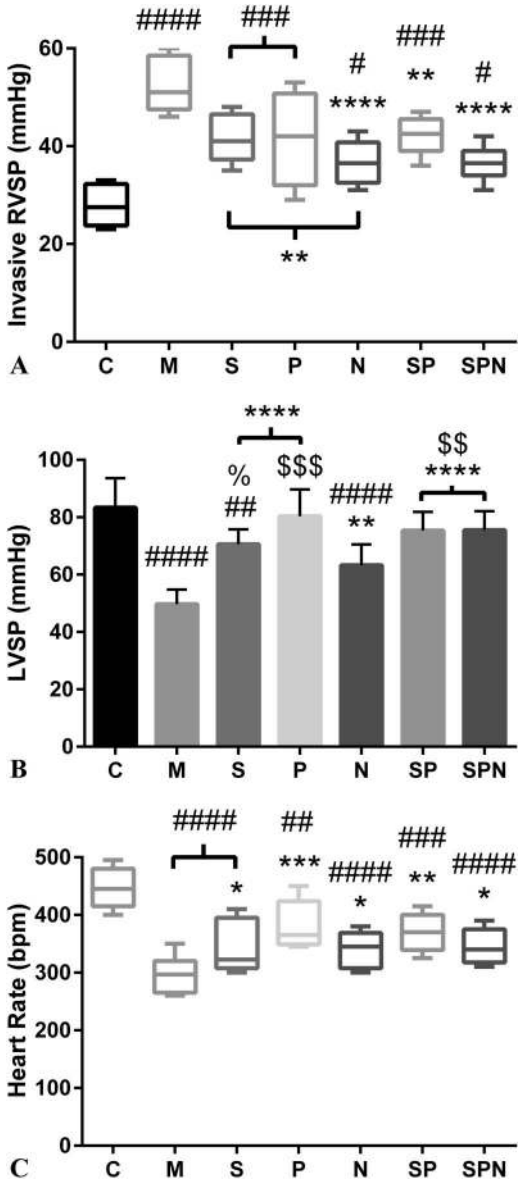

FIGURE 5. Invasive measurements and ECG after 3 weeks administration of sildenafil, pimobendan, nicorandil, and their combination on (A) right ventricular systolic pressure (RVSP), (B) left ventricular systolic pressure (LVSP), and (C) heart rate. ##$P < 0.01$, ####$P < 0.0001$ versus control group; **$P < 0.01$, ****$P < 0.0001$ versus model group; \$\$$P < 0.01$, \$\$\$$P < 0.001$, \$\$\$\$$P < 0.0001$ versus N group, %$P < 0.05$ versus P group. C, normal control (n = 6); M, MCT injection only (n = 5); S, sildenafil (n = 6); P, pimobendan (n = 6); N, nicorandil (n = 4); SP, sildenafil and pimobendan in combination (n = 6); SPN, sildenafil, pimobendan, and nicorandil in combination (n = 6).

long-term survival) when administered to patients with primary PAH whose heart failure had previously failed to respond favorably to prostacyclin therapy.[62,63] In summary, depending on the severity of RV dysfunction and baseline hemodynamic state, the reduction of afterload (PAP) alone may not be sufficient to improve RV function, resulting in subsequent right-sided heart failure.[5,8] However, PAH is not likely to be diagnosed before the overt signs of disease become evident, and the prevention or improvement of RV dysfunction is essential for therapeutic strategies.[8]

Limitations

This study used a small number of rats and administered fixed doses of the 3 vasodilator agents in all tested groups, which limited the evaluation of the additional effects of each drug. Histopathological analysis was not performed, despite its importance in corroborating the echocardiographic findings after the administration of the drugs under investigation. Although echocardiographic parameters are reliable indicators of RV function, simultaneous pressure–volume measurements would be useful to demonstrate global RV performance and confirm echocardiographic-derived RVSP data. However, a simultaneous study would be technically difficult to perform. The 3-dimensional echocardiographic assessment of RV systolic function has shown fewer reproducibility errors, but we are aware that no ultrasound technique is exempt from criticism. In addition, analysis of multiple parameters may require a multiple comparison adjustment; however, it was not performed in this study. Nonetheless, our study aimed to demonstrate the echocardiographic changes of RV function using parameters of clinical utility to evaluate different therapies. Additionally, neurohormonal activation states and adverse factors generally induce individual variation, even when experimental conditioning is performed to minimize these differences, and may have thus influenced the echocardiographic parameters analyzed.

CONCLUSIONS

In summary, the single or combined administration of sildenafil, pimobendan, and nicorandil showed similar effects without significant differences on RV systolic function between treatment groups of rats with MCT-induced PH. Sildenafil in combination with pimobendan, as well as a combination of sildenafil, pimobendan, and nicorandil, further ameliorated global function and cardiovascular hemodynamics in this model of PH. LVSP was also improved in the group treated with the 3-drug combination despite the hypotensive synergistic action of the association of sildenafil and nicorandil, which may have been counteracted by positive inotropic effect of pimobendan. Further studies on the combination of these 3 drugs at lower doses and over longer periods are necessary to establish the optimum therapeutic measures in the treatment of patients with severe PAH and RV dysfunction.

REFERENCES

1. Galiè N, Torbicki A, Barst R, et al; Task Force. Guidelines on diagnosis and treatment of pulmonary arterial hypertension. The Task Force on Diagnosis and Treatment of Pulmonary Arterial Hypertension of the European Society of Cardiology. *Eur Heart J.* 2004;25:2243–2278.
2. Hoeper MM, Barberà JA, Channick RN, et al. Diagnosis, assessment, and treatment of non-pulmonary arterial hypertension pulmonary hypertension. *J Am Coll Cardiol.* 2009;54:S85–S96.
3. Gupta H, Ghimire G, Naeije R. The value of tools to assess pulmonary arterial hypertension. *Eur Respir Rev.* 2011;20:222–235.

4. Lee KS, Abbas AE, Khandheria BK, et al. Echocardiographic assessment of right heart hemodynamic parameters. *J Am Soc Echocardiogr.* 2007; 20:773–782.
5. Vonk Noordegraaf A, Galiè N. The role of the right ventricle in pulmonary arterial hypertension. *Eur Respir Rev.* 2011;20:243–253.
6. Brierre G, Blot-Souletie N, Degano B, et al. New echocardiographic prognostic factors for mortality in pulmonary arterial hypertension. *Eur J Echocardiogr.* 2010;11:516–522.
7. Voelkel NF, Quaife RA, Leinwand LA, et al. Right ventricular function and failure: report of a National Heart, Lung, and Blood Institute working group on cellular and molecular mechanisms of right heart failure. *Circulation*. 2006;114:1883–1891.
8. Bogaard HJ, Abe K, Noordegraaf AV, et al. The right ventricle under pressure. Cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. *Chest*. 2009;135:794–804.
9. Vanderpool RR, Pinsky MR, Naeije R, et al. RV-pulmonary arterial coupling predicts outcome in patients referred for pulmonary hypertension. *Heart*. 2014; pii: heartjnl-2014–306142.
10. Champion HC, Michelakis ED, Hassoun PM. Comprehensive invasive and noninvasive approach to the right ventricle-pulmonary circulation unit: state of the art and clinical and research implications. *Circulation*. 2009;120:992–1007.
11. Van de Veerdonk MC, Kind T, Marcus JT, et al. Progressive right ventricular dysfunction patients with pulmonary arterial hypertension responding to therapy. *J Am Coll Cardiol.* 2011;58:2511–2519.
12. Maclean MR, Johnston ED, McCulloch KM, et al. Phosphodiesterase isoforms in the pulmonary arterial circulation of the rat: changes in pulmonary hypertension. *J Pharmacol Exp Ther.* 1997;283: 619–624.
13. Murray F, MacLean MR, Pyne NJ. Increased expression of the cGMP-inhibited cAMP-specific (PDE3) and cGMP binding cGMP-specific (PDE5) phosphodiesterases in models of pulmonary hypertension. *Br J Pharmacol.* 2002;137:1187–1194.
14. Jeffery TK, Wanstall JC. Phosphodiesterase III and V inhibitors on pulmonary artery from pulmonary hypertensive rats: differences between early and established pulmonary hypertension. *J Cardiovasc Pharmacol.* 1998;32:213–219.
15. Matot I, Gozal Y. Pulmonary responses to selective phosphodiesterase-5 and phosphodiesterase-3 inhibitors. *Chest*. 2004;125:644–651.
16. Lobato EB, Beaver T, Muehjschlegel J, et al. Treatment with phosphodiesterase inhibitors type III and V: milrinone and sildenafil is an effective combination during thromboxane-induced acute pulmonary hypertension. *Br J Anaesth.* 2006;96:317–322.
17. Rabe KF, Tenor H, Dent G, et al. Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. *Am J Physiol.* 1994;266:L536–L543.
18. Atkinson KJ, Fine DM, Thombs LA, et al. Evaluation of pimobendan and N-terminal probrain natriuretic peptide in the treatment of pulmonary hypertension secondary to degenerative mitral valve disease in dogs. *J Vet Intern Med.* 2009;23:1190–1196.
19. Böhm M, Morano I, Pieske B, et al. Contribution of cAMP-phosphodiesterase inhibition and sensitization of the contractile proteins for calcium to the inotropic effect of pimobendan in the failing human myocardium. *Circ Res.* 1991;68:689–701.
20. Von Der Leyen H, Mende U, Meyer W, et al. Mechanism underlying the reduced positive inotropic effects of the phosphodiesterase III inhibitors pimobendan, adibendan and saterinone in failing as compared to non-failing human cardiac muscle preparations. *Naunyn Schmiedebergs Arch Pharmacol.* 1991;344:90–100.
21. Wilkins MR, Wharton J, Grimminger F, et al. Phosphodiesterase inhibitors for the treatment of pulmonary hypertension. *Eur Respir J.* 2008;32: 198–209.
22. Nagendran J, Archer SL, Soliman D, et al. Phosphodiesterase type 5 is highly expressed in the hypertrophied human right ventricle, and acute inhibition of phosphodiesterase type 5 improves contractility. *Circulation*. 2007;116:238–248.
23. Takimoto E, Champion HC, Li M, et al. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med.* 2005;11:214–222.
24. Fisher PW, Salloum F, Das A, et al. Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. *Circulation*. 2005;111:1601–1610.
25. Taira N. Similarity and dissimilarity in the mode and mechanism of action between nicorandil and classical nitrates: an overview. *J Cardiovasc Pharmacol.* 1987;10:S1–S9.
26. Blaise G, Langleben D, Hubert B. Pulmonary arterial hypertension: pathophysiology and anesthetic approach. *Anesthesiology*. 2003;99: 1415–1432.
27. Li J, Long C, Cui W, et al. Iptakalim ameliorates monocrotaline-induced pulmonary arterial hypertension in rats. *J Cardiovasc Pharmacol Ther.* 2013;18:60–69.
28. Hongo M, Mawatari E, Sakai A, et al. Effects of nicorandil on monocrotaline-induced pulmonary arterial hypertension in rats. *J Cardiovasc Pharmacol.* 2005;46:452–458.
29. Sahara M, Sata M, Morita T, et al. Nicorandil attenuates monocrotaline-induced vascular endothelial damage and pulmonary arterial hypertension. *PLoS One*. 2012;7:e33367.
30. Zuo XR, Wang Q, Cao Q, et al. Nicorandil prevents right ventricular remodeling by inhibiting apoptosis and lowering pressure overload in rats with pulmonary arterial hypertension. *PLoS One.* 2012;7:e44485.
31. Bardou M, Goirand F, Marchand S, et al. Hypoxic vasoconstriction of rat main pulmonary artery: role of endogenous nitric oxide, potassium channels, and phosphodiesterase inhibition. *J Cardiovasc Pharmacol.* 2001; 38:325–334.
32. Haddad F, Hunt SA, Rosenthal DN, et al. Right ventricular function in cardiovascular disease, part I: anatomy, physiology, aging, and functional assessment of the right ventricle. *Circulation*. 2008;117:1436–1448.
33. Rudski LG, Lai WW, Afilalo J, et al. Guidelines for the echocardiographic assessment of the right heart in adults: a report from the American Society of Echocardiography endorsed by the European Association of Echocardiography, a registered branch of the European Society of Cardiology, and the Canadian Society of Echocardiography. *J Am Soc Echocardiogr.* 2010;23:685–713.
34. Zoghbi WA, Enriquez-Sarano M, Foster E, et al; American Society of Echocardiography. Recommendations for evaluation of the severity of native valvular regurgitation with two-dimensional and Doppler echocardiography. *J Am Soc Echocardiogr.* 2003;16:777–802.
35. Lindqvist P, Calcutteea A, Henein M. Echocardiography in the assessment of right heart function. *Eur J Echocardiogr.* 2008;9:225–234.
36. Faul JL, Nishimura T, Berry GJ, et al. Triptolide attenuates pulmonary arterial hypertension and neointimal formation in rats. *Am J Respir Crit Care Med.* 2000;162:2252–2258.
37. Braun MU, Szalai P, Strasser RH, et al. Right ventricular hypertrophy and apoptosis after pulmonary artery banding: regulation of PKC isozymes. *Cardiovasc Res.* 2003;59:658–667.
38. Nishii Y, Gabazza EC, Fujimoto H, et al. Protective role of protein C inhibitor in monocrotaline-induced pulmonary hypertension. *J Thromb Haemost.* 2006;4:2331–2339.
39. Fisher MR, Forfia PR, Chamera E, et al. Accuracy of Doppler echocardiography in the hemodynamic assessment of pulmonary hypertension. *Am J Respir Crit Care Med.* 2009;179:615–621.
40. Jones JE, Mendes L, Rudd MA, et al. Serial noninvasive assessment of progressive pulmonary hypertension in a rat model. *Am J Physiol Heart Circ Physiol.* 2002;283:H364–H371.
41. Stenmark KR, Meyrick B, Galie N, et al. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol.* 2009;297:L1013–L1032.
42. Gomez-Arroyo JG, Farkas L, Alhussaini AA, et al. The monocrotaline model of pulmonary hypertension in perspective. *Am J Physiol Lung Cell Mol Physiol.* 2012;302:L363–L369.
43. Ferlinz J. Right ventricular diastolic performance: compliance characteristics with focus on pulmonary hypertension, right ventricular hypertrophy, and calcium channel blockade. *Cathet Cardiovasc Diagn.* 1998;43:206–243.
44. Haddad F, Doyle R, Murphy DJ, et al. Right ventricular function in cardiovascular disease, part II: pathophysiology, clinical importance, and management of right ventricular failure. *Circulation*. 2008;117:1717–1731.
45. Boissiere J, Gautier M, Machet MC, et al. Doppler tissue imaging in assessment of pulmonary hypertension-induced right ventricle dysfunction. *Am J Physiol Heart Circ Physiol.* 2005;289:H2450–H2455.
46. Hardziyenka M, Campian ME, de Bruin-Bon HA, et al. Sequence of echocardiographic changes during development of right ventricular failure in rat. *J Am Soc Echocardiogr.* 2006;19:1272–1279.

47. Koskenvuo JW, Mirsky R, Zhang Y, et al. A comparison of echocardiography to invasive measurement in the evaluation of pulmonary arterial hypertension in a rat model. *Int J Cardiovasc Imaging.* 2010;26:509–518.
48. Walker LA, Buttrick PM. The right ventricle: biologic insights and response to disease: updated. *Curr Cardiol Rev.* 2013;9:73–81.
49. Das A, Xi L, Kukreja RC. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. *J Biol Chem.* 2005;280:12944–12955.
50. Shan X, Quaile MP, Monk JK, et al. Differential expression of PDE5 in failing and nonfailing human myocardium. *Circ Heart Fail.* 2012;5: 79–86.
51. Guazzi M, Vicenzi M, Arena R, et al. PDE5 inhibition with sildenafil improves left ventricular diastolic function, cardiac geometry, and clinical status in patients with stable systolic heart failure: results of a 1-year, prospective, randomized, placebo-controlled study. *Circ Heart Fail.* 2011;4:8–17.
52. Dahlöf B, Pennert K, Hansson L. Reversal of left ventricular hypertrophy in hypertensive patients. A meta-analysis of 109 treatment studies. *Am J Hypertens.* 1992;5:95–110.
53. Pamboukian SV, Carere RG, Webb JG, et al. The use of milrinone in pre-transplant assessment of patients with congestive heart failure and pulmonary hypertension. *J Heart Lung Transplant.* 1999;18:367–371.
54. Fujino K, Sperelakis N, Solaro RJ. Sensitization of dog and guinea pig heart myofilaments to Ca2+ activation and the inotropic effect of pimobendan: comparison with milrinone. *Circ Res.* 1988;63:911–922.
55. Pagel PS, Hettrick DA, Warltier DC. Influence of levosimendan, pimobendan, and milrinone on the regional distribution of cardiac output in anaesthetized dogs. *Br J Pharmacol.* 1996;119:609–615.
56. Yoshiyuki R, Nakata TM, Fukayama T, et al. Pimobendan improves right ventricular myocardial contraction and attenuates pulmonary arterial hypertension in rats with monocrotaline-induced pulmonary arterial hypertension. *J Med Ultrason.* 2014;14:173–180.
57. Tissier R, Chetboul V, Moraillon R, et al. Increased mitral valve regurgitation and myocardial hypertrophy in two dogs with long-term pimobendan therapy. *Cardiovasc Toxicol.* 2005;5:43–51.
58. Burniston JG, Ellison GM, Clark WA, et al. Relative toxicity of cardiotonic agents: some induce more cardiac and skeletal myocyte apoptosis and necrosis in vivo than others. *Cardiovasc Toxicol.* 2005; 5:355–364.
59. Chetboul V, Lefebvre HP, Sampedrano CC, et al. Comparative adverse cardiac effects of pimobendan and benazepril monotherapy in dogs with mild degenerative mitral valve disease: a prospective, controlled, blinded, and randomized study. *J Vet Intern Med.* 2007;21:742–753.
60. Walter M, Liebens I, Goethals H, et al. Pimobendane (UD-CG 115 BS) in the treatment of severe congestive heart failure. An acute haemodynamic cross-over and double-blind study with two different doses. *Br J Clin Pharmacol.* 1988;25:323–329.
61. Effects of Pimobendan on Chronic Heart Failure Study (EPOCH Study). Effects of pimobendan on adverse cardiac events and physical activities in patients with mild to moderate chronic heart failure: the effects of pimobendan on chronic heart failure study (EPOCH study). *Circ J.* 2002;66:149–157.
62. Watanabe E, Shiga T, Matsuda N, et al. Low-dose systemic phosphodiesterase III inhibitor pimobendan combined with prostacyclin therapy in a patient with severe primary pulmonary hypertension. *Cardiovasc Drugs Ther.* 2003;17:375–379.
63. Sahara M, Takahashi T, Imai Y, et al. New insights in the treatment strategy for pulmonary arterial hypertension. *Cardiovasc Drugs Ther.* 2006;20:377–386.
64. Tei C, Dujardin KS, Hodge DO, et al. Doppler echocardiographic index for assessment of global right ventricular function. *J Am Soc Echocardiogr.* 1996;9:838–847.
65. Yeo TC, Dujardin KS, Tei C, et al. Value of a Doppler-derived index combining systolic and diastolic time intervals in predicting outcome in primary pulmonary hypertension. *Am J Cardiol.* 1998;81:1157–1161.
66. Holzmann S. Cyclic GMP as possible mediator of coronary arterial relaxation by nicorandil (SG-75). *J Cardiovasc Pharmacol.* 1983;5:364–370.
67. Gori T, Sicuro S, Dragoni S, et al. Sildenafil prevents endothelial dysfunction induced by ischemia and reperfusion via opening of adenosine triphosphate-sensitive potassium channels: a human in vivo study. *Circulation.* 2005;111:742–746.
68. Lepore JJ, Maroo A, Bigatello LM, et al. Hemodynamic effects of sildenafil in patients with congestive heart failure and pulmonary hypertension: combined administration with inhaled nitric oxide. *Chest.* 2005; 127:1647–1653.
69. Ishizuka N, Saito K, Akima M, et al. Hypotensive interaction of sildenafil and nicorandil in rats through the cGMP pathway but not by K(ATP) channel activation. *Jpn J Pharmacol.* 2000;84:316–324.
70. Nootens M, Kaufmann E, Rector T, et al. Neurohormonal activation in patients with right ventricular failure from pulmonary hypertension: relation to hemodynamic variables and endothelin levels. *J Am Coll Cardiol.* 1995;26:1581–1585.
71. Kiely DG, Cargill RI, Wheeldon NM, et al. Haemodynamic and endocrine effects of type 1 angiotensin II receptor blockade in patients with hypoxaemic cor pulmonale. *Cardiovasc Res.* 1997;33:201–208.
72. Schrier RW, Abraham WT. Hormones and hemodynamics in heart failure. *N Engl J Med.* 1999;341:577–585.
73. Fan TH, Liang CS, Kawashima S, et al. Alterations in cardiac beta-adrenoceptor responsiveness and adenylate cyclase system by congestive heart failure in dogs. *Eur J Pharmacol.* 1987;140:123–132.
74. Bristow MR, Minobe W, Rasmussen R, et al. Beta-adrenergic neuroeffector abnormalities in the failing human heart are produced by local rather than systemic mechanisms. *J Clin Invest.* 1992;89:803–815.
75. Wang GY, McCloskey DT, Turcato S, et al. Contrasting inotropic responses to alpha1-adrenergic receptor stimulation in left versus right ventricular myocardium. *Am J Physiol Heart Circ Physiol.* 2006;291: H2013–H2017.
76. McClanahan A, Guglin M. Right ventricular dysfunction compromises accuracy of echocardiographic diagnosis of pulmonary hypertension in heart failure. *J Card Fail.* 2011;17:1023–1027.
77. Arcasoy SM, Christie JD, Ferrari VA, et al. Echocardiographic assessment of pulmonary hypertension in patients with advanced lung disease. *Am J Respir Crit Care Med.* 2003;167:735–740.
78. Rich S, D'Alonzo GE, Dantzker DR, et al. Magnitude and implications of spontaneous hemodynamic variability in primary pulmonary hypertension. *Am J Cardiol.* 1985;55:159–163.
79. Sahay S, Tonelli AR. Pericardial effusion in pulmonary arterial hypertension. *Pulm Circ.* 2013;3:467–477.