Effects of Citrulline Combined with Tadalafil on Monocrotaline-Induced Pulmonary Hypertension in Rats Compared with Arginine

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Abstract

Purpose: Tadalafil, phosphodiesterase5 (PDE5) inhibitor, was reported to have a therapeutic effect on pulmonary hypertension (PH) and citrulline is an amino acid to dilate arteries as an extracellular supplement to improve nitric oxide (NO) production. Therefore, it is expected that combination of tadalafil and citrulline exert a stronger effect for PH. The aim of this study is to evaluate the combination effects of tadalafil and citrulline on monocrotaline (MCT) -induced PH in rats compared with the combination effects of tadalafil and arginine.

Methods: We used 4-week-old male SD rats which developed PH by 5-times subcutaneous injection of MCT. We evaluated heart function by echocardiography and calculated the ratio of RV to LV weight of heart before and after 6-times administration per two weeks of saline, tadalafil alone, citrulline alone and tadalafil and citrulline or arginine.

Results: The survival rate of tadalafil and citrulline administration was 91.7%, which was higher than other groups. The ratio of RV to LV weight in a tadalafil group and a tadalafil+citrulline group were significantly lower than other 3 groups.

Conclusion: Combination therapy of tadalafil and citrulline useful to prevent deterioration of pulmonary hypertension and improve survival rate compared with concomitant use of arginine.

Keywords: Pulmonary hypertension; Nitric oxide; Pulmonary heart disease; Citrulline; Tadalafil

Abbreviation: MCT: Monocrotaline; PDE5: Phosphodiesterase5; PH: Pulmonary Hypertension; NO: Nitric Oxide; NOS: Nitric oxide synthase; PA: Pulmonary artery; Vmax: Maximum PA velocity; AT: Acceleration Time; ET: Ejection time; AT/ET: The ratio of AT to ET; RVW: Right Ventricular Free Wall Weight; LVW+SEPW: Left Ventricular Weight Including the Intraventricular Septum

Introduction

Single subcutaneous administration of monocrotaline (MCT), a pyrrolizidine alkaloid, has been reported to cause pulmonary hypertension in rats [1,2]. Although this model is very useful for studying the pathophysiology and treatment of pulmonary hypertension, it is very difficult to evaluate the degree of pulmonary hypertension and right ventricular pressure overload. Many studies have evaluated histological samples after sacrificing small animals [3-6]. Echocardiographic evaluation could be useful for evaluating heart function noninvasively, but the small size of the heart and rapid heart rates offer limited echocardiographic assessment. A high-frequency transducer recently became available to evaluate the heart of small animals and we established a rat model of pulmonary hypertension with sufficient tricuspid regurgitation [7].

Tadalafil, phosphodiesterase5 (PDE5) inhibitor, was reported to have a therapeutic effect on pulmonary hypertension (PH) and now PDE5 inhibitors are the first line to treat a patients with PH. However, a direct NO donor, such as nitrolygicerin is contraindication of concomitant use with PDE5 inhibitors. Arginine is a physiological substrate for Nitric Oxide Synthase (NOS). Dietary citrulline can be converted to arginine by argininosuccinate synthetase, and could work as a NO donor with PDE5 inhibitor. There are some studies demonstrating the therapeutic effect of arginine and citrulline on PH [8-10], however, there is no study of combining effect of these amino acids and PDE inhibitor on PH.

Substantial intestinal and hepatic metabolism of arginine to ornithine and urea by arginase makes oral delivery of arginine very ineffective [11]. In contrast, citrulline is not metabolized in the intestine or liver [11]. Citrulline entering the kidney, vascular endothelium and other tissues can be readily converted to arginine, thus raising plasma and tissue levels of arginine and enhancing NO production [11]. Citrulline exhibits various physiological activities through NO production, such as vasodilation [12-15], neurotransmission [16-19] and immunostimulation [20]. Therefore, combination of citrulline with PDE inhibitor may promise as a therapeutic adjunct in PH.

The aim of this study is to evaluate the combination effects of tadalafil and citrulline on monocrotaline (MCT) -induced PH in rats compared with the combination effects of tadalafil and arginine.

Materials and Methods

Materials

A total of 60 four-week-old male Sprague-Dawley rats were purchased from Japan SLC Inc. (Shizuoka, Japan). The rats were divided into 5 groups, a vehicle, a tadalafil alone, a citrulline alone, a tadalafil and citrulline, a tadalafil and arginine group. The rats were

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housed in a temperature-controlled environment and free access to food and water.

All rats were induced pulmonary hypertension by 5 time’s subcutaneous injection of MCT (10 mg/kg/day) within 2 weeks. Next, we orally administrated tadalafil (0.4 mg/kg/day) or saline (vehicle group) for 6 times every other day. Citrulline and arginine (KYOWA HAKKO BIO CO., LTD., Tokyo, Japan) were administered from water supply (10 g/L) (Figure 1).

This study was approved by the Osaka University Medical School Animal Care and Use Committee and was in compliance with the Osaka University Medical School Guidelines for the Care and Use of Laboratory Animals. All institutional and national guidelines for the care and use of laboratory animals were followed.

**Estimation of cardiac function by echocardiography**

Echocardiography was performed at 6-weeks and 8-weeks. Rats were anesthetized with sodium pentobarbital (40 mg/kg, ip). Right and left ventricular end-diastolic area were measured at the papillary muscle level using SONOS5500 (Philips Medical Systems, USA) with an s12 probe (frequency: 5-12 MHz, frame rate: >120 Hz) (Figure 2). At the aortic valve level in the short axis view, Pulmonary artery (PA) flow velocities were recorded and maximum PA velocity (Vmax), acceleration time (AT), and ejection time (ET) of PA flow velocity tracing were measured, and the ratio of AT to ET (AT/ET) was calculated according to the method of Kato et al. [21] (Figure 3). Three to five cardiac cycle data sets were averaged to measure each parameter.

**Pathological studies**

After echocardiographic examination or death, the heart was removed from rats. The heart was dissected to remove the right and left atrium. Right ventricular free wall weight (RVW) and left ventricular weight including the intraventricular septum (LVW+SEPW) were measured, and the ratio of RVW and LVW+SEPW to body weight (BW) were calculated.

**Statistical analysis**

Data are shown as mean ± SD. Statistical comparisons between the each group values were analyzed using Bonferroni’s test following one-way ANOVA. A p-value of less than 0.05 was considered to indicate significance. Statistical analyses of survival curves were carried out using log-rank analyses of Kaplan-Meier curves. Statistical analysis was performed with Statcel software for Windows (Version 2, OMS Publishing, Inc., Saitama, Japan) and the ystat 2000 Statistical Program File (Igaku Tosho Shuppan, Tokyo, Japan).

**Results**

**Survival rate**

The survival rate of a tadalafil and citrulline group is much higher than other 4 groups (Figure 4). However, The survival rate of a tadalafil and arginine group is almost same as that of a tadalafil group.

**Two-dimensional echocardiographic parameters**

At 6 weeks, the ratio of right to left ventricular end-diastolic area in all groups was almost same. At 8 weeks, the ratio of right to left ventricular end-diastolic area in 4 groups (tadalafil, citrulline, tadalafil and citrulline, tadalafil and arginine) was lower than that in a vehicle group (Figure 5).

**Pulmonary artery flow**

Maximum flow velocity of the pulmonary artery at 8 weeks in 4 groups except for a tadalafil+arginine group is lower than at 6
weeks (Figure 6a). The AT/ET of the pulmonary artery at 8 weeks in all 5 groups is lower than that at 6 weeks (Figure 6b).

Tricuspid regurgitation

We evaluated tricuspid regurgitation by the color Doppler method and recorded the flow profile of TR from which maximum regurgitant velocity was measured and pulmonary artery pressure was estimated using the modified Bernoulli equation from the four-chamber view. Tricuspid regurgitation was recognized in many surviving rats. Estimated pulmonary artery pressure in a tadalafil+citrulline group seems to be lower than those in other groups (Figure 7).

Pathological changes

After the echocardiographic examination or immediately after death, we measured heart weight, and calculated the ratio of RV to LV weight. The ratio of RV to LV weight in a tadalafil group and a tadalafil+citrulline group were significantly lower than other 3 groups (Figure 8).

Discussion

The purpose of this study is to evaluate the combination effects of tadalafil and citrulline on monocrotaline (MCT) -induced PH in rats compared with the combination effects of tadalafil and arginine. Here we clarified that combination therapy of tadalafil and citrulline might be useful to prevent deterioration of pulmonary hypertension compared with concomitant use of arginine.
Expression of PDE5 was found to be greater in lung tissues from patients with PH compared with controls [22]. Highly expression of PDE5 in lung tissues causes excess contraction of the pulmonary artery and leads to PH. Now PDE5 inhibitors are the first line to treat patients with PH. PDE5 is the major negative regulator of cGMP. Its expression bioavailability ratio is associated with higher systolic pulmonary artery pressure and higher central venous pressure [25]. In a study of orally ingested arginine, arginine is extensively metabolized by arginase. In contrast, citrulline is not metabolized in the gut wall or liver, and is much higher than other 4 groups involving in tadalafil and arginine. The survival rate of a tadalafil and arginine group is almost same as that of a tadalafil+citrulline group. The survival rate and the ratio of RV to LV weight could be the most reliable data. In this study, we didn’t have blood pressure and histopathological data. It has been reported that oral administration of citrulline suppressed the increase of blood pressure and lung histopathological changes in pulmonary hypertensive rats [8,10]. Therefore, the efficacy of tadalafil + citrulline in preventing deterioration of pulmonary hypertension is likely to be partially mediated by these effects.

We tried to establish a rat model of pulmonary hypertension with sufficient tricuspid regurgitation by 15 times injections of 5 mg/kg/day of MCT. In this model, we obtained sufficient tricuspid regurgitation in 78% of rats. Other parameters, such as right ventricle area and right ventricle or lung weight, in the PH group were greater than those in the control group, and AT/ET and VTI in the PH group were smaller than those in the control group. The findings were very almost similar to those in other reports [6,7,21].

It is more effective to administrate tadalafil every day, but to be performed forced oral dosage is very stressful for rats and affects survival rate. Therefore we orally administrated tadalafil for 6 times every other day in this study. In this condition, the combination of tadalafil and citrulline demonstrated survival benefit. It is possible that administrating tadalafil with citrulline every day is more efficient therapeutic effect on PH.

Conclusion
Combination therapy of tadalafil and citrulline might be useful to prevent deterioration of pulmonary hypertension and improve survival rate compared with concomitant use of arginine.

Conflict of interest
Fuminobu Ishikura, Mai Egawa, Yuri Takano, Kota Kumagai, Takashi Suzuki and Masahiko Morita declare that have no conflict of interest.

References


