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Molecular, Biological, Physiological and Clinical Aspects of Melatonin
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Melatonin as a preventive and curative therapy against pulmonary hypertension

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Abstract: Pulmonary hypertension (PH) is characterized by elevated pulmonary arterial pressure, which leads to right ventricular (RV) hypertrophy and failure. The pathophysiological mechanisms of PH remain unclear but oxidative stress is believed to contribute to RV dysfunction. Melatonin is a powerful antioxidant and is cardioprotective against ischemia–reperfusion injury and hypertension. Therefore, we hypothesized that a chronic treatment with melatonin, given as a curative or preventive therapy, may confer cardiovascular benefits in PH. PH was induced in Long Evans rats ($n \geq 6$ per group), with a single subcutaneous injection of monocrotaline (MCT, 80 mg/kg). Melatonin was given daily in the drinking water, with the treatment starting either on the day of the injection of MCT (dose testing: melatonin 75 ng/L and 6 mg/kg), 14 days after the injection of MCT (curative treatment: 6 mg/kg), or 5 days before the injection (preventive treatment: 6 mg/kg). The development of PH was assessed by measuring RV hypertrophy, RV function, cardiac interstitial fibrosis, and plasma oxidative stress. Compared with controls, MCT-treated rats displayed RV hypertrophy and dysfunction, increased interstitial fibrosis, and elevated plasma oxidative stress. A chronic melatonin treatment (75 ng/L or 6 mg/kg) reduced RV hypertrophy, improved RV function and reduced plasma oxidative stress. Curative and preventive treatment improved RV functional and plasma oxidative stress parameters and reduced cardiac interstitial fibrosis. Our data demonstrate that melatonin confers cardioprotection in this model of PH. As melatonin is an inexpensive and safe drug, we propose that clinical investigation of the effects of melatonin on RV function in patients with PH should be considered.

Key words: cardioprotection, melatonin, monocrotaline, oxidative stress, pulmonary hypertension

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Introduction

Pulmonary hypertension (PH) is a disorder characterized by mean pulmonary arterial pressure greater than 25 mmHg at rest [1, 2]. It is associated with infectious diseases such as human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), schistosomiasis, and viral hepatitis, as well as chronic non-communicable diseases including sickle cell disease, systemic sclerosis, congenital heart disease, and chronic obstructive pulmonary disease [2, 3]. Currently, the overall prevalence of PH remains elusive due to a lack of large epidemiological studies with appropriate diagnostic methods to confirm the diagnosis of PH [4]. Pulmonary hypertension is thought to develop due to endothelial damage which initiates remodeling and narrowing of the pulmonary vasculature, leading to increased right ventricular (RV) afterload and subsequent RV hypertrophy/failure [5–9]. The exact interplay of the pathologic pathways underlying the pathophysiology of PH remains elusive [1, 5, 10, 11]. Studies have highlighted the inadequacy of existing PH treatments indicating that there is a need for either a cure, novel therapies or improved treatment strategies [5, 6, 10–13]. Such strategies include combination therapy or adjunctive, cardioprotective treatments that complement current PH treatments [7, 14–16].

In recent years, oxidative stress has been indicated as a major contributor in the development of PH, suggesting that antioxidant therapy may be considered as a successful therapy against the disease [17–23]. Melatonin is a powerful antioxidant which can limit damage in cardiovascular disease [24–28]. This indoleamine hormone synthesized in the pineal gland and gastrointestinal system, is also present in plants, and is produced during the fermentation of wine [29–31]. It is a lipophilic compound that can readily cross-cell membranes and exert multiple physiological

functions after binding to its receptors or independent of its receptors [32–34]. Melatonin is beneficial in many different pathophysiological conditions including irregular sleep–wake cycle, in vitro tumor growth, cardiac hypertrophy, ischemia/reperfusion injury, and hypertensive heart disease [24, 26, 27, 35–39]. Very recently, melatonin has been suggested to be beneficial in animal models of hypoxic-PH and PH of newborn sheep [40, 41]. In line with this, we therefore investigated whether chronic melatonin treatment, given at a therapeutic dose (6 mg/kg) or at the concentration obtained via nutrition (75 ng/L), may improve RV function in an experimental rat model of PH induced with monocrotaline (MCT). Our results suggest that melatonin, used as a preventive or curative therapy, confers cardioprotection by reducing oxidative stress, normalizing antioxidant enzyme activity and reducing cardiac interstitial fibrosis in our rat PH model.

Material and methods

Animals

Animal care and experimental protocols for this study were approved by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town, South Africa. All experiments were conducted in male Long Evans rats (150–175 g), housed in the University of Cape Town's animal unit, exposed to a 12-hr dark/light cycle. Room temperature was kept at 22°C, humidity at 40%, and the rats had ad libitum access to food and drinking water. The rats were randomly divided into four groups: control plus water (C), monocrotaline (MCT) plus water, control plus melatonin (C+MEL), and monocrotaline plus melatonin (MCT+MEL). Rats received either a single subcutaneous injection of MCT (80 mg/kg) or a sterile saline injection (0.9% sterile saline) and developed PH and RV hypertrophy in 28 days. The C groups and MCT groups only received drinking water without melatonin, while C+MEL and MCT+MEL groups received melatonin in their drinking water.

Drug preparation

Monocrotaline (Sigma-Aldrich, Cape Town, South Africa) was dissolved in 1N HCl, neutralized with 1N NaOH to pH 7.38 and diluted with sterile 0.9% saline to achieve a final concentration of 14 mg/mL. Melatonin (Sigma-Aldrich) was dissolved in absolute ethanol to a final concentration of 0.05% (v/v) as previously described [39]. Melatonin was tested at a low, dietary concentration of 75 ng/L and a higher pharmacological dose of 6 mg/kg body weight or vehicle (ethanol diluted in drinking water, 0.05% v/v) [38, 39, 42]. Melatonin was placed in water bottles covered with aluminum foil for protection from light and a fresh batch was prepared daily [39].

Treatment protocols

Rats were injected with either MCT or sterile saline on the first day (day 0). One group of rats (MCT and C) received melatonin (75 ng/L) and a separate group received melatonin (6 mg/kg/day). Melatonin was given daily from day 0 until day 28 (Fig. 1). For the preventive treatment group, melatonin treatment (6 mg/kg/day) was started 5 days before the MCT injection, given daily until day 28 (Fig. 1). For the curative treatment, rats were injected with MCT on day 0 and the treatment with melatonin (6 mg/kg/day) commenced on day 14 and continued daily until day 28 (Fig. 1).

Isolated heart perfusion

Rats were anaesthetized with 4% isoflurane (SAFELINE Pharmaceuticals (Pty) Ltd., Cape Town, Western Cape, South Africa) and maintained at 2% isoflurane in room air supplemented with 100% O_2 (Afrox, Cape Town, South Africa). Once they were fully unconscious, the chest cavity was opened and the heart was rapidly excised and placed in ice cold Krebs–Henseleit buffer as previously described [43]. Within 3 min, the heart was cannulated via the aorta onto the Langendorff perfusion apparatus and perfused retrograde [43]. Perfusion was performed at a constant pressure of 100 cm H_20 at 37°C, with Krebs–Henseleit buffer (pH 7.4) equilibrated with oxygen/carbon dioxide at a ratio of 95:5% (Afrox). A water-filled, latex balloon was inserted in the left ventricle and connected to a pressure transducer, and the data were recorded on a Power laboratory, Lab Chart computerized data acquisition program (ADI Instruments, Lasec, South Africa). The temperature of the heart was measured with a fine thermocouple wire (Physitemp, Clifton, NJ, USA) and monitored with a Digitron 2600T temperature sensor (2026P7 Pressure and Temperature Meter, Torquay, UK). End diastolic pressure was adjusted and maintained at 8–9 mmHg, and the heart was perfused for 30 min (stabilization period). Left ventricular (LV) and RV functional parameters were measured including heart rate, systolic, and developed pressures (developed pressure = systolic pressure minus diastolic pressure). As an index of RV hypertrophy, the RV was separated from the LV+septum and their weights were expressed as a ratio of RV/LV+septum, as reported previously [40]. Biometric measurements included body weight, heart, lung and liver weights, and tibia lengths.

Blood sample collection

After the heart was excised for perfusions, blood samples were immediately collected from the thoracic cavity. Blood was transferred into a BD vacutainer blood collection tube (containing EDTA) (Lasec, South Africa), gently tilted twice and immediately placed on ice. The tubes were transferred to a precooled (4°C) bench centrifuge and centrifuged for 10 min at 1500 g. Plasma (300-μL aliquots) was stored at −80°C until assessment of oxidative stress, antioxidant enzyme activity, and protein determination.

Thiobarbituric acid reactive substance (TBARS) assay

The TBARS assay was used to measure oxidative stress in the form of lipid peroxidation based on the Jentzsch

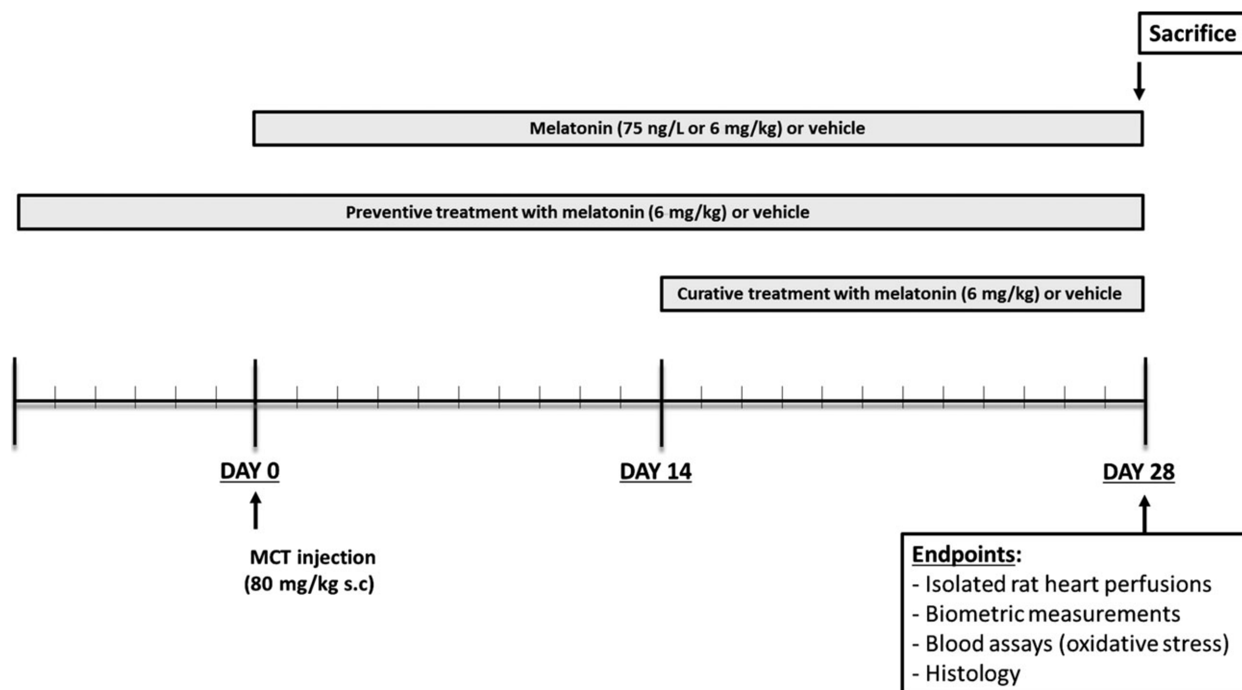

Fig. 1. Melatonin treatment protocol. The 75 ng/L concentration and 6 mg/kg melatonin treatment commenced on day 0 and continued until day 28. Preventive melatonin treatment (6 mg/kg) commenced on day minus 5 (5 days before MCT injection) and curative melatonin treatment (6 mg/kg) on day 14 (14 days after MCT injection) and continued until day 28. On the day of sacrifice (day 28), blood samples were collected, isolated rat heart perfusions and blood assays were performed. A separate group of rats were (n = 4) used for the collection and preparation of hearts for histological staining. Assays included TBARS, ORAC, catalase and SOD activity. s.c: subcutaneous, mg/kg: milligram per kilogram, MCT: monocrotaline, TBARS: thiobarbituric acids reactive substances, ORAC: oxygen radical absorbance capacity, SOD: superoxide dismutase.

method [44]. Blood plasma samples were assayed for lipid peroxidation during which the concentrations of TBARS were measured by a spectrophotometer (SPECTRA-maxPLUS-384; Molecular Devices Corporation, Labotec Industrial Technologies, Cape Town, South Africa). Absorbance was read at 532 nm, with a microplate data acquisition program (SoftMax® Pro 4.8; Molecular Devices Corporation, Labotec Industrial Technologies). The Beer–Lamberts law was used to calculate the concentration of malonic dialdehyde in the samples with an extinction coefficient of 1.54×10^5 M/cm. The final values were expressed in μmol malonic dialdehyde/mL plasma.

Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was employed to measure the inherent antioxidant capacity of blood plasma samples as previously described [45]. Blood plasma samples were deproteinized and microfuged at 11,000 g, for 5 min. The supernatant was diluted (736× dilution) with phosphate buffer ($K_2HPO_4{\cdot}3H_2O/NaH_2PO_4{\cdot}H_2O$, 0.075 mol/L, pH 7.4). The final sample was pipetted into a 96-well, white flat bottomed, microtitre plate (Polysorp®-Nunc, Lasec, South Africa) with phosphate buffer, fluorescein (95.7 nmol/L) and AAPH (0.33 mol/L). Readings were recorded at an excitation wavelength of 485 nm and emission wavelength of 520 nm, with a micro plate data acquisition program (Advanced Reads®; Cary Eclipse WinFLR, SSM Instruments, Set Point Technology, Cape Town, South Africa). Values were expressed as nmol/L trolox equivalents.

Catalase activity assay

The catalase (CAT) activity assay is based on the principle that CAT catalyzes the reduction of hydrogen peroxide into water [46, 47]. All blood plasma samples were diluted (1 in 10) with phosphate buffer ($K_2HPO_4{\cdot}3H_2O/NaH_2PO_4{\cdot}H_2O$, 0.075 mol/L, pH 7.4) and pipetted into a Costar, 96-well plate (Lasec, South Africa). Measurements were taken at 240 nm, at an initial time point of zero, and 1 min later, with a spectrophotometer (SPECTRA-maxPLUS-384). The final CAT activity was expressed as international units (IU) hydrogen peroxide per milligram (mg) protein (IU/mg protein).

Superoxide dismutase assay

Plasma superoxide dismutase (SOD) activity was measured using a spectrophotometric method described by Dalloz et al. [46] This assay is based on the principle that xanthine oxidase catalyzes the oxidation of xanthine to uric acid and in the process super oxide is produced. During this chemical reaction, cytochrome C is also reduced by super oxide and this reaction is diminished by SOD competing for super oxide. The reduction rate is measured at a wavelength of 550 nm. Readings were taken at time point zero with a micro plate data acquisition program

(SoftMax® Pro 4.8), and another reading was taken 3 min later. The final plasma SOD activity was calculated with the use of a SOD standard curve, and final values were expressed in IU/mg protein.

Total protein determination of blood plasma

Total blood plasma protein concentrations were determined by using the Qubit® assay kit and Quant-iT fluorometer (Invitrogen, Cape Town, South Africa). Plasma samples were diluted with buffers provided with the kit and after 15 min of incubation at room temperature, a reading was recorded at 470 nm excitation/590 nm emission. The final protein concentration was expressed as mg protein/μL plasma.

Histological staining: Cardiac interstitial fibrosis

After hemodynamic measurements of the heart in the isolated apparatus, the RV was separated from the LV+septum and washed with 1% phosphate-buffered saline. The LV+septum was sliced into four equally sized cross sections, the RV remained unsliced and was fixed for 2 days with 4% paraformaldehyde (Sigma-Aldrich). Subsequently, the sections were washed with and placed in 70% ethanol and embedded in paraffin. Thereafter, the slices were sectioned (transverse) at a thickness of 3 μm for histological analysis [48]. The RV sections were stained with 0.1% Sirius red in picric acid (Sigma-Aldrich) for the determination of collagen deposition. Five focus fields were examined with a Nikon Eclipse 90i microscope using a 20 times objective. Images were captured with the NIS-Elements Basic Research software (Nikon, Instech Co. Ltd., Tokyo, Japan) and quantified with the Visiopharm Integrator System (Leica Microsystem, Wetzlar, Germany). Cardiac interstitial fibrosis was expressed as percentage collagen deposition per total area sectioned [48].

Statistical analyses

Unless stated otherwise, all data were expressed as mean ± standard error of the mean (S.E.M.). For comparative studies, Student's *t*-test (unpaired) or two-way ANOVA (with Bonferroni post-test, if $P < 0.05$) were used, respectively. The experimental groups used for the fibrosis determination were statistically analyzed by one-way ANOVA and Newman–Keuls post hoc test for multiple comparisons. All statistical analyses were performed with GraphPad Prism-5 (La Jolla, CA, USA), and a $P < 0.05$ was considered statistically significant.

Results

A single subcutaneous injection of MCT resulted in increased weight of the heart, RV/tibia length, lung weight, liver weight, reduced LV+septal weight, and a decrease in body weights (Table 1). Melatonin treatment at a dietary concentration of 75 ng/L, increased body weight compared to the MCT group ($P < 0.02$), but had no effect on the heart weight over body weight ratio (n.s.) (Table 1). Melatonin (75 ng/L) also reduced RV weight/tibia length in comparison with the MCT group ($P < 0.01$), increased LV+septal weight/heart weight ($P < 0.001$, reduced lung weight ($P < 0.02$), and reduced liver weight ($P < 0.001$) (Table 1). Melatonin at a pharmacological dose of 6 mg/kg, increased body weight compared to the MCT group ($P < 0.02$), and reduced the heart weight to body weight ratio ($P < 0.05$) (Table 1). Melatonin (6 mg/kg) also reduced RV weight/tibia length in comparison with the MCT group ($P < 0.01$), increased LV+septal weight/heart weight ($P < 0.001$), reduced lung weight ($P < 0.02$), and reduced liver weight ($P < 0.001$) (Table 1).

Furthermore, melatonin (6 mg/kg) given as a preventive treatment increased body weight compared to the MCT group ($P < 0.001$) and reduced the heart weight to body

Table 1. Effects of melatonin treatment (75 ng/L and 6 mg/kg) on biometric measurements and cardiac function. Melatonin added to drinking water from day 0 (on the same day as MCT injection) until day 28. Values are means ± S.E.M. Sample size is ≥6. C, control; MCT, monocrotaline; C+MEL, control plus melatonin; MCT+MEL, monocrotaline plus melatonin; g, gram; g/cm, gram per centimetre; %, percentage; mL, millilitre; bpm, beats per minute; mmHg, millimetre mercury. *P: Significance of MCT versus C, $^{\#}P$: Significance of MCT+MEL (75 ng/L) versus MCT, $^{\$}P$: MCT+MEL (6 mg/kg) versus MCT

			75 ng/L melatonin		6 mg/kg melatonin	
	C	MCT	C+MEL	MCT+MEL	C+MEL	MCT+MEL
Weights						
Body weight (g)	323.0 ± 5	218.80 ± 18*	320.20 ± 14	297.30 ± 11$^{\#}$	334.60 ± 5	265.60 ± 5$^{\$}$
Heart weight (g)	1.36 ± 0.10	1.62 ± 0.10*	1.34 ± 0.10	1.63 ± 0.11	1.37 ± 0.08	1.28 ± 0.09$^{\$}$
Heart weight/body weight	0.36 ± 0.02	0.57 ± 0.02*	0.38 ± 0.03	0.58 ± 0.03	0.43 ± 0.02	0.41 ± 0.02$^{\$}$
RV weight/tibia length	0.55 ± 0.03	1.30 ± 0.10*	1.50 ± 0.07	1.10 ± 0.05$^{\#}$	0.57 ± 0.05	0.98 ± 0.10$^{\$}$
LV+septal weight/heart weight	0.80 ± 0.03	0.65 ± 0.01*	0.82 ± 0.01	0.82 ± 0.10$^{\#}$	0.83 ± 0.01	0.74 ± 0.04$^{\$}$
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13*	1.63 ± 0.14	2.87 ± 0.17$^{\#}$	1.50 ± 0.05	2.65 ± 0.03$^{\$}$
Liver weight (g)	15.81 ± 1	19.60 ± 1*	15.51 ± 1	11.30 ± 1$^{\#}$	15.28 ± 1	12.77 ± 1$^{\$}$
Isolated heart perfusions						
Heart rate (beats/minute)	272.10 ± 14	289.40 ± 12	274.90 ± 15	283.90 ± 14	303.20 ± 15	318.40 ± 16
LV systolic pressure (mmHg)	99.19 ± 6.6	103.80 ± 4.1	115.20 ± 2.5	99.19 ± 6.6	113.30 ± 2.7	109.70 ± 2.2
LV developed pressure (mmHg)	96.18 ± 5.2	96.08 ± 6.7	106.30 ± 2.4	96.18 ± 5.2	98.41 ± 3.9	107.50 ± 1.5
RV systolic pressure (mmHg)	54.35 ± 2.3	87.53 ± 4.8*	50.44 ± 5.3	87.91 ± 5.5	54.50 ± 3.7	64.34 ± 2.6$^{\$}$
RV developed pressure (mmHg)	45.87 ± 2.1	81.22 ± 2.8*	48.15 ± 2.2	71.95 ± 5.1$^{\#}$	48.72 ± 5.2	60.38 ± 3.1$^{\$}$

Table 2. Effects of melatonin treatment (6 mg/kg), at various time points, on biometric measurements and cardiac function. Melatonin added to drinking water either on day minus 5 (5 days before MCT injection), or on day 14 (started 14 days after MCT injection). Values are means ± S.E.M., n = 6 in all groups. C, control; MCT, monocrotaline; C+MEL, control plus melatonin; MCT+MEL, monocrotaline plus melatonin; g, gram, g/cm, gram per centimetre; %, percentage; mL, millilitre; bpm, beats per minute; mmHg, millimetre mercury. **P*: Significance of MCT versus C, #*P*: Significance of MCT+MEL (pre-treatment) versus MCT, $*P*: MCT+MEL (therapeutic treatment) versus MCT

			Preventive treatment (6 mg/kg)		Curative treatment (6 mg/kg)	
	C	MCT	C+MEL	MCT+MEL	C+MEL	MCT+MEL
Weights						
Body weight (g)	323.0 ± 5	218.80 ± 19*	317.80 ± 16	300.00 ± 28	276.20 ± 25	304.00 ± 21
Heart weight (g)	1.36 ± 0.10	1.62 ± 0.10*	1.44 ± 0.08	1.52 ± 0.08	1.32 ± 0.10	1.28 ± 0.11
Heart weight/body weight	0.36 ± 0.02	0.57 ± 0.02*	0.41 ± 0.01	0.49 ± 0.01#	0.42 ± 0.05	0.62 ± 0.08
RV weight/tibia length	0.55 ± 0.03	1.30 ± 0.10*	0.54 ± 0.03	0.90 ± 0.05#	0.54 ± 0.06	0.95 ± 0.13$
LV+septal weight/heart weight	0.80 ± 0.03	0.65 ± 0.01*	0.85 ± 0.01	0.77 ± 0.02#	0.83 ± 0.01	0.75 ± 0.02$
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13*	1.41 ± 0.07	2.44 ± 0.11#	1.33 ± 0.11	2.39 ± 0.22$
Liver weight (g)	15.81 ± 1	19.60 ± 1*	16.56 ± 1	14.08 ± 1#	12.36 ± 1	12.28 ± 1$
Isolated heart perfusions						
Heart rate (beats/minute)	272.10 ± 14	299.40 ± 13	302.60 ± 15	318.70 ± 9	284.80 ± 11	279.20 ± 18
LV systolic pressure (mmHg)	103.80 ± 4.1	103.80 ± 4.1	106.20 ± 1.8	99.19 ± 6.6	109.20 ± 2.0	99.15 ± 3.5
LV developed pressure (mmHg)	96.18 ± 5.2	96.08 ± 6.7	115.60 ± 3.1	108.80 ± 3.3	94.70 ± 3.8	92.62 ± 2.3
RV systolic pressure (mmHg)	54.35 ± 2.3	87.53 ± 4.8*	57.80 ± 3.5	68.65 ± 3.8#	50.96 ± 4.2	69.18 ± 5.2$
RV developed pressure (mmHg)	45.87 ± 2.1	81.22 ± 2.8*	49.04 ± 3.3	59.85 ± 3.8#	42.62 ± 4.1	56.67 ± 2.5$

weight ratio ($P < 0.05$) (Table 2). Melatonin preventive treatment also reduced RV weight/tibia length in comparison with the MCT group ($P < 0.01$), increased LV+septal weight/heart weight ($P < 0.03$), reduced lung weight ($P < 0.02$), and reduced liver weight ($P < 0.001$) (Table 2). Melatonin (6 mg/kg), given as a curative treatment, increased body weight compared to the MCT group ($P < 0.001$), but had no effect on the heart weight to body weight ratio ($P > 0.05$) (Table 2). Melatonin given as a curative treatment also reduced RV weight/tibia length in comparison with the MCT group ($P < 0.01$), increased LV+septal weight/heart weight ($P < 0.03$), reduced lung weight ($P < 0.02$), and reduced liver weight ($P < 0.001$) (Table 2).

We assessed LV and RV function with the Langendorff perfusion apparatus 28 days after MCT injection. Heart rates, LV systolic pressure, and LV developed pressure were similar between the MCT and control group ($P > 0.05$) (Table 1). RV systolic pressure was significantly increased in the MCT group compared to untreated controls ($P < 0.001$) (Table 1). A similar increase was seen in RV developed pressure ($P < 0.04$). In the MCT+MEL group, melatonin treatment (75 ng/L) had no effect on RV systolic pressure ($P > 0.05$) but reduced RV developed pressure ($P < 0.04$) compared to the untreated MCT group (Table 1). Melatonin treatment (6 mg/kg) reduced RV systolic pressure ($P < 0.001$) and reduced RV developed pressure ($P < 0.04$) in the MCT+MEL group compared to the untreated MCT group (Table 1). Similarly, melatonin given as a preventive treatment (6 mg/kg) reduced RV systolic pressure ($P < 0.001$) and RV developed pressure ($P < 0.001$) (Table 2) of the MCT+MEL group compared to the untreated MCT group. Melatonin, given as a curative treatment (6 mg/kg), decreased RV systolic pressure ($P < 0.001$) and RV developed pressure ($P < 0.001$) (Table 2) compared to the untreated MCT group.

The injection of monocrotaline resulted in a significant increase in the plasma levels of TBARS ($P < 0.001$), a reduction of the inherent antioxidant capacity of the blood plasma ($P < 0.02$), an increase in plasma CAT and SOD activities ($P < 0.001$) after 28 days, compared with the control group ($P < 0.01$) (Fig. 2). TBARS levels were reduced by with a chronic treatment with melatonin 75 ng/mL ($P < 0.002$) or 6 mg/kg ($P < 0.005$). Antioxidant capacity remained unaffected by 75 ng/L melatonin ($P > 0.05$) but improved by 6 mg/kg melatonin ($P < 0.01$). CAT activity was reduced by 75 ng/L melatonin ($P < 0.001$) as well as 6 mg/kg ($P < 0.001$) (Fig. 2). SOD activity was reduced by 75 ng/L melatonin ($P < 0.02$) and 6 mg/kg ($P < 0.01$) (Fig. 2). Most importantly, plasma CAT activity was reduced to similar levels of the control group, with melatonin given as a preventive treatment ($P < 0.001$) or a curative treatment ($P < 0.004$) (Fig. 3). Lastly, plasma SOD activity was increased to similar levels of the control group, with melatonin given as a preventive treatment ($P < 0.001$) or a curative treatment ($P < 0.002$) (Fig. 3).

Monocrotaline rats had a higher percentage of fibrosis in RV sections compared to controls ($P < 0.04$) (Fig. 4). Melatonin given as a preventive treatment or a curative treatment reduced cardiac interstitial fibrosis compared to the untreated MCT group ($P < 0.04$) (Fig. 4).

Discussion

In the present study, we investigated whether a chronic treatment with melatonin may improve RV function in the MCT model of PH. We successfully established the experimental model of MCT-induced PH in the Long Evans rats strain with a single subcutaneous injection of MCT. The rats displayed RV hypertrophy, cardiac dysfunction, increased plasma oxidative stress, and cardiac interstitial fibrosis 28 days after the injection of MCT. Most importantly, our data strongly suggest that melatonin, given at a therapeutic dose or at a concentration as found in food, protects against PH when given as a curative or a

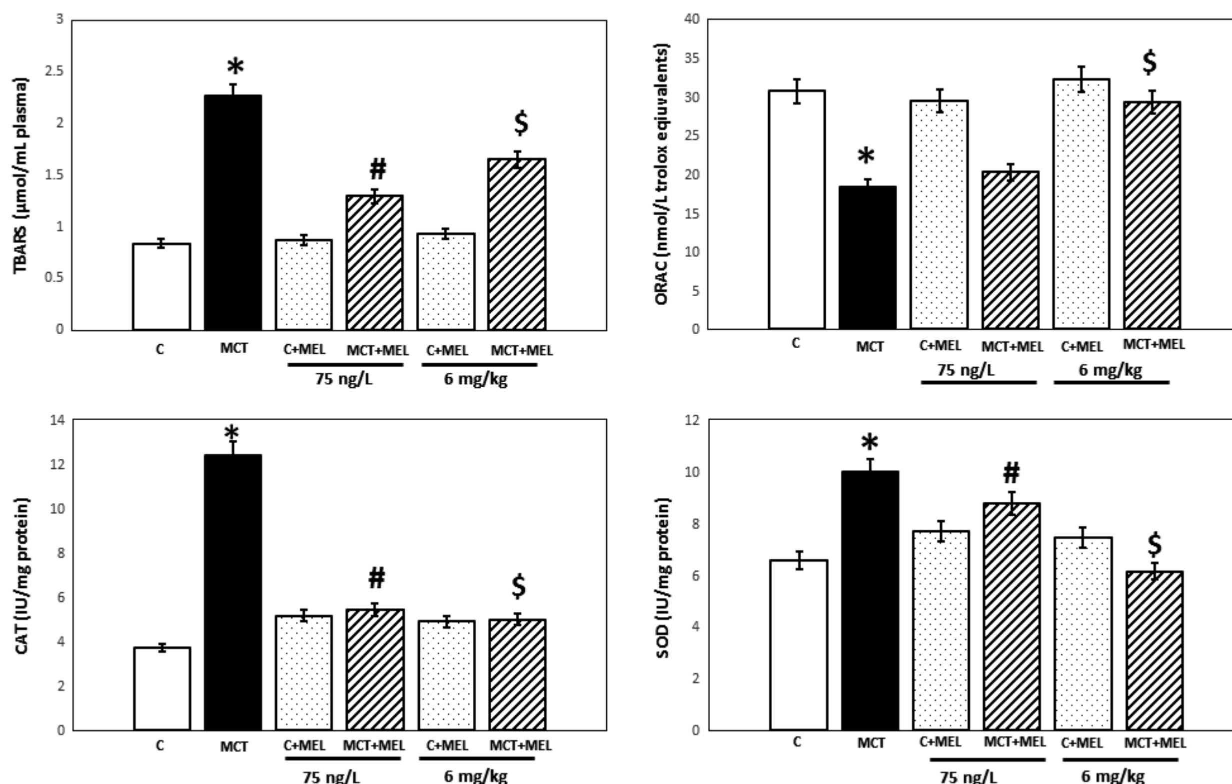

Fig. 2. Effect of melatonin treatment (75 ng/L and 6 mg/kg) on blood plasma oxidative stress and antioxidant enzyme activities. Melatonin was added to drinking water daily, from day 0 (on the same day as MCT injection) and continued until day 28. Values are means ± S.E.M., n = 6 in all groups. These bar graphs show the plasma TBARS, reduced ORAC and increased CAT and SOD activities of the various groups *: Significance of MCT versus C, #: Significance of MCT+MEL (75 ng/L) versus MCT, $: MCT+MEL (6 mg/kg) versus MCT. C: control, MCT: monocrotaline, C+MEL: control plus melatonin and MCT+MEL: monocrotaline plus melatonin.

preventive treatment. Indeed, melatonin reduced RV hypertrophy, plasma oxidative stress and cardiac interstitial fibrosis and improved RV function in rats with PH.

With regard to the model of monocrotaline-induced PH, our findings were consistent with previous reports using the same protocol with a different strain of rats [49–51]. A single injection of monocrotaline in Long Evans rats resulted in alteration of RV function, as observed in Wistar or Sprague Dawley rats [48–50]. Our study found that lung weights were significantly higher in the MCT rats, which could be due to extensive and proliferative pulmonary response to edema [50]. Our MCT rats also had significantly higher liver weights, similar to findings by Handoko et al. [49] The reason for this liver weight increase is unclear but it has been reported that MCT induces damage of sinusoidal endothelial cells, central venular endothelial cells, and hepatic parenchymal cells [52]. Although these effects of MCT on the liver may not completely explain the increase in liver weights, they do provide enough grounds for speculation that MCT may have caused hepatic damage associated with edema. Cardiac dysfunction of our MCT rats was underlined by RV hypertrophy, LV+S atrophy and interstitial fibrosis.

In our rats, RV hypertrophy, LV atrophy, and cardiac dysfunction were associated with an increase in plasma TBARS, reduced total antioxidant capacity (ORAC), and increased antioxidant enzyme (CAT and SOD) activity. Other mechanisms that underlie cardiac alteration and dysfunction in PH include pressure overload, cardiomyocyte microtubule proliferation, LV atrophic remodeling, and fibrosis due to reactive oxygen species (ROS)-dependent activation of matrix metalloproteinase [53, 54]. There is an abundance of evidence which suggest that oxidative stress plays a crucial role in the pathogenesis of PH, in both patients and experimental models [17–23]. Oxidative stress occurs due to the detrimental effect of excessive cellular ROS production on (i) proteins important for cardiac excitation-contraction coupling, (ii) mitochondrial deoxyribonucleic acid, and (iii) hypertrophic signaling kinases, transcription factors, and matrix metalloprotease activities [55]. These adverse effects of ROS can cause cardiomyocyte apoptosis, remodeling of the extracellular matrix, and impaired cardiac contractile function [55]. In ventricular failure, ROS suppresses enzymes involved in excitation–contraction coupling and poly-nitrosylates ryanodine receptors which also contributes to cardiac dysfunction [7]. Ultimately, oxidative stress has become increasingly recognized as a major contributor to cardiac dysfunction and remodeling in PH and the need for a powerful antioxidant therapy in PH has been largely reviewed [5, 13, 56, 57].

In our study, chronic melatonin supplementation (75 ng/L and 6 mg/kg) given in the drinking water, improved biometric parameters, including body, lung and liver weights. Melatonin also reduced RV hypertrophy and improved RV function as evident by the reduced RV/

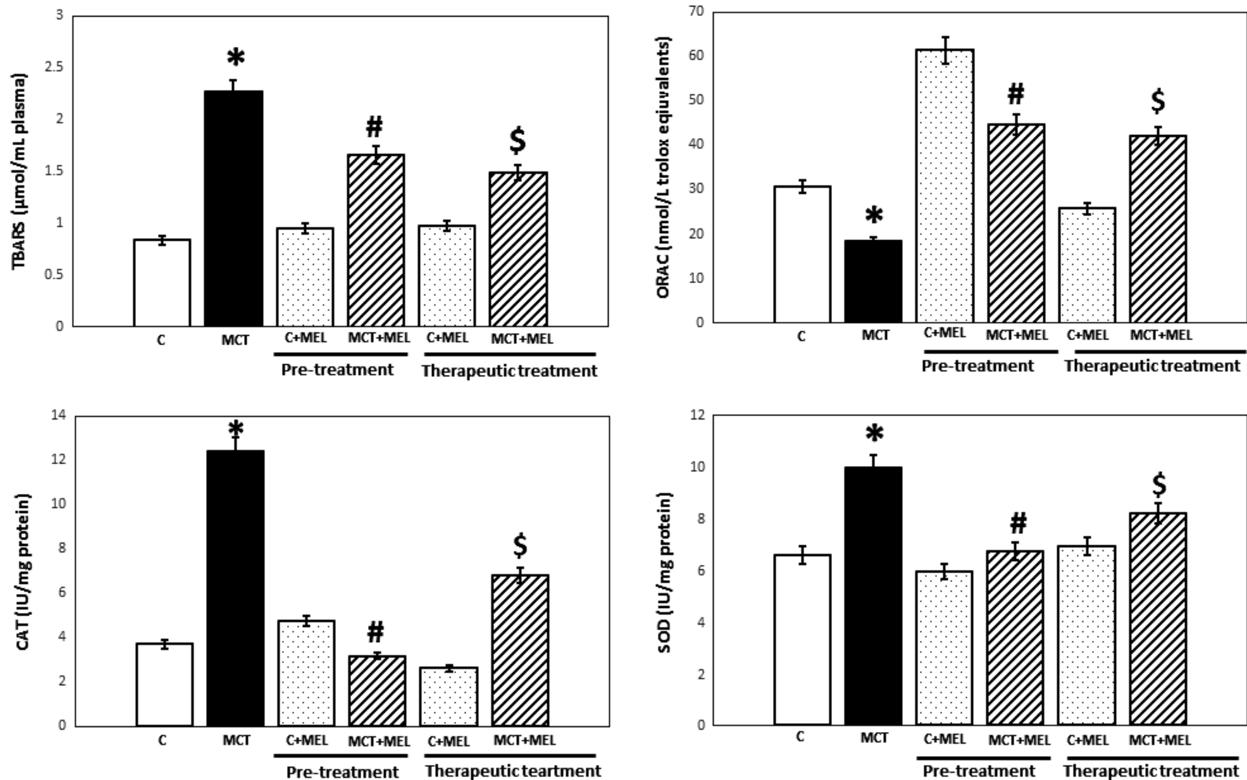

Fig. 3. Effect of melatonin preventive treatment (6 mg/kg, 5 days before MCT injection) or curative treatment (6 mg/kg, 14 days after MCT injection) on blood plasma oxidative stress and antioxidant enzyme activities. Melatonin was added to drinking water either 5 days before MCT injection, or 14 days after MCT injection. Values are means ± S.E.M., n = 4 in all groups. These bar graphs show the plasma TBARS, reduced ORAC and increased CAT and SOD activities of the various groups. *: Significance of MCT versus C, #: Significance of MCT+MEL (preventive treatment) versus MCT, $: MCT+MEL (curative treatment) versus MCT. C: control, MCT: monocrotaline, C+MEL: control plus melatonin and MCT+MEL: monocrotaline plus melatonin.

tibia length and improvement of RV functional parameters. Based on our results, it is interesting to note that the low-dietary concentration (75 ng/L) of melatonin, as found in the regular consumption of 2–3 glasses of wine per day, and the higher pharmacological dose (6 mg/kg) of melatonin improved these parameters in a similar fashion. Although the higher dose of melatonin has been successfully used in preclinical studies [28, 39–41], it is important to note that this dose is equivalent to 80 mg/kg for an 80 kg adult subject [58] and is indeed quite high in face of the regular amounts of melatonin given in a clinical setting (2–5 mg/day).

It is believed that the antihypertrophic effects of melatonin are mediated via its receptors, which activates anti-hypertrophic pathways and modulates cardiac gene transcription [26, 27, 59]. Our study did not include assessments of these pathways but future studies to investigate these parameters may be warranted. It is most likely that the dietary concentration of melatonin could have achieved its effects either directly or by binding to melatonin receptors [26, 27, 32, 33, 59] while the higher dose of melatonin does probably not exert its effects via the melatonin receptors [58, 60]. Further beneficial effects of melatonin treatment (75 ng/L and 6 mg/kg) were shown via the reduced levels of plasma TBARS, increased antioxidant capacity and normalized antioxidant enzyme (CAT and SOD) activity. What constitutes a physiological level of melatonin is debatable and it has been demonstrated by Venegas et al. [60], that high doses of melatonin (10–200 mg/kg) are necessary to reach subcellular concentrations sufficient to exert curative effects. However, our data show that melatonin at a low concentration of only 75 ng/L given chronically was sufficient to modulate antioxidant capacity and antioxidant enzyme activities.

We further investigated the cardioprotective effects of melatonin (6 mg/kg) by daily administration of melatonin, either as a preventive treatment (started 5 days before MCT injection) or a curative treatment (started 14 days after MCT injection) to better mimic the clinical setting. In both treatment protocols, melatonin stunted all the adverse effects of MCT-induced PH and conferred significant cardioprotection. In these protocols, melatonin also markedly reduced RV interstitial fibrosis compared to the untreated MCT group. These findings are in agreement with data from Simko et al. [26], who showed that in a model of hypertension, melatonin reduced interstitial fibrosis when given at dose of 10 mg/kg. The cardioprotective effect of melatonin has been ascribed to its powerful antioxidant properties and its ability to activate pro-survival signaling pathways including reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) [38, 39]. Campian et al. [61] showed that collagen deposition in MCT rat hearts is induced by

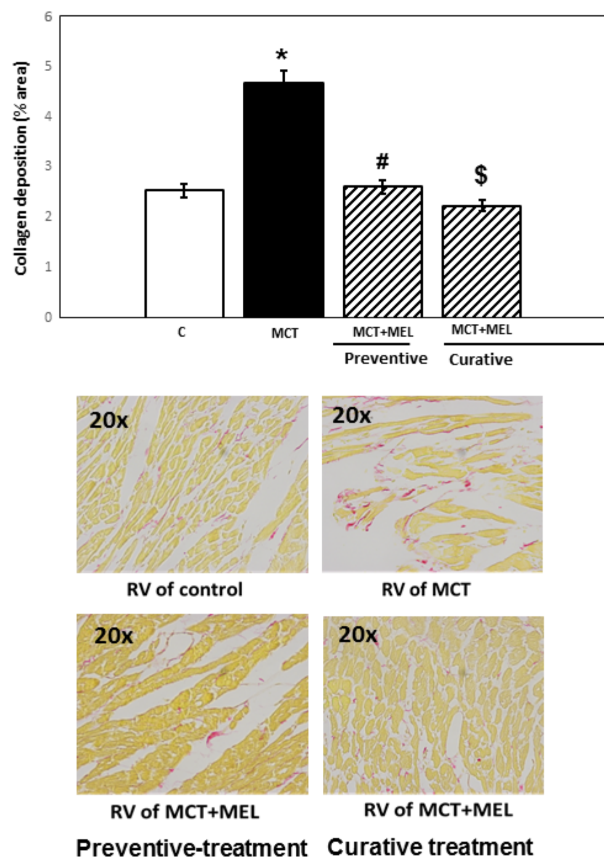

Fig. 4. Effect of melatonin preventive treatment (6 mg/kg, 5 days before MCT injection) or curative treatment (6 mg/kg, 14 days after MCT injection) on RV collagen deposition. Collagen deposition is expressed as percentage area of the sample assayed. Values are means ± S.E.M., n = 6 in all groups. *: Significance of MCT versus C, #: Significance of MCT+MEL (preventive treatment) versus MCT, $: MCT+MEL (curative treatment) versus MCT. C: control, MCT: monocrotaline and MCT+MEL: monocrotaline plus melatonin.

angiotensin-II and mediated via growth factor pathways and activation of the angiotensin receptor-1. Therefore, it is possible that melatonin could modulate these pathways, as shown by others who suggest that the antifibrotic effect of melatonin is mediated via its antioxidant effects as well as modulation of the sympathetic nervous and renin–angiotensin systems [26]. Although we did not investigate these specific mechanisms, our results are corroborated by Ersahin et al. [62], who showed that melatonin reduced oxidative stress and cardiac remodeling and improved systolic function in rats with renovascular hypertension.

An interesting finding in our study was that the preventive treatment of melatonin also increased the blood plasma antioxidant capacity in healthy rats, to more than double the levels of control rats who did not receive melatonin. This important observation highlights melatonin's ability to increase the antioxidant capacity of blood plasma in healthy animals and thus, could potentially protect against future disease associated with an oxidative stress. In fact, similar findings were generated by Piechota et al. [63], who administered 6 mg of melatonin orally to healthy individuals. However, administering preventive doses of melatonin to individuals should be performed with caution. Although antioxidant therapy in experimental PH and RV remodeling is strikingly beneficial, this therapy is challenging, as ROS production also plays an important physiological role in both health and disease conditions [5, 13]. If ROS are completely scavenged by antioxidants, this could have detrimental effects on the heart, as physiological low levels of ROS are required to maintain normal cellular function [13].

In our study, a curative treatment with melatonin also effectively reduced RV hypertrophy, cardiac dysfunction, and fibrosis in our model of PH. These results are very promising as they display the powerful cardioprotective effect of melatonin, even in an advanced cardiac stage alteration with MCT-induced PH. Melatonin has been widely tested in small clinical studies to protect against various diseases, and it has shown great efficacy in correcting sleeping patterns and hypertension [35, 64, 65]. It has no reported toxic side effects and therefore, given our findings, we suggest that melatonin should be tested in patients with PH who currently lack effective therapy.

In conclusion, our data demonstrate that a chronic treatment of melatonin confers cardioprotection in a model of MCT-induced PH. As melatonin is inexpensive, safe, and available over the counter in many countries, we propose that melatonin could be considered for clinical trials as a novel preventive/curative therapy to limit cardiac dysfunction in patients with PH. The low, dietary concentration of melatonin (75 ng/L) conferred striking cardiac benefits and revives the possibility that cardioprotection could be achieved by low, dietary intake of melatonin. The cardioprotective efficacy of melatonin, in our study, highlights its potential benefit as an adjunctive antioxidant cardioprotective therapy in patients with PH.

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Author contributions

Gerald Maarman

Dr. Maarman is the first and corresponding author, who performed all the experiments reported in this manuscript. He collected data, contributed to the concept and design of experiments, performed the data analysis and interpretation. As the first author he prepared the various draft versions of the manuscript and combined the contributions of the co-authors to produce the final version of the manuscript.

Dee Blackhurst

Dr. Blackhurst supervised the blood plasma experiments to measure antioxidant capacity, lipid peroxidation, and

catalase and superoxide dismutase activity. She also supervised the analysis of data generated from these experiments, the statistical analyses and interpretation. Lastly, she contributed to critical revision and approval of the manuscript and made recommendations about the layout of the data figures and tables.

Friedrich Thienemann

Dr. Thienemann, treats pulmonary hypertension patients on a daily basis and conceptually contributed to the research project from a clinical perspective. He helped with the interpretation of the data, analyses, overall display of the data in the manuscript and critically reviewed the manuscript.

Lori Blauwet

Prof. Blauwet trained the first author in echocardiography procedures and contributed to the correct analysis of results and the interpretation of the final data. Furthermore, she contributed by reviewing the manuscript to achieve the final version and helped by incorporating a clinical component into the discussion of the findings.

Ghazwan Butrous

Prof. Butrous is a world-renowned pulmonary hypertension physician and conceptually contributed to the overall research project. He facilitated the training of the first author on the use of the monocrotaline model of pulmonary hypertension and right ventricular failure. He also reviewed the manuscript, helped interpret the data and added clinical relevance to the discussion of data.

Neil Davies

Dr. Davies supervised the histology staining experiments, preparation of heart samples, data collection, analyses and final interpretation. Furthermore, he also gave critical input into interpretation of echocardiography data and he reviewed the manuscript.

Karen Sliwa

Prof. Sliwa was the co-supervisor of the research project and gave major input into the concept and design of the study. She critically reviewed the manuscript, contributed to the interpretation of the data and adding a clinical component to the discussion of the data.

Sandrine Lecour

Prof. Lecour was the main supervisor of the research project and gave major input into the concept and design of the study. She also gave major input into all the experiments performed in this study, methods of data collection, treatment protocols, data analyses, interpretation, layout of data, figures and tables, overall formatting and critical review of the manuscript.

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