MELATONIN AS A NOVEL CARDIOPROTECTIVE THERAPY IN PULMONARY HYPERTENSION

GERALD JEROME MAARMAN

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Supervisor: Associate Prof. Sandrine Lecour

Co-Supervisor: Prof. Karen Sliwa

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DECLARATION

I, **GERALD JEROME MAARMAN** hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicated otherwise). I declare that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion

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CONTENTS

LIST OF FIGURES

PAGE:

SECTION A:

SECTION E:

75ng/L melatonin:

LIST OF TABLES

PAGE:

ABBREVIATIONS

Adenosine tri-phosphatase	**ATPase**
Akt-mammalian target of rapamycin	**Akt/mTOR**
Ammonium phosphate	**NH_2PO_4**
Angiopoeitin-1	**Ang-1**
Approximately	**~**
Azobis-2-methyl-propanimidamide di-hydrochloride	**AAPH**
B-cell lymphoma-2	**bcl-2**
Beta	**β**
Calcium chloride	**$CaCl_2.2H_20$**
Cardiac output	**CO**
Chronic obstructive pulmonary disease	**COPD**
Control plus melatonin	**CON+MEL**
Control	**CON**
Degrees Celsius	**°C**
Deoxyribonucleic acid	**DNA**
Di-potassium hydrogen phosphate	**$K_2HPO_4 \cdot 3H_2O$**
Endoglin gene-heterogeneous mutation	**$Eng^{+/-}$**
Ethylenediamine-tetra-acetic acid	**EDTA**
Fibroblast growth factor-2	**FGF-2**
Flavin adenine dinucleotide	**FADH**
Fraction of inspired oxygen	**FiO_2**
Greater than or equal to	**≥**
Greater than	**>**
Heart rate	**HR**
Helix-loop-helix transcription factor-d	**dHAND**
Helix-loop-helix transcription factor-e	**eHAND**
Histone acetylases	**HDACs**
Homeo-domain containing transcription factor for cardiogenesis	**Nkx-2.5/Csx**
Human immune virus/acquired immune deficiency syndrome	**HIV/AIDS**
Insulin like growth factor	**IGF-1**
International units	**IU**
International units per milligram per minute	**IU/mg/min**
International units per millilitre per minute	**IU/mL/min**

International units per millilitre	**IU/mL**
International units/millilitre	**IU/mL**
Interventricular septal thickness at end-diastole	**IVSd**
Intraperitoneal	**i.p**
Kilogram per meters squared	**Kg/m^2**
Left ventricle	**LV**
Left ventricular developed pressure	**LVDP**
Left ventricular internal dimensions at end-diastole	**LVIDd**
Left ventricular internal dimensions at end-systole	**LVIDs**
Less than	**<**
Less than or equal to	**≤**
Litre	**L**
Magnesium sulphate hepta-hydrate	**$MgSO_4.7H_2O$**
Manganese-superoxide dismutase	**Mn-SOD**
Mean pulmonary artery pressure	**MPAP**
Megahertz	**MHz**
Messenger ribonucleic acid	**mRNA**
Microlitre	**µL**
Micromolar	**µL/mL**
Microgram	**µg**
Micrometre	**µm**
Micro-ribonucleic acids	**miRs**
Milligram	**mg**
Milligram per 100 gram	**mg/100g**
Milligram per day	**mg/day**
Milligram per kilogram	**mg/kg**
Millilitre per minute	**mL/min**
Millilitre	**mL**
Millimetres mercury	**mmHg**
Millimolar	**mM**
Millimole	**mmol**
Millisecond	**Ms**
Molar	**M**
Monocrotaline	**MCT**

Monopotassium phosphate	**KH_2PO_4**
Myocyte enhancer factor-2	**MEF-2**
Nanogram per kilogram per minute	**ng/kg/min**
Nanogram per litre	**ng/L**
Nanometre	**nm**
Nanomole	**nmol**
Nicotinamide adenine dinucleotide phosphate	**NADPH**
Nicotinamide adenine dinucleotide phosphate-oxidase	**NADPH-OX**
Nicotinamide adenine dinucleotide	**NADH**
Oxygen radical absorbance capacity	**ORAC**
Oxygen	**O_2**
Parts per million	**ppm**
Percentage	**%**
Picogram per litre	**pg/L**
Posterior wall thickness at end-diastole	**PWTd**
Potassium chloride	**KCl**
Protein kinase-B	**Akt**
Protein kinase-B/Akt	**PKB/Akt**
Pulmonary arterial smooth muscle cells	**PASMC's**
Pulmonary artery acceleration time	**PACT**
Pulmonary hypertension	**PH**
Pulmonary vascular resistance	**PVR**
Reactive oxygen species	**ROS**
Revolutions per minute	**rpm**
Ribonucleic acid	**RNA**
Right ventricle	**RV**
Right ventricular developed pressure	**RVDP**
Right ventricular dimension	**RVD**
Right ventricular wall thickness	**RVWT**
Small mothers against decapentaplegia	**smad**
Sodium bicarbonate	**$NaHCO_3$**
Sodium chloride	**NaCl**
Sodium hydrogen phosphate	**$NaH_2PO_4 \cdot H_2O$**
Sodium-hydrogen-exchange	**NHE-1**

Subcutaneous	**s.c**
Thiobarbituric acid reactive substance	**TBARS**
Transcriptional activator-4 that binds to the sequence 5'-AGATAG-3	**GATA-4**
Transforming growth factor-beta	**TGF-β**
Tricuspid annular plane systolic excursion	**TAPSE**
Ultraviolet	**UV**
Vascular endothelial growth factor	**VEGF**
Water	**H_2O**

ABSTRACT

Melatonin as a novel cardioprotective therapy in pulmonary hypertension

Introduction:

Pulmonary hypertension (PH) is characterized by elevated pulmonary arterial pressure which leads to right ventricular hypertrophy and failure. The mechanism involved in the pathophysiology of the disease remains unclear but it is suggested that oxidative stress may trigger cardiovascular dysfunction associated with the disease. To date, there is no efficient therapy against PH and novel therapies are urgently needed. Melatonin is a powerful antioxidant that can confer benefit against ischemia-reperfusion injury and hypertension. We therefore hypothesised that melatonin may confer cardiovascular benefits against PH.

Methods:

Oxidative stress (plasma lipid peroxidation, antioxidant capacity and antioxidant enzyme activity) was assessed in healthy (n=10), in patients with PH (n=10), in Long Evans rats (n≥6) or in a rat model of PH induced 28 days after the injection of monocrotaline (MCT, 80mg/kg, subcutaneous) (n≥6). Melatonin (75ng/L, nutritional concentration), 4mg/kg or 6mg/kg (therapeutic dose) was given daily in the drinking water of rats, with the treatment started 5 days before the injection of MCT, on the day of the injection or 14 days after the injection of MCT. The development of PH was measured by assessing right ventricular hypertrophy, cardiac fibrosis, oxidative stress and cardiac function (via echocardiography and the isolated heart Langendorff perfusion model).

Results:

Plasma oxidative stress was increased in both patients and rats with PH compared with their respective controls. A chronic treatment with melatonin (75ng/L, 4mg/kg or 6mg/kg) starting on the day of the injection with MCT in rats with PH reduced right ventricular hypertrophy, cardiac dysfunction and plasma oxidative stress compared with control rats. Furthermore, the beneficial effect of melatonin (6mg/kg) could be observed when given as a preventive (5 days prior to the injection of MCT) or as a curative therapy (14 days after the injection of MCT).

Conclusions:

Our data demonstrate that chronic treatment of melatonin confers cardioprotection in a rat model of PH. As melatonin is inexpensive, safe (no reported side effects) and already available over the counter in many countries, we propose that melatonin should be considered as a novel preventive/curative therapy to limit cardiac dysfunction in patients with PH.

SECTION A

INTRODUCTION

1. The global burden of cardiovascular disease

Noncommunicable diseases, including cardiovascular disease, are the leading cause of global mortality, accounting for more deaths annually than all other causes combined [1,2]. In 2008, noncommunicable diseases were responsible for approximately 36 million deaths (63%) [2]. Among these, cardiovascular disease, including heart attacks and strokes, were responsible for 17.3 million deaths, contributing to nearly a half of noncommunicable disease-related deaths [2]. Furthermore, it is projected that the annual number of deaths due to cardiovascular disease will increase from 17 million in 2008 to 25 million in 2030 [3]. Despite major pharmaceutical developments, the largest proportion of noncommunicable disease-related deaths is still due to cardiovascular disease (48%), followed by cancers (21%), chronic respiratory diseases (12%), other noncommunicable disease (7.75%), digestive disorders (7.75%) and diabetes (3.5%) (Figure 1). Thus, the global burden of cardiovascular disease paints a grim picture and generates great concern for the public health sector. The prevention and management of cardiovascular disease is complicated by its divergent aetiologies including hypertension, atherosclerosis, obesity, and congenital defects, and in particular, pulmonary hypertension which is the main focus of this research study.

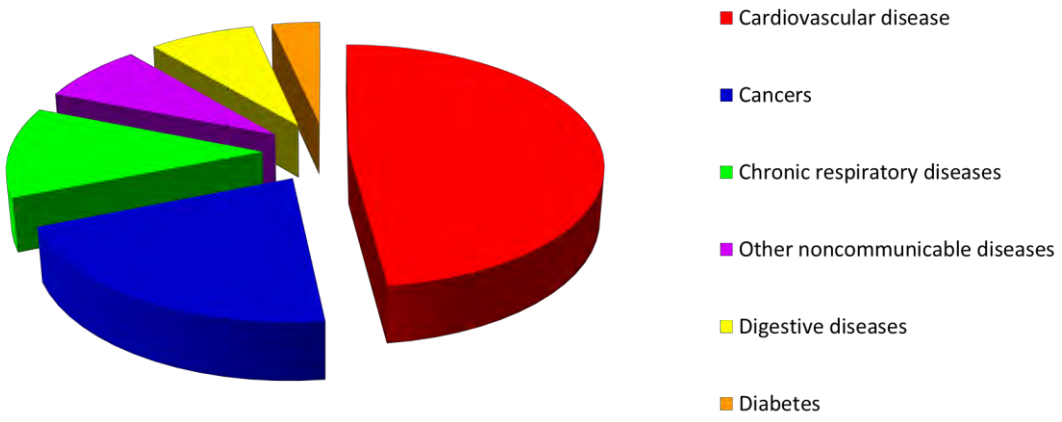


Figure 1. Proportion of global deaths caused by cardiovascular disease. A representation of the proportion of global noncommunicable deaths under the age of 70 (Adapted from WHO Global status report, 2010). **WHO**: world health organisation.

2. Pulmonary hypertension

Though often referred to as a disease, pulmonary hypertension (PH) is more accurately described as a pathophysiological parameter defined by certain haemodynamic measurements, including pulmonary arterial systolic pressure ≥ 35 mmHg, mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest, pulmonary capillary wedge pressure ≤ 15 mmHg and a pulmonary vascular resistance (PVR) ≥ 3 woods units [4,5]. PH is classified into various groups depending on the aetiology: group 1 (pulmonary arterial hypertension), group 2 (PH with left heart disease), group 3 (PH due to lung diseases/hypoxia), group 4 (Chronic thromboembolic PH) and group 5 (PH with indistinct, multifactorial mechanisms) [5]. Due to the wide diversity of aetiologies with PH, the haemodynamic measurements which are used to make a diagnosis, are also diverse and dependent on which group of PH is present in a patient. PH develops due to endothelial damage and consequent pathological remodelling of the pulmonary vasculature, resulting in the narrowing of vessels in the lung. This causes increased blood pressure in the pulmonary vasculature, which is normally a low pressure system. Consequently, the right ventricular (RV) afterload increases and induces hypertrophic remodelling of the RV [6-8]. PH is a heterogeneous disorder due to its aetiology, gender differences (in response to treatment) and variations in mortality/survival rates [8-12]. Furthermore, it is associated with infectious diseases such as human immune virus/acquired immune deficiency syndrome (HIV/AIDS), schistosomiasis, and viral hepatitis, as well as chronic noncommunicable conditions including sickle cell disease, systemic sclerosis, congenital heart disease and chronic obstructive pulmonary disease (COPD) [6].

2.1. Prevalence of pulmonary hypertension

Currently, the prevalence of PH in general, remains elusive due to a lack in large epidemiological studies with appropriate diagnostic methods [13]. Many studies that reported on the prevalence of PH, in any given patient population, based PH diagnosis on suboptimal measurements during echocardiography, such as tricuspid valve regurgitant jet velocity determined with Doppler echocardiography [5,14]. MPAP is one of the main parameters used to diagnose PH and this is calculated from the tricuspid regurgitant velocity by the echocardiographer [14,15]. An insufficient Doppler or tricuspid regurgitation jet is could be measured, which can results in an underestimated mPAP measurement [14,15]. This may yield misleading results, usually with an underestimation of the prevalence of PH. Nevertheless, this can be addressed by confirming echocardiographic diagnosis with right heart

catheterization which is considered to be the "gold standard" for the diagnosis of PH [16]. One should therefore be cautious when interpreting studies that investigate the prevalence of PH solely in one patient population, as this prevalence is likely to be true for that particular population but may not necessarily be extrapolated to other patient populations. From the literature, it is clear that there are no statistics for the overall prevalence of PH, either in a specific country or globally. In many publications, the prevalence of PH is often recorded for a specific patient population who have PH associated with diseases such as HIV/AIDS, congenital heart disease or COPD.

Prevalence of pulmonary hypertension in human immune virus/acquired immune deficiency syndrome

HIV/AIDS is an independent risk factor for PH, and in 2012 it was estimated that 35.3 million people were infected with HIV, with 70% of new HIV infections occurring in Sub-Saharan Africa [17]. Furthermore, within this time period, the incidence of PH in HIV infected individuals was estimated to be approximately a thousand times higher than in the general population [17]. In 80% of cases, where PH is associated with HIV, the prevalence is 0.5% in patients with plexiform lesions [18]. Nevertheless, the prevalence of PH in HIV infected individuals in African countries has been estimated to be 0.6% to 5% [19,20]. However, considering the depressingly large percentage of HIV infected individuals and the impact of suboptimal diagnostic methods, the prevalence is likely to be much higher.

Prevalence of pulmonary hypertension in congenital heart disease

It has been estimated that worldwide approximately 600 000 babies are born annually with congenital heart disease and 50% or more will die of infection and heart failure during infantile development [21]. Globally, three million children are at risk for the development of pulmonary vascular disease due to congenital heart disease [21]. On the other hand, the adult congenital heart disease patient population is increasing and requires lifelong tertiary medical care, with approximately five to ten percent developing PH [22]. In a retrospective longitudinal cohort study conducted by Lowe and colleagues, an adult patient population with congenital heart disease, was investigated from 1983 to 2005 [23]. A total of 38,430 adults (living with congenital heart disease in 2005) were included in the study and 2,212 were diagnosed with PH. This study calculated the prevalence of PH as 58 in a 1000 patients with

congenital heart disease [23]. However, this prevalence is likely to differ for various countries and patient populations.

Prevalence of pulmonary hypertension in chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide with PH considered as a major complication [24]. The prevalence of PH in COPD is unknown due to a lack of large epidemiological studies [13]. However, depending on which studies one looks at, most studies estimate the prevalence of PH in COPD to be approximately 30% to 70% [13]. In a study by Scharf et al, they evaluated 120 patients with severe COPD and defined PH as an mPAP > 20mmHg [25]. These authors determined that 90.8% of patients had PH [25].

The PH registry in France shows that the prevalence of PH from 2002 to 2003, was 15 cases per million and it varied among regions in France, from 5 to 25 per million [26]. In South Africa, a clinical registry, the Heart of Soweto cohort study, was created to capture data from 5328 de novo presentations of heart disease at the Cardiology Unit, Chris Hani Baragwanath Hospital in Soweto [27,28]. There were more females than males in the study (379 versus 318), while more men presented with RV failure. The study was conducted from 2000 to 2008. There was a total of 2505 cases of heart failure (47% of total cohort) while 697 (28%) were diagnosed with RV failure. Possible pathways which could have caused the RV failure in these patients included chronic lung disease (26%) and PH (20%). Furthermore, women were almost two times more likely to present with PH and those with low levels of education and originating from Soweto were less likely to present with PH, compared with the rest of the cohort [27,28]. The Heart of Soweto cohort study is a very unique study as it showed that PH in African communities is not such a rare disease as once thought.

All these studies have shown the heterogeneous nature of PH and how this, together with insufficient diagnostic methods, can influence the estimated prevalence. Therefore, whether or not PH is truly as rare as once thought is unclear as not enough is known about the underlying pathophysiology and PH continues to be a fatal disorder which affects patients globally.

2.2. Pathophysiology of pulmonary hypertension

It was previously thought that the development of PH was solely due to an imbalance of vasodilator and vasoconstrictor substances/responses which led to the pulmonary vascular remodelling observed in PH [29-31]. However, this has been proved incorrect and we now know that with PH there are certain molecules (hypoxia, sheer stress and toxins) that trigger and initiate endothelial injury which leads to (i) increased/disorganized proliferation of endothelial cells and increased endothelial cells apoptosis, (ii) increased proliferation of pulmonary arterial smooth muscle cells (PASMC's) and resistance of PASMC's to apoptosis [29,30].

These proliferated cell masses, are better described as thickening of the muscle layers (intima, media and adventitia) of small pulmonary arteries, as well as intimal fibrosis, in situ thrombosis and plexogenic arteriopathy (plexiform lesions), as seen in severe forms of PH [18,30,32,33]. All of these proliferative processes embody the pathological remodelling of the pulmonary vasculature, evident by narrowing and obliteration of the vascular lumen (Figure 2).

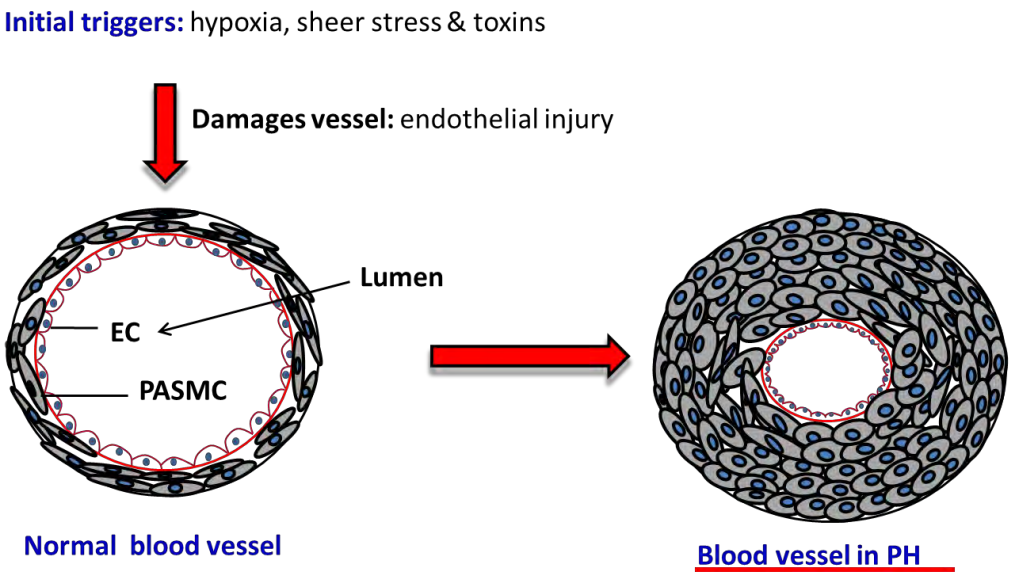


Figure 2. Pulmonary vascular remodelling in PH. A simplified representation of the remodelling of the pulmonary vasculature in PH. Here it is clear how the lumen of the vessels are constricted which ultimately results in increased pressure (Adapted from Wilkins MR. 2012). **EC**: endothelial cell, **PASMC**: pulmonary artery smooth muscle cell.

The pulmonary vascular remodelling in PH is exacerbated by biologically active inflammatory cytokines, growth factors, elastases and vasoconstrictors, such as thromboxane-A2, endothelin-1 and serotonin [18,30,33]. Other contributing factors to this remodelling process include activated endothelial cell-colony forming cells, reduced circulation of vasodilators (such as prostacyclin, nitric oxide and vascular endothelial peptide) and bone morphogenetic protein receptor-2 (BMPR-2) mutations [30, 34, 45, 49, 50, 57] (Figure 3).

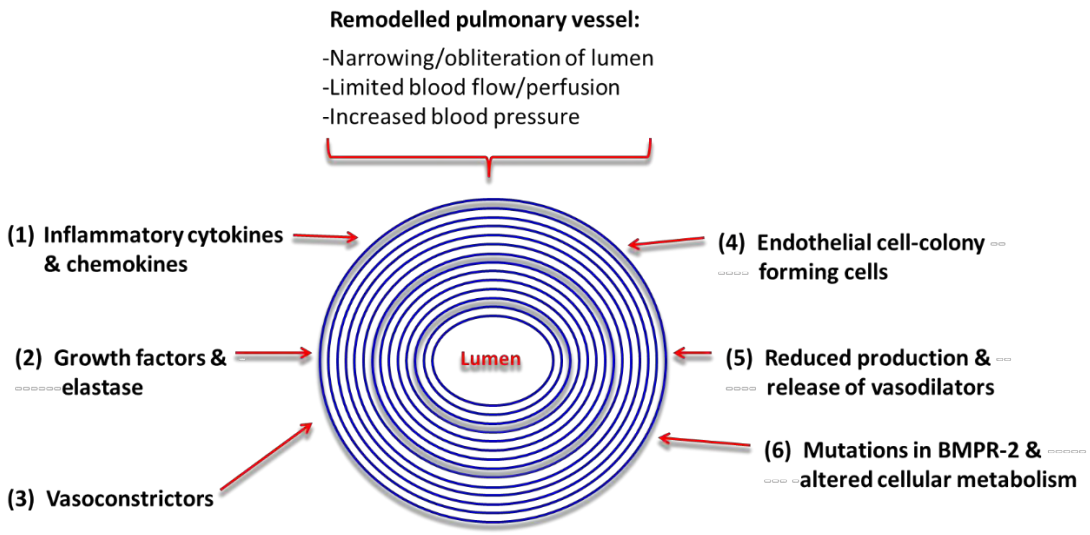


Figure 3. Contributors to pulmonary vascular remodelling in PH. Factors that possibly contribute to the remodelling of the pulmonary vasculature in PH. **BMPR-2**: bone morphogenetic protein receptor-2.

Inflammatory cytokines in pulmonary vascular remodelling

Inflammation has been defined as a complex series of interactions among soluble factors and cells that can arise in response to trauma, infection, ischemia, toxicity or autoimmune injury [18]. With PH, there is histological evidence of both innate and adaptive immunity involvement in the pulmonary vasculature and heart, as indicated by infiltration of mononuclear cells, B & T-cells, macrophages, dendritic cells, mast cells, cytokines and chemokines (such as interleukin-1β, interleukin-6, monocyte chemo attractantprotein-1 and chemokine ligand) [18]. This may be evidence, that inflammation plays an integral role in the development of PH and RV remodelling. However, the exact mechanism of how inflammation contributes to the pathogenesis of PH is currently unknown [18,34].

Growth factors and elastases in pulmonary vascular remodelling

The balance of proteases and anti-proteases regulates the constant turnover of the cellular matrix and the availability of growth factors, including platelet- derived growth factor, epidermal growth factor, vascular endothelial growth factor and fibroblast growth factor [29,34]. Various studies ascribed the changes in pulmonary vascular wall construction, to alterations in the circulating and tissue levels of growth factors and extracellular matrix molecules which control migration, proliferation and differentiation of endothelial cells, PASMC's and fibroblasts, as well as regulate vascular branching morphogenesis [34]. Furthermore, increased levels of platelet- derived growth factor have been found in blood and lung tissue samples of PH patients [35]. It is known that, platelet- derived growth factor induces the Akt-mammalian target of rapamycin (Akt/mTOR) signalling pathway in order to bring about PASMC proliferation and pulmonary vascular remodelling [,36].

On the other hand, elastase activity is increased in the isolated pulmonary arteries from the monocrotaline (MCT) and chronic hypoxia rat models of PH [37-39]. It is an enzyme that degrades elastin and proteoglycans which serve as storage sites for growth factors such as fibroblast growth factor-2 and transforming growth factor-beta (TGF-β) [40,41]. The sequence of events which precedes increased elastase activity is believed to be as follows: toxin and disease compromising the endothelial barrier and serum factors (such as apolipoprotein-A1) accumulation in the sub-endothelium [40,41]. This sequence of events induces the expression of endogenous vascular elastase in vascular smooth muscle cells by binding to elastin on the PASMC surface [40,41]. Elastin binds to elastin binding protein and increases the activity of elastase, which is also associated with activity of mitogen-activated protein kinase and focal adhesion kinase [40,41]. This augmented elastase activity has been linked to the induction of PASMC proliferation via association with the glycoprotein, tenascin [40,41]. The importance of elastase has been proven by the experimental model of elastase-induced pulmonary emphysema and its ability to attenuate cigarette smoke-induced remodelling of lung vessels in guinea pigs [42,43]. It is therefore clear, that growth factors and elastase play a key role in pulmonary vascular remodelling observed in PH.

Vasoconstrictors: Thromboxane-A2, endothelin-1 and serotonin in pulmonary vascular remodelling

Thromboxane-A2 is generated by thromboxane-synthase, stimulates vasoconstriction and platelet aggregation [44]. Thromboxane-A2 levels are increased in lung tissue of PH patients [45]. Endothelin-1 is elevated in circulation and lung tissue of patients with PH associated with chronic thromboembolic disease and COPD [46-48]. It is a well-known endothelium derived vasoconstrictor peptide that stimulates PASMC proliferation and vasoconstriction upon activation of its receptors [30,33,44]. In the MCT and chronic hypoxia rat models of PH, circulating levels of endothelin-1 have also been shown to be increased [30,33]. Lastly, serotonin, also known as 5-hydrotryptamine, is reportedly reduced in platelets and plasma of patients with PH [30]. It is a vasoconstrictor that promotes PASMC hyperplasia and hypertrophy [30]. Collectively, this suggests that increased levels of these vasoconstrictors may contribute to pulmonary vascular remodelling in PH.

Activation of endothelial cell-colony forming cells in pulmonary vascular remodelling

Endothelial cells are normally quiescent, but injury, inflammation and hypoxia can initiate angiogenesis via mobilization of pro-angiogenic cells from the bone marrow and proliferation of local endothelial cells [49]. These pro-angiogenic cells are myeloid in origin, found in the pulmonary vessel wall and known as endothelial cell-colony forming cells [49]. They reside among pulmonary artery endothelial cells and possibly contribute to angiogenesis via cell proliferation, but it is unclear whether they are unipotent or multipotent stem cells or fully undifferentiated with high proliferative potential [49]. More research is required to elucidate their exact function in PH as this may be instrumental in delineating our understanding of the disease pathogenesis. Nevertheless, these cells may be a contributing factor in the development of pulmonary vascular remodelling in PH.

Reduced production of vasodilators: Prostacyclin, nitric oxide and vascular intestinal peptide in pulmonary vascular remodelling

Prostacyclin is generated by prostaglandin-I-2 synthase which is expressed in pulmonary vascular endothelium, where it relaxes smooth muscle and inhibits platelet aggregation [44]. In patients with PH, there is a decrease in prostacyclin levels and the production of prostacyclin-I2 synthase in the small- and medium- sized pulmonary arteries [45]. On the other hand, nitric

oxide is produced by endothelial nitric oxide synthase, is a vasodilator and an inhibitor of platelet activation and PASMC proliferation [30,50,51]. With vascular endothelial injury, nitric oxide synthase produces nitric oxide via the activation of soluble-guanalate-cyclase [51]. Subsequently, guanosine-triphosphate is converted to cyclic-guanosine-monophosphate which acts as a second messenger and induces smooth muscle relaxation or vasodilatation [51].

In the pulmonary vasculature of PH patients, endothelial nitric oxide synthase expression has been shown to be decreased [52]. However, its expression is increased in plexiform lesions where it is thought to promote pulmonary vascular endothelial cell proliferation [30,34,52]. Impaired nitric oxide synthesis and vasodilatation have been observed in an animal model of chronic hypoxia-induced PH and this was associated with impaired cyclic-guanosine monophosphate dependent mechanisms [34]. Vascular intestinal peptide is a neuropeptide that act as a vasodilator and has the ability to reduce mPAP and pulmonary vascular resistance [53]. Patients with PH have decreased serum levels of vascular intestinal peptide which is thought to also influence remodelling [54-56]. Thus, it is evident that an imbalance between vasoconstrictor/vasodilator responses has merit as a contributing factor to the development of PH. This gives more value to the current understanding that vascular remodelling in PH is most probably due a combination of factors and pathological pathways.

Mutations in the gene encoding for bone morphogenetic protein receptor-2 in pulmonary vascular remodelling

PH also occurs in a rare idiopathic form with a prevalence of 90% for sporadic PH and 10% for familial PH [34]. A mutation in the gene encoding for BMPR-2 is associated with 60% of familial cases of PH, while 80% of family members who carry the mutation, never present with the PH phenotype [34]. BMPR-2 is highly expressed on the endothelium of pulmonary arteries [57]. It is also expressed at a lower level in PASMC's and fibroblasts and possibly contributes to PH, pulmonary fibrosis and COPD [58]. It has been shown that, lung samples of patients with PH have mutations in genes encoding for BMPR-2 [59-62]. BMPR-2 forms part of the TGF-superfamily of proteins where TGF-β signalling is initiated by signals transmitted from small mothers against decapentaplegia (Smad)-molecules that facilitate the binding of TGF-β ligands to the TGF-β-receptor [63-65]. Subsequently, Smad 2, 3 or Smad 1, 5, 8 are phosphorylated and co-localize with Smad 4 which translocate to the nucleus where it modulates transcription of target genes (Figure 4).

The general understanding is that, signalling via TGF-β/Smad 2, 3 induce PASMC proliferation and resistance to apoptosis, whereas BMPR-2/Smad 1, 5, 8 induce the opposite effects [63,64]. This means that neither BMPR-2 nor TGF-β act independently, but form part of the TGF-β/ BMPR-2/Smad signalling axis, which controls apoptosis and PASMC proliferation [63,64]. Studies in the animal model of MCT-induced PH have shown that the expression of TGF-β receptor-activin-A receptor-like kinase-1 and Smad 3, 4, is reduced in the lungs. In the MCT model, the toxin induces PH by initiating endothelial injury in the lung and smooth muscle cell proliferation which leads to narrowing of the pulmonary arterioles [165, 324]. This was associated with a decreased expression of the full BMPR-2 gene in the lungs of these rats [63,64]. This may indicate that TGF-Smad and BMPR-2 signalling is impaired in PH and contributes to remodelling via PASMC proliferation and resistance to apoptosis.

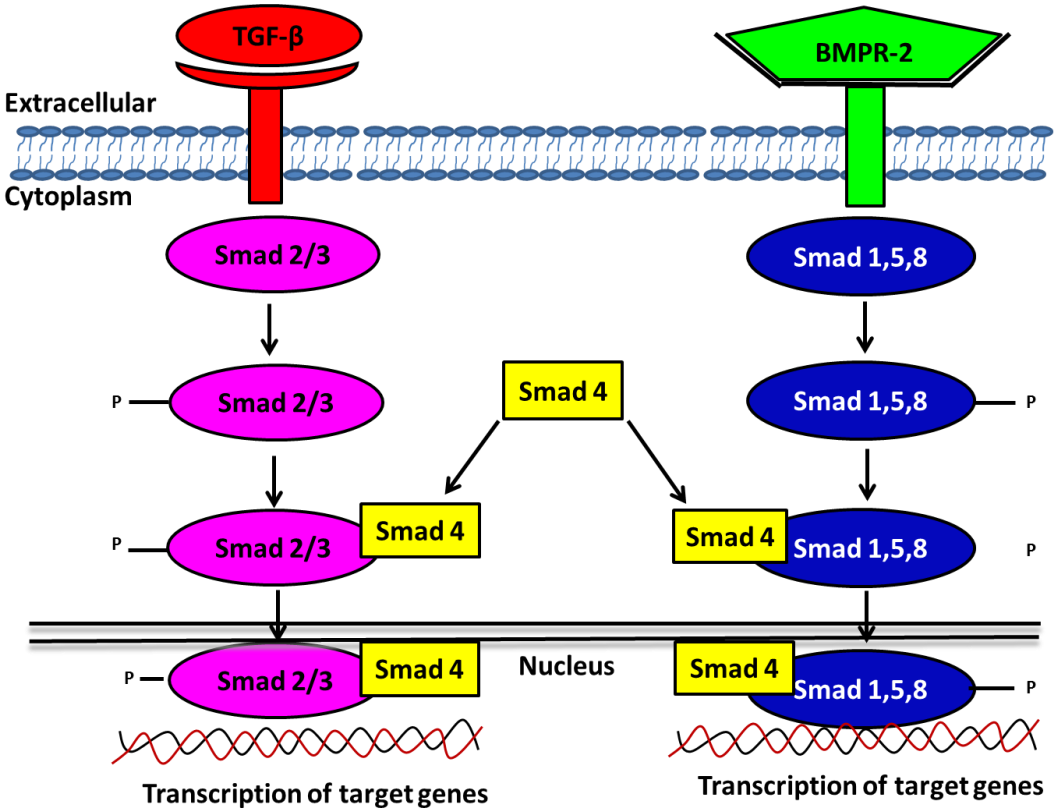


Figure 4. The significance of the TGF-β/BMPR-2 signaling axis in PH. This figure is a simplified schematic of the TGF-β and BMPR-2 signalling pathway underlying the pathogenesis of PH. TGF-β and BMPR-2 binds to their receptors respectively where TGF-β activates smad 2, 3 and BMPR-2 activates smad 1, 5 and 8 (Santibanez et al. 2011). Upon phosphorylation of these smad molecules, smad 4 is activated by both pathways, translocates to the nucleus where it promotes transcription of target genes involved in cell growth, proliferation and apoptosis. Abbreviations: **BMPR-2**: bone morphogenetic protein receptor-2, **TGF-β**: transforming growth factor-beta, **smad**: small mothers against decapentaplegia.

2.3. Pulmonary hypertension and cardiac alterations

General overview of the right ventricle: before and after pathology

Under normal circumstances, the RV is structurally, geometrically and mechanically distinct from the left ventricle (LV) [6,7]. It has a thinner wall than the LV and is half-moon shaped which reflects the low pressures in the pulmonary circulation (See figure 4). The RV also has a greater compliance compared to the LV and has an ability to adapt to changes in volume load more rapidly [66]. Furthermore, the RV is different from the LV with regards to (i) its highly trabeculated endocardial surface, (ii) presence of the moderator band and (iii) lower oxygen requirement at rest/during exercise [67]. When the RV, as in the case of PH, is exposed to the increased afterload and possibly even decreased coronary perfusion, it undergoes hypertrophy (Figure 5). This is also known as adaptive RV remodelling, as the heart attempts to adapt to the higher RV afterload and increased wall stress based on Laplace's principle [6]. However, if the increased afterload persists, the RV will eventually dilate and fail to pump blood to the lungs for oxygenation (i.e. RV failure) [6,68].

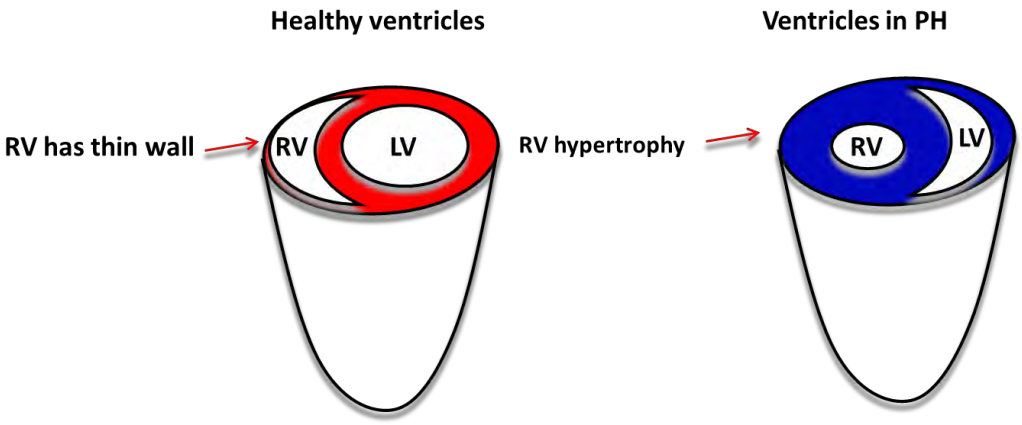


Figure 5. Ventricular remodelling in PH. A simplified and graphic representation of the thin walled RV under normal circumstances and RV hypertrophy in PH due to increased RV afterload (adapted from Voelkel et al. 2006). **RV**: right ventricle or right ventricular, **LV**: left ventricle or ventricular, **PH**: pulmonary hypertension

RV dysfunction also occur in progressed RV remodelling (hypertrophy and dilatation) and is the main determinant of prognosis of PH patients and a strong predictor of mortality [66,69,70]. However, the relationship between RV function and afterload is complex. This is often seen in patients with idiopathic PH who have low load or low pressure, but yet have a worse prognosis compared to patients with congenital heart disease with high load or high pressure and, yet, often have a better prognosis [66,69,70]. Therefore, it is not the load per se which causes death in PH but the RV failure after prolonged RV hypertrophy [69]. On the molecular level, RV hypertrophy is achieved by increased protein synthesis and increased cell size, whilst the progressive process from RV hypertrophy to RV failure is potentially modified by a variety of contributing factors [6,66]. These factors include (i) neurohormonal activation, (ii) increased reactive oxygen species (ROS) production, (iii) infiltration of inflammatory cells and disturbed balance of cell loss (apoptosis) and new cell formation (due to cardiac stem cells inherent to the myocardium), (iv) degradation of the extracellular matrix, (v)alterations in contractile and regulatory proteins, (vi) alterations in ion channels and (vii) mitochondrial defects (Figure 6) [6,69].

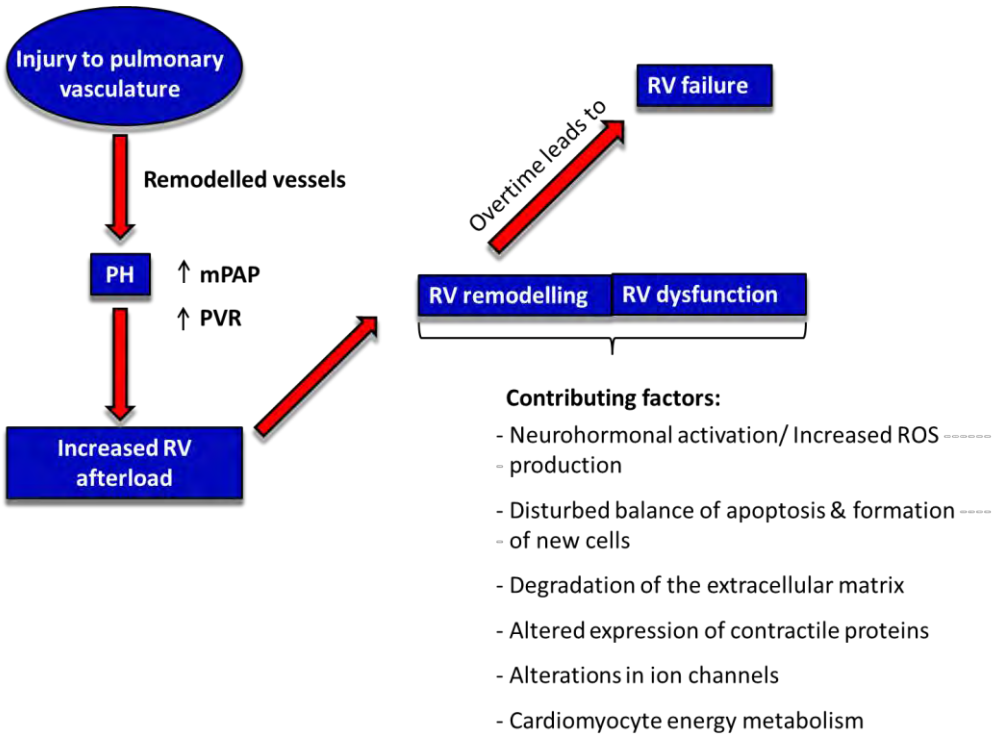


Figure 6. Factors contributing to ventricular remodelling in PH. A summary of PH induced RV remodelling and a list of factors that contribute to the remodelling process. **mPAP**: mean pulmonary artery pressure, **PVR**: pulmonary vascular resistance, **PH**: pulmonary hypertension, **RV**: right ventricle/ventricular, **ROS**: reactive oxygen species.

Neurohormonal activation in right ventricular remodelling

In ventricular failure (LV or RV) neurohormonal pathways are activated due to reduced tissue perfusion caused by decreased cardiac output to compensate for depressed cardiac function [6,69,71,72]. This may very well be the case in PH-induced RV failure when neurohormonal activation (via angiotensin-II, aldosterone, and atrial natriuretic peptide and brain natriuretic peptide) may attempt to maintain blood pressure and renal perfusion [6,69]. Furthermore, the renin-angiotensin-system has been shown to be involved in the RV remodelling process in the pulmonary artery banding animal model of pressure overload-induced RV remodelling [73,74]. This is in line with current knowledge, that with heart failure, the renin-angiotensin-system (angiotensin-II) is upregulated and directly affects cardiomyocyte growth and proliferation, myocardial fibrosis and contractile dysfunction [6]. Thus, the initial activation of neurohormonal pathways may be beneficial but eventually lead to ventricular remodelling and decreased cardiac function [6].

Increased reactive oxygen species production in right ventricular remodelling

It is known that all cells in the heart can produce reactive oxygen species (ROS) in the absence and presence of pathology [75,76]. In ventricular remodelling, ROS damages (i) proteins important for cardiac excitation-contraction coupling, (ii) mitochondrial deoxyribonucleic acid, as well as (iii) activity of hypertrophic signalling kinases, transcription factors and matrix metalloproteases [77]. These adverse effects of ROS cause cardiomyocyte apoptosis, remodelling of the extracellular matrix and impaired cardiac contractile function [77]. Furthermore, in ventricular failure, ROS suppresses enzymes involved in excitation-contraction coupling and poly-nitrosylates ryanodine receptors which also contribute to cardiac dysfunction [6]. Therefore, ROS plays an instrumental role in RV remodelling and dysfunction associated with PH.

Disturbed balance of cell loss and new cell formation in right ventricular remodelling

Cardiomyocyte apoptosis is increased in the RV of rats that underwent pulmonary artery banding-induced RV remodelling [73]. This has also been proven in animal models of heart failure due to mechanisms involving mechanical stretch, increased ROS production, pro-inflammatory cytokines and angiotensin-II release [6,73]. A rather interesting development in cardiac research is the evidence that the mammalian heart harbours clonogenic, multipotent cardiac stem cells that are able to differentiate into cardiomyocytes [6]. The mere fact that the heart has these cells suggests an inherent ability to balance cell loss with *de novo* cell production [6]. It is also possible, that, with ventricular remodelling, there may be an imbalance between apoptosis and the formation for new cells [6]. However, although an interesting notion, this area of research is still highly experimental. Other factors that contribute to RV remodelling include excessive degradation of the extracellular matrix via matrix metallo protease activity, alterations in contractile and regulatory protein expression, alterations in enzymes and ion channels involved in cardiomyocyte excitation-contraction coupling, as well as the modification of cardiomyocyte energy metabolism [6].

Degradation of the extracellular matrix in right ventricular remodelling

In PH-induced RV remodelling, there is an increase in mast cells which activates matrix metalloproteases by secreting tumour necrosis factor-alpha, tryptase and chymase that is linked to TGF-β signalling [78-80]. This leads to degradation of the extracellular matrix and increased collagen content which may exacerbate ventricular remodelling [6,78,80].

Alterations in contractile and regulatory protein expression in right ventricular remodelling

In the rat model of MCT-induced PH, it has been shown that sarco (endo) plasmic reticulum calcium-ATPase and phosphorylated phospholamban levels are increased and total-phospholamban levels decreased [81]. This was associated with impaired intracellular calcium handling. Another contributor to ventricular remodelling is alterations in the expression of transcription factors including GATA-4, Nkx-2.5/Csx, MEF-2 and the HAND family (eHAND and dHAND) [7]. In patients with idiopathic PH there is a decrease in the cardiac expression of α-myosin heavy chain and an increase in β-myosin heavy chain which is regulated by GATA-4 [7]. This is part and parcel of the phenomenon called the recapitulation of the foetal gene pattern [7]. The β-myosin heavy chain isoform has lower adenosine triphosphatase activity compared to α-myosin heavy chain and, therefore, the increase in the expression of β-myosin heavy chain impairs systolic RV function [6]. These alterations in the expression of proteins, which are imperative for myocardial function, play a key role in ventricular remodelling [6].

Alterations in ion channels in right ventricular remodelling

It has been reported, that in cardiac pathology there are alterations in the messenger ribonucleic acid expression of transient outward ion channels (such as the voltage gated potassium channels-4.2 and 4.3 and the sodium-hydrogen-exchanger-1 or NHE-1) [82]. These channels are involved in cardiomyocyte excitation-contraction coupling and the downregulation of their expression contributes to RV dysfunction in the rat model of MCT-induced PH, representative of group 1 PH. [82]. This is supported by data showing that cardiac remodelling is associated with stage dependent changes in the transient outward current and L-type calcium channel current [83]. LV remodelling is known to be dependent on sodium-hydrogen-exchange, in particular the activity of the NHE-1 [84-86]. In MCT-induced PH, the RV has increased expression of NHE-1 [84]. Collectively, this suggests that alterations in the expression of ion channels play an important role in RV remodelling.

Cardiomyocyte energy metabolism in right ventricular remodelling

In cardiomyocytes, phospholipids are situated in the mitochondrial membrane which is essential for mitochondrial biogenesis and optimal mitochondrial activity [87]. In PH, there is a reduction in the levels of these phospholipids and thus defective mitochondrial activity [87]. Cardiac tissue samples from animal models and patients with PH have increased glycolysis in RV remodelling, as opposed to fatty acid oxidation under normal circumstances [88,89]. This choice of fuel to generate energy is insufficient to maintain the increased RV energy requirement (as with RV hypertrophy) and results in impaired RV contractility [88,89].

Some of the multiple factors that possibly contribute to the development of pulmonary vascular remodelling and RV remodelling in PH have now been discussed. Furthermore, there are some other factors that also play a role in PH and these factors should be taken into consideration. These include: diagnosis of PH when the disease may already be in a far progressed stage, differences in aetiology that possibly precede or modulate PH, differential response to treatments, gender differences and varying mortality rates (Figure 7). All these factors accompany PH, perhaps varying in the extent to which they contribute, but together they prove PH to be a very complex disorder. However, the exact mechanistic interplay of these factors is not fully elucidated and the complete understanding of the pathophysiology of PH appears to evade experts at present. Therefore, due to the incomplete understanding of PH in general, fully effective treatments are also currently non-existent. Sadly, PH remains a fatal, global health threat without any cure or effective treatments.

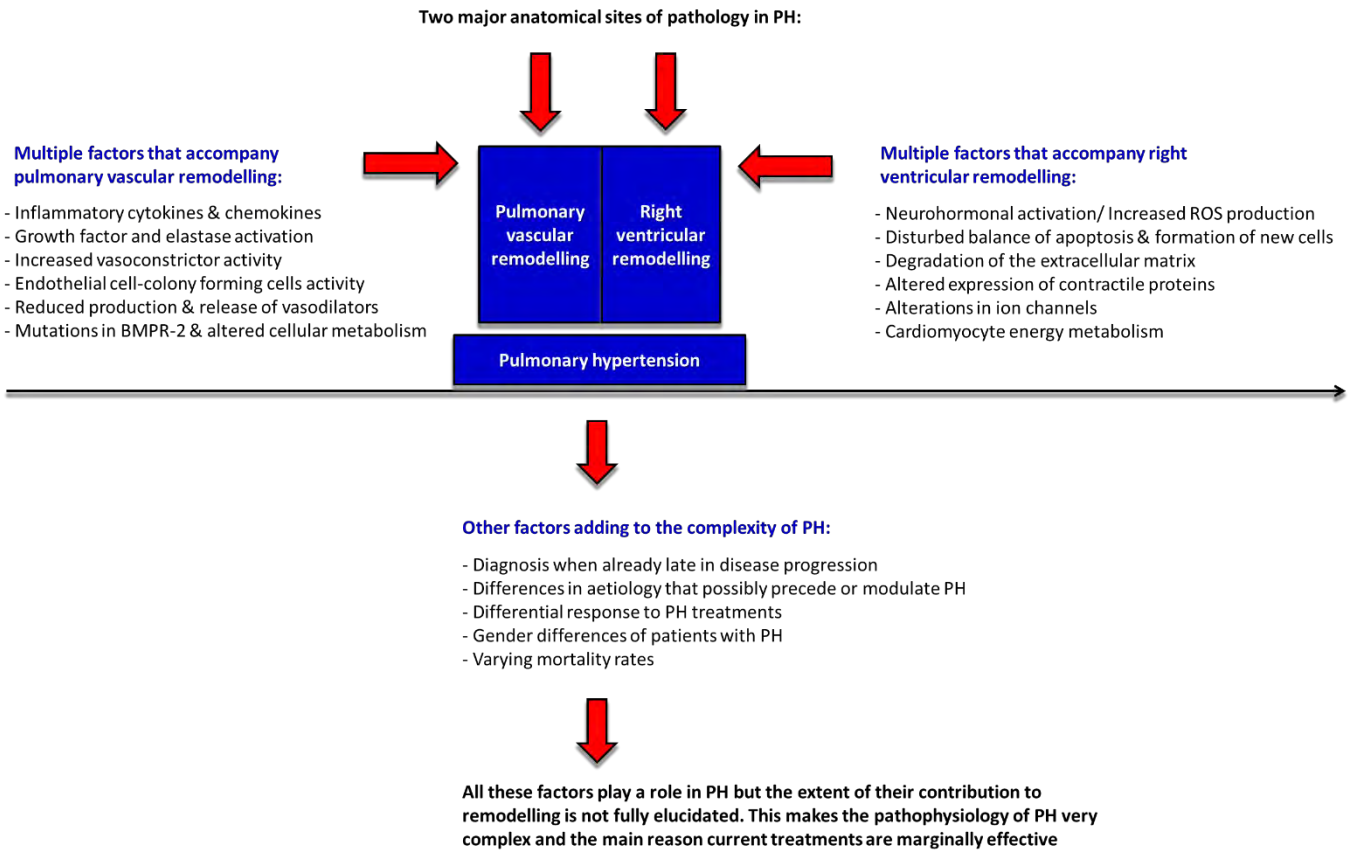


Figure 7. The complex nature of PH. A graphical representation of the two major anatomical sites of pathology in PH. This figure also outlines the multiple factors that accompanyboth pulmonary vascular remodelling and RV remodelling. This shows how complex PH is. **PH**: pulmonary hypertension, **RV**: right ventricular

3. Current treatments for pulmonary hypertension

Overall treatment for PH includes the primary treatment (digoxin, supplemental oxygen therapy, diuretics and anticoagulants) and targeted treatment (endothelin-1 receptor antagonists, prostacyclin analogues and phosphodiesterase type-5 inhibitors) [90-92]. As mentioned earlier, the pathophysiology of PH is complex as it arises due to the culmination of various pathological pathways and treatment of PH thus comprises of a variety of treatment modalities [90-92].

3.1. Primary treatments

The first step in the treatment of PH is the baseline assessment of the severity of PH, followed by treatment directed at the underlying cause of the PH and this is usually referred to as primary treatments [90]. Primary treatments include supplemental oxygen therapy, diuretics and anticoagulants [90].

Supplemental oxygen therapy

In a study with 203 patients with PH and hypoxemic COPD, supplemental oxygen therapy was shown to be beneficial with minimal improvements in the severity of PH at rest or during exercise [95]. In this particular study, the pulmonary vascular resistance (PVR), mPAP and stroke volume-index were improved after continuous oxygen therapy. Improved PVR was associated with improved cardiac function as displayed by an increase in the baseline and exercise stroke volume-index. However, the response of pulmonary haemodynamics to oxygen therapy did not allow the authors to make predictions regarding the long-term effects of supplementary oxygen therapy on patient survival [95]. Furthermore, right heart catheterization was performed in 16 patients with severe hypoxemic COPD before and after nineteen months of supplemental oxygen therapy [96]. This long term oxygen therapy, decreased mPAP and for two years after therapy, a similar decrease was observed annually. Lastly, short term supplementary oxygen therapy during right heart catheterisation in 104 patients with PH reduced heart rate, cardiac output, mPAP and PVR [97]. The heart rate in response to the short term oxygen therapy was associated with a better prognosis in patients with PH associated with chronic thromboembolism and chronic lung disease.

Collectively all these studies show that supplemental oxygen therapy has a favourable effect on pulmonary haemodynamics and a modest impact on survival in patients with PH

secondary to COPD. However, there is a lack of randomized studies that test the efficacy of long-term oxygen therapy in PH and, even though oxygen therapy is advised to correct hypoxemia, it may increase the risk of aggravated pulmonary vasoconstriction [90-92].

Diuretics and anticoagulants

Diuretics diminish hepatic congestion and peripheral oedema and are useful in treating fluid retention in patients with PH and RV failure [90]. It also improves cardiac contractility via reduction of RV preload and dilatation and is therefore an important component of primary therapy for PH [98-100]. On the other hand, anticoagulation therapy such as warfarin is often recommended to prevent thrombosis and stroke in patients with PH associated with chronic thromboembolic disease [92]. These patients are prone to thromboembolism due to tardy pulmonary blood flow, dilated RV, venous insufficiency and physical inactivity [92]. Anticoagulation therapy is crucial in such patients and has also been shown to prolong life in patients with idiopathic PH [92,101]. Apart from treating the underlying cause of PH, targeted treatment should be started promptly. Very often patients with idiopathic PH either don't respond to primary treatment or the PH may already be too severe. In such cases targeted treatment is mandatory (Figure 8).

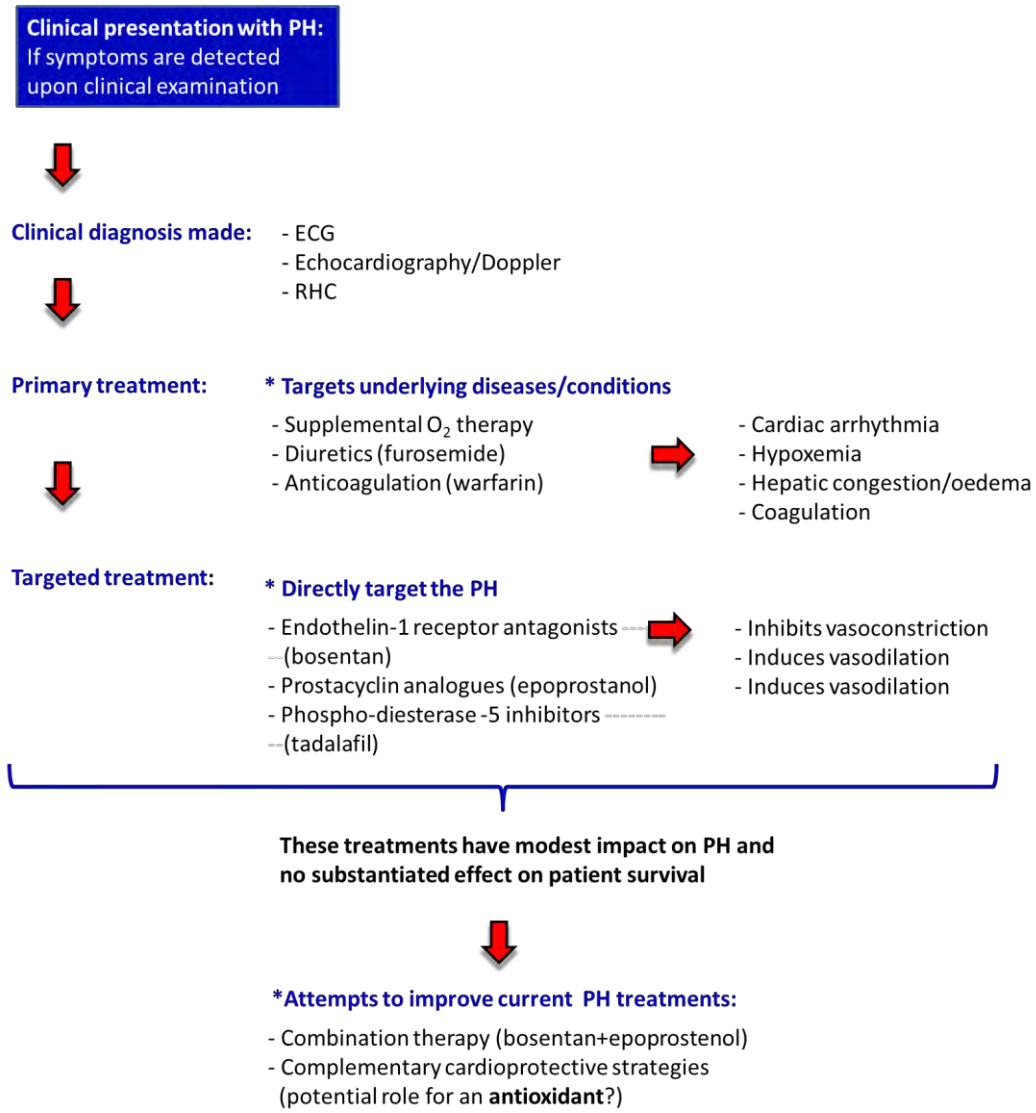


Figure 8. Treatment strategies against PH. A schematic of how the various treatment strategies of PH are employed from the point of clinical examination to advance treatment options in severe cases. The figure display the fact that current treatments for PH is only modestly effective with no impact on patient survival. Attempts to improve current treatment strategies are being made and these includes antioxidant therapy with a potential role for melatonin. **ECG**: electrocardiogram, **RHC**: right heart catheterization, **O_2**: oxygen, **PH**: pulmonary hypertension

3.2. Targeted treatments

Targeted treatments are the second step in treating PH, when PH is either unresponsive to primary treatment or present at a severely progressed stage. These treatments include endothelin-1 receptor antagonists, prostacyclin analogues, and phosphodiesterase type-5 inhibitors) on which ample of research has been done over the years [90-92].

Endothelin-1 receptor antagonists

Endothelin-1 is a peptide that regulates vascular tone by modulating signalling via endothelin receptor-A and B which are located on PASMC's [44]. Activation of receptor-A cause PASMC proliferation and vasoconstriction while activation of receptor-B induces nitric oxide and prostacyclin production from pulmonary endothelial cells [99,100,102]. Both PASMC proliferation and vasoconstriction play a key role in PH, therefore endothelin-1 receptor antagonists have been developed as a treatment for patients with PH [33,90,99,103]. There are various endothelin-1 receptor antagonists, including bosentan (a dual receptor antagonist), ambrisentan and sitaxentan (which are selective receptor antagonists) [33,99,103,104]. In a retrospective data analysis in 82 PH patients with end-stage heart failure, 54 received oral bosentan treatment twice a day (for a period of fifteen months), while the remaining 28 patients received standard medical treatment [105]. Bosentan reduced mPAP and PVR compared to the control patients. Interestingly, the authors noted that after one year of treatment, the survival was approximately 20% higher in the bosentan-treated group [105].

In a randomized placebo-controlled study by Channick and colleagues, 33 PH patients were randomly assigned to placebo or bosentan treatment [106]. The starting dose of bosentan was 62.5mg twice daily for four weeks, which was then up titrated to 125mg twice daily for twelve weeks. Patients who received bosentan treatment displayed a dramatic increase of 76 metres walking distance during the six-minute walk test (a measure of exercise capacity and severity of PH). This was associated with improved cardiac output, PVR and mPAP [106]. Furthermore, in a one-year follow up study, 213 PH patients received bosentan treatment at a dose of 62.5mg twice daily for four weeks and thereafter 125mg or 125mg twice daily for one year [107]. Bosentan increased the walking distance during the six-minute walk test to 44 metres and improved the time to clinical worsening [107]. In a recent publication, bosentan displayed similar benefit with longevity of benefit for up to 4 years after treatment [108]. All these studies show that bosentan treatment is effective in improving cardiopulmonary haemodynamic parameters and endpoints such as the six minute walk distance.

The selective endothelin receptor antagonists include ambrisentan and sitaxentan [99]. Ambrisentan increased the six minute walk distance and decreased circulating brain natriuretic peptide levels in 224 patients with PH associated with various diseases [109]. Which was corroborated by data that ambrisentan provided modest benefit in patients with idiopathic PH [109]. In two randomized, double- blind, placebo-controlled studies, the so called ARIES-1 and ARIES-2 trials, ambrisentan treatment was tested in 202 and 192 patients with PH [104]. In the ARIES-1 study, patients were assigned to placebo or oral ambrisentan treatment respectively (5 or 10mg) and in ARIES-2 study, doses were 2.5 or 5mg daily for 12e weeks. The six minute walk distance increased in all ambrisentan treated groups, with approximately 20 metres. Further improvements were seen in the functional class, dyspnoea score and natriuretic peptide-type B levels. Thus, ambrisentan modestly improved the exercise capacity of these patients [104]. Furthermore, in a single centre cohort study with 17 patients with PH associated with congenital heart disease, ambrisentan improved exercise capacity but had no convincing effect on functional class [110]. Lastly, D'Alto and colleagues conducted a single centre, non-placebo controlled study during which they investigated the long term effects of ambrisentan treatment in patients with PH [111]. Ambrisentan treatment for 12 months improved the six minute walk distance, functional class and cardiopulmonary haemodynamics. These improvements were associated with a reduction in the pro-natriuretic peptide type-B levels [111]. It is thus clear, that ambrisentan provides cardiopulmonary benefit similar to bosentan however, the other selective endothelin receptor antagonist, sitaxentan, generated very interesting results.

Sitaxentan is a very potent endothelin receptor antagonist with a long duration of action [112]. In a study by Barst, patients with idiopathic PH were randomized to placebo, oral sitaxsentan 100mg or oral sitaxsentan 300mg once daily for 12 weeks [113,114]. Both the 100mg and 300mg dose increased six minute walk distance; functional class, cardiac index and PVR, compared to placebo [113,114]. Similar results were generated in other studies that tested the effects of sitaxentan in patients with PH [115-120]. However, in the study by Barst et al, they noticed that the incidence of elevated aminotransferase values were three times higher than normal and this appeared to be dose dependent [113,121]. Although this finding was not given much thought at the time, in retrospect, it was a subtle indication of liver toxicity associated with sitaxentan treatment. In 2012, Chin and colleagues published a case report of a 61-year old female with sitaxentan-induced acute liver failure [122]. This was further corroborated by evidence of a similar toxic effect of sitaxentan in 7 cases of severe hepatitis-type drug reactions associated with sitaxentan [114,123-126]. Consequently, sitaxentan was withdrawn from the market.

Prostacyclin analogues

Epoprostenol was the first prostacyclin analogue used in the treatment of PH [127]. In a randomized, open label, controlled trial, intravenous epoprostenol treatment (12 weeks) improved exercise capacity, haemodynamics, functional class and dyspnoea scores in patients with moderate/severe PH [128]. In a separate study, patients with primary PH received poprostenol infusion for 3 months and those with a history of RV failure, persistent functional class two/three and the absence of a decrease in the PVR were all associated with poor survival [107]. In these studies, epoprostenol improved certain endpoints but it has a very short life and thus treprostinil has been considered as a better option [129].

In a study by Gomberg-Maitland, 27 patients were switched from intravenous epoprostenol to intravenous treprostinil over 24 to 48 hours, for a period of 12 weeks [129]. At week 12, the six minute walk distances and dyspnoea scores were maintained with intravenous treprostinil. These results were associated with decreased PVR, mPAP and cardiac index. This suggested that the transition from intravenous epoprostenol to intravenous treprostinil is safe and effective [129,130]. The bulk of data indicate that treprostinil may be a first line treatment for patients with PH [130]. Similar success has been shown with other prostacyclin analogues such as beraprost and iloprost [131,132].

Phosphodiesterase type-5 inhibitors

One of the most well-known phosphodiesterase type-5 inhibitor is sildenafil and its efficacy in PH well documented, for its both oral and intravenous forms [133,134]. In a double-blinded, placebo-controlled study of 278 idiopathic PH patients, oral sildenafil treatment (20, 40, or 80mg, three times daily for a period of 12 weeks) increased six minute walk distance, reduced mPAP and improved functional class [135]. Furthermore, in an open label pilot study 14 children with primary and secondary PH were assigned to oral sildenafil treatment at a dose of 0.25 to 1mg/kg four times daily [136]. After treatment, the mean distance walked increased, while both the mPAP and PVR decreased [136]. Similarly, oral sildenafil (75mg/day for 12 weeks) increased the six minute walking distance and improved dyspnoea scores; functional class and cardiopulmonary haemodynamics [137].All of these studies corroborate the efficacy of sildenafil as treatment for PH.

Another phosphodiesterase type-5 inhibitor is tadalafil [138]. In a double-blind, 52 week, uncontrolled extension study, 293 PH patients received tadalafil treatment at doses 20mg or 40mg [138]. Long term tadalafil treatment improved the six minute walk distance and functional

class. Aggarwal and colleagues conducted a prospective study with thirteen PH patients treated with tadalafil (10mg for ten weeks and thereafter up titrated to 20mg for two more weeks) [139]. After 4 weeks of therapy, all patients had improved exercise capacity, functional class, haemodynamic parameters and mPAP. The PVR and pulmonary blood flow were also improved. Lastly, in a 16 week, double-blind, placebo controlled study, 405 patients with idiopathic PH were randomized to placebo or oral tadalafil treatment at doses of 2.5, 10, 20, or 40mg once daily [16]. Tadalafil increased the six minute walk distance in a dose dependent manner while the highest dose of tadalafil (40mg) improved the time to clinical worsening, incidence of clinical worsening and quality of life. Tadalafil appears to be an effective and a safe treatment for patients with PH by improving exercise capacity, clinical status, haemodynamic parameters and quality of life [140].

3.3. Efficacy of current treatments against pulmonary hypertension

Despite a plethora of research displaying positive results and the significant advancements that have been made in the treatment of PH in the last 12 years, the disease remains fatal [141,142]. As seen in the above mentioned studies, all the targeted treatments strategies, with the exception of tadalafil, show modest improvement in endpoints such as clinical status and patient survival, together with cardiopulmonary haemodynamic benefit. Therefore, current treatments are only successful to a certain extent but do not provide a permanent reduction of mPAP or reversal of pulmonary vascular remodelling [143-147]. Furthermore, there is a lack of adequately powered, multicentre studies that show the effects of treatment on patient survival, after long term treatment and for these reasons there is a great need for either an improvement of current PH treatments or a cure [6,148]. In the last decade, there has been an increasing amount of research studies which, if carefully studied, display certain trends in treatment strategies. These trends comprise various ways to improve the limited impact of current treatments on PH and patient survival.

3.4. Novel trends pulmonary hypertension treatment research

3.4.1. Combination therapy

One of the most prominent trends in PH research is the combination of various existing PH treatments. The first rationale for combination therapy is based on the hope of achieving greater and longer lasting benefit with PH treatment [16]. The second is based on the fact that several pathological pathways are involved in the pathogenesis of PH and, thus, combination therapy has the benefit of targeting multiple pathways simultaneously [16]. Many studies have emerged that support this concept of combination therapy. One such study is the so called BREATHE-2 trial (bosentan randomised trial of endothelin antagonist therapy for PH trial-two) [99,104,149].

The BREATHE-2 trial was a randomized, double-blinded, placebo-controlled study that investigated the effects of combined of bosentan and epoprostenol in patients with severe PH [99]. In this trial, 33 patients received epoprostenol treatment at doses of 2ng/kg/min (starting dose), up to 14ng/kg/min (at week sixteen). Patients were then randomized for bosentan treatment (62.5mg twice daily for 4 weeks and thereafter 125mg) or placebo. Thus, there were two groups; one group that received the placebo plus epoprostenol and another group that received bosentan combined with epoprostenol. After 16 weeks, the exercise capacity, haemodynamics and functional class improved in both groups. The group that received the combination therapy showed a trend for a greater improvement in all measured haemodynamics but this was not statistically significant. The BREATH-2 trial data gave some indication of a possible greater clinical benefit with combination therapy although not statistically significant at the time [99]. Other smaller studies, which were not blinded or placebo controlled, corroborated this notion.

In one such retrospective, single centre study, 7 patients with PH received continuous inhalation of nitric oxide (10ppm) and iloprost (10µg) [150]. Combined treatment decreased PVR and mPAP which was associated with an improvement in RV function. In a study by Hoeper, a combination of bosentan and sildenafil improved the six minute walk distances in 9 patients with severe idiopathic PH [151,152]. However, this was not sustained and, after 12 months, the walk distance declined. At this point, sildenafil was administered in addition to bosentan treatment. After an additional 3 months, the six minute walk distances increased and patients remained stable for the following 9 months [151,152]. Similar success was reported with a combination of prostacyclin and tadalafil [153]. However, in another study, PH patients who underwent valvular heart surgery did not show any improvement after combination

therapy with oral sildenafil and beraprost [154]. In this particular study, combination therapy was administered 15 minutes prior to heart surgery and resulted in loss of pulmonary selectivity and no additional pulmonary vasodilation or favourable perioperative haemodynamics. Nevertheless, in retrospect, the concept of combination therapy is plausible but needs to be corroborated by larger, randomized, placebo-controlled, multicentre studies [155-157].

3.4.2. Complementary cardioprotective strategies for pulmonary hypertension

Another trend in PH treatment research is the investigation of possible cardioprotective treatments that may protect the RV in PH. In theory, such a cardioprotective strategy can be given complementary to existing PH treatments and may further improve the quality of life and survival of patients with PH (See figure 8) [6,7,69,158]. This type of cardioprotective or RV protective treatment is even more relevant, as RV function is the main predictor of mortality in patients with PH [66,69,70].

Many studies indicate that RV remodelling in PH is similar to that of the LV with regards to energy substrate utilization, beta-adrenergic receptor density and cardiomyocyte growth response [88,159-162]. Therefore, because RV remodelling potentially shares certain pathophysiological mechanisms with LV remodelling it can, in theory, be treated with therapies that have been successful in treating LV remodelling [67,69,163]. However, more research is needed before such treatments can officially be implemented in patients with PH-induced RV remodelling [67,69,70].

Unfortunately, due to such a small number of studies that investigate complementary cardioprotective strategies, one is obliged to revisit data from LV remodelling studies and constantly draw parallels between RV protective treatments and those for LV remodelling. Based on our current knowledge of LV remodelling, a variety of signalling pathways are activated to initiate this remodelling process [164,165]. These pathways are well studied in LV pathology, but whether they are involved in RV remodelling, to the same extent, is not completely elucidated [7,70]. There are a number of possible cardioprotective treatments or therapeutic targets including cardiomyocyte apoptosis, epigenetic control of cardiac gene transcription, cardiac gene expression and micro-RNA's (miRs), nuclear factor kappa-B modulation of notch signalling, energy metabolism of RV cardiomyocytes, the sodium-hydrogen-exchanger-1 and cardiac resynchronization therapy (Figure 9).

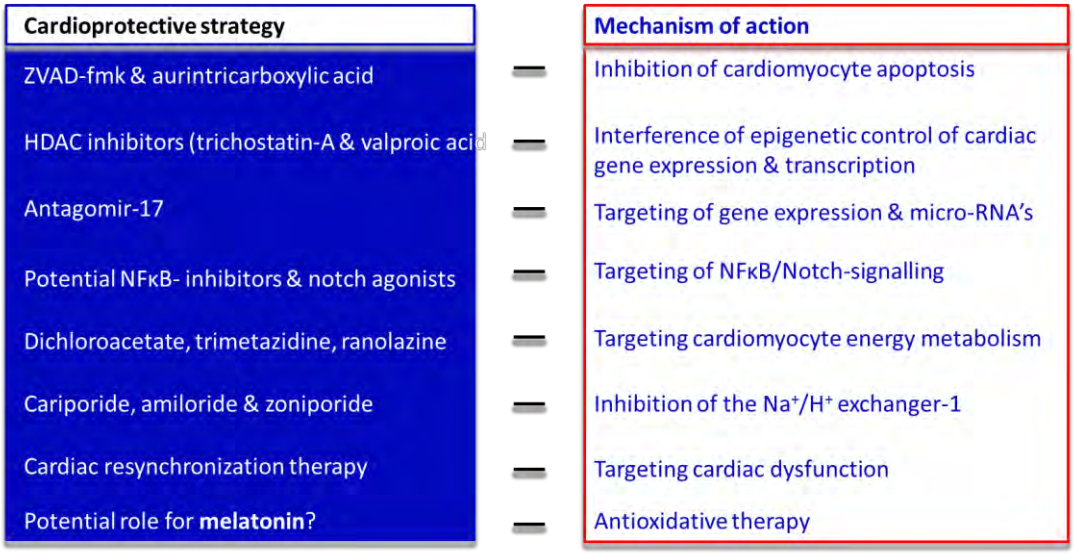


Figure 9. Potential complementary cardioprotective strategies in PH. A summary of potential cardioprotective strategies that may protect the right ventricle in PH. The rationale is that cardioprotective therapies may improve survival with PH if administered as a complementary therapy to current PH treatments. **ZVAD-fmk**: z-val-ala-asp(ome)-CH_2K, **HDAC**: histone deacetylase, **NFκB**: nuclear factor kappa-B, **RNA**: ribonucleic acid, **Na^+**: sodium, **H^+**: hydrogen, **PH**: pulmonary hypertension

Inhibition of cardiomyocyte apoptosis

With LV failure, cardiomyocyte apoptosis is increased which is triggered by activation of G-protein-coupled receptors, cytokines and increased ROS [165]. Because of the regulated and ordered nature of apoptosis, it is possible to intervene at an early stage and prevent it [165,166]. Many proteins are implicated in apoptosis with caspases being the most prominent and important [166 ,167]. Z-Val-Ala-Asp (Ome)-CH2K (ZVAD-fmk) is a tripeptide inhibitor of the caspase-interleukin-1β-converting enzyme family of cysteine proteases [166] . In a rat model of ischemia/reperfusion injury, ZVAD-fmk (3.3mg/kg) reduced myocardial ischemia/reperfusion injury associated and partially attenuated cardiomyocyte apoptosis [166] .

Another apoptosis targeting agent is aurintricarboxylic acid, an inhibitor of endonucleases which is situated downstream in the apoptotic pathway and facilitates DNA strand breaks [168,169]. Aurintricarboxylic acid has been shown to reduce the number of necrotic cells in the perinecrotic region of the damaged myocardium in a dog model of ischemia/reperfusion [168,169]. Furthermore, mitofusion-2, a factor that is a major determinant of oxidative stress, mediates cardiomyocyte apoptosis and is thought to be beneficial in overt heart failure [170]. In a rat model of MCT-induced PH and right ventricular remodelling, rat hearts have been shown to have increased RV cardiomyocyte apoptosis [171]. Ecarnot-Laubriet, showed that in the same model, the RV is vulnerable to apoptosis with concomitant activation of the cell

cycle [162]. In this study, RV cardiomyocyte apoptosis was evident with an increased expression of caspase-3 and down regulation of the anti-apoptotic protein B-cell lymphoma-2 (bcl-2) [162]. Taken together, these data suggest that inhibition of various steps in the apoptotic signalling pathway may be a therapeutic target in developing a RV protective treatment in PH as apoptosis play an important role in ventricular remodelling.

Interference with the epigenetic control of cardiac gene transcription

A fairly novel approach in PH research is the pharmacological inhibition of histone deacetylases (HDACs) [141,172,173]. HDACs are enzymes that add acetyl groups to histone proteins and cause relaxation of the chromatin structure, enhances the accessibility of histone proteins to DNA binding as well as transcription [172,174]. HDACs have a regulatory function in ventricular remodelling and can be suppressed with HDAC inhibitors (such as trichostatin-A and valproic acid) to attenuate LV remodelling [172,175]. Bogaard et al investigated whether interference with the epigenetic control of gene transcription, by means of HDAC inhibitors, may also attenuate RV hypertrophy [148].

Male rats were subjected to pulmonary artery banding surgery to constrict the main pulmonary artery; increase RV afterload and cause RV hypertrophy [69,88,148]. Four weeks after surgery, when RV hypertrophy was evident, trichostatin-A treatment was initiated (450µg/kg intraperitoneal, 5 times per week for a period of 2 weeks). Interestingly, trichostatin-A was ineffective in attenuating RV dysfunction and decreased the mRNA expression of phosphorylated protein kinase-B, vascular endothelial growth factor, angiopoeitin-1 and insulin-like growth factor-1. Furthermore, trichostatin-A significantly decreased the endothelial nitric oxide synthase gene expression [148]. The authors did not know the reason for this but speculated that the rats developed RV dilatation after the pulmonary artery banding surgery that was associated with capillary rarefaction. This may have caused RV ischemia and hypoxic stabilization of hypoxia inducible factor-1-alpha expression [148].

Another HDAC inhibitor, valproic acid, has been shown to block RV hypertrophy in both the pulmonary artery banding and monocrotaline models of RV failure [176]. However, valproic acid is a nonspecific inhibitor of HDACs and with a wide range of pharmacological actions such as regulation of ion channels, expression of glycogen synthase kinase-3β and mitogen activated protein kinases [177]. Therefore, due to the nonspecific nature of valproic acid, these results should be taken with caution [177]. On the other hand, selective class-1 inhibition of HDAC's attenuates RV hypertrophy in the chronic hypoxia model of PH [173]. Benzamide, a selective

HDAC inhibitor (MGCD0103) decrease caspase-3/7 activity; interleukin mRNA expression (IL-1β, IL-2) and reduce RV hypertrophy [173]. However, HDAC inhibitors need further investigation as potential RV protective treatments but remain attractive targets for RV remodelling in PH.

Gene expression and micro ribonucleic acids

Micro-RNA's (miR's) are short, noncoding ribonucleic acids that regulate gene expression at posttranscriptional level [165,178]. They are activated by cellular stress signals and their regulatory function is achieved by inhibiting protein translation or promoting the degradation of target messenger-RNA's [165,178-180]. MiR's are highly conserved and involved in biological processes such as cardiovascular development and pathology [165,178-181]. More specifically, they have important functions in the regulation of cardiomyocyte apoptosis, differentiation and proliferation [182]. In heart failure, a number of miR's are either up or down regulated, including miR-17, miR-20a and miR-24 [165,178,182,183]. In 2007, Care at el, published a study where they elegantly showed the instrumental role of miR's in 3 different models of LV hypertrophy, including transverse aortic constriction, transgenic mice with selective cardiac overexpression of Akt kinase [184]. Here, overexpression of miR-133 inhibited LV hypertrophy while antagomir-133 caused sustained LV hypertrophy. This was suggestive of the regulatory role of this specific miR in LV remodelling. Furthermore, In vivo antagomir silencing of miR-24 were done in mice with LV hypertrophy after aortic constriction [185]. MiR-24 silencing prevented the transition to decompensated cardiac hypertrophy. In the chronic hypoxia (representative of group 2 PH) and MCT models of PH (representative of group 1 PH), inhibition of miR-17 with the antagomir-17 improved lung and heart function [182]. Furthermore, antagomir-20a significantly reduced RV hypertrophy in a mouse model of chronic hypoxia induced pulmonary vascular remodelling and RV hypertrophy [186]. Understandingly, the miR's gained much attention as novel therapeutic targets for cardiovascular disease due to their cardiac specific nature and therefore they may also be applicable in the development of RV protective treatments for PH [165,181].

Nuclear factor kappa-B modulation of notch signalling

Nuclear factor kappa-B (NFκB) is a key modulator of the notch signalling pathway and has been shown to be involved in the development of PH [187]. This is a complex juxtacrine signalling pathway initiated by interaction between the notch transmembrane receptors (notch1-4) and their ligands (jagged-1 and 2) [188,189]. Notch signalling is widely implicated in cardiovascular development and pathology [188-191]. In a study by Gude et al, mice were subjected to coronary artery ligation and an adenoviral vector expressing notch intracellular domain, was injected into the myocardium after coronary artery ligation [190]. This improved haemodynamic function of infarcted mice hearts with enhanced Akt-signalling, induced by notch activation [190].

The NFκB /notch signalling pathway has been shown to be involved in RV remodelling in transgenic mice over expressing a cardiac specific dominant-negative-IκB gene [187]. These mice were in subcutaneously injected with MCT (60mg/kg) and transgenic over expression of the IκB gene inhibited myocardial NFκB activation [187]. The inactivation of NFκB prevented RV hypertrophy despite the presence of PH [187]. This was associated with a reduced activation of NFκB genes, restoration of the expression of bone morphogenetic protein (BMP) signalling molecules and alteration in notch and smad signalling [187]. These changes were observed in cardiac and in lung tissue. Activation of NFκB, decreased the expression of notch-3, BMP-2 and BMPR-2, inhibitory of differentiation (Id), Smad-2 & 8, increased Smad-4 expression, as well as RV hypertrophy [187]. These observations were abolished in the transgenic mice with inhibited cardiac NFκB activation [187]. Collectively, these studies suggest that pharmacological modulation of the NFκB /notch signalling axis may be useful in developing a RV protective treatment for PH.

Cardiomyocyte energy metabolism

When cardiomyocytes are exposed to decreased oxygen levels they shift their energy metabolism from oxidative phosphorylation to glycolysis and reverse this shift when oxygen levels return to normal [159,192-195]. In response to pressure overload, cardiomyocytes develop a metabolic phenotype known as the glycolytic shift characterized by increased glycolysis [88,196]. The general concept holds that increased glucose oxidation might ameliorate cardiac function [159,192-195]. In 2000, Wambolt, subjected rats to suprarenal abdominal aortic constriction in order to induce cardiac hypertrophy [197]. The hearts were isolated and subjected to 20 minutes of global ischemia and 60 minutes reperfusion. Throughout the

perfusion protocol, the hearts were perfused with a buffer containing dichloroacetate. In this study, dichloroacetate improved the function of the hypertrophied hearts.

Furthermore, Piao et al, showed that promotion of glucose oxidation with dichloroacetate partially prevented RV hypertrophy in the MCT and pulmonary artery banding animal models [88]. Dichloroacetate treatment increased the expression of glucose transporter-1, pyruvate dehydrogenase and restored the expression of the voltage gated potassium channels (Kv1.5 and Kv4.2) [88]. This led to increased RV glucose oxidation, cardiac work, improved RV repolarisation and RV function [88]. It has also been shown that partial inhibition of fatty acid oxidation with trimetazidine and ranolazine increased cardiac output and exercise capacity in the pulmonary artery banding model [198]. However, although these pharmacological treatments are effective, one should bear in mind that they are not cardiac specific and may have widespread systemic effects [196]. Nevertheless, these studies suggest that dichloroacetate, trimetazidine and ranolazine may be cardioprotective targets in PH induced RV remodelling.

Inhibition of the sodium-hydrogen-exchanger-1

The sodium hydrogen exchanger-1 (NHE-1) is an integral membrane glycoprotein, ubiquitously expressed in mammalian cells and responsible for the removal of intracellular protons at the expense of the sodium ions [85,86,199]. It is regulated by hormonal, paracrine/autocrine regulators, ribosomal-S-6-kinase and mechanical stretch [181,199,200]. The involvement of NHE-1 in the cardiac hypertrophic response is thought to be initiated by mechanical stretch that stimulates NHE-1 activity via the activation of kinases such as phoshokinase-C or mitogen activated protein kinases [199]. The pro-hypertrophic activity of NHE-1 also involves calcineurin, calmodulin-dependent-kinase-II and ROS [199]. Furthermore, the inhibition of phosphorylation dependent NHE-1 activation in cardiomyocytes has been shown to occur via increased dephosphorylation by the catalytic subunit of protein phosphatase-2-A [199,201].

NHE-1 has been implicated in cardiac remodelling and evidence suggests that inhibitors of NHE-1 are beneficial against cardiac pathology [85,86,199]. Such inhibitors include cariporide, amiloride & zoniporide and have been widely tested in LV remodelling [202,203]. Seven days after left coronary artery ligation in female rats, cariporide treatment was commenced and continued for 52 days [204]. Interestingly, cariporide treatment did not affect hypertrophy post infarction but did improve cardiac contractility. In another study, rabbits were subjected to

myocardial infarction and received cariporide treatment for nine weeks [205]. Here cariporide improved LV dilatation and improved cardiac function. The anti-hypertrophic effects of cariporide treatment were more outspoken in a study where it attenuated LV hypertrophy and improved cardiac function in aortic constriction mice [206].

Seven days of pre-treatment with cariporide in the MCT model of RV failure attenuated RV hypertrophy compared to the hearts of MCT treated rats [84]. Cariporide also reduced the cardiac response to pulmonary vascular damage and the beneficial cardiovascular effects were independent of the pulmonary vascular system [84]. In general, the cardioprotective effects of this form of NHE-1 inhibition are thought to be based on two mechanisms, including the prevention of cytosolic sodium and calcium overload and the prevention of mitochondrial permeability transition pore opening [207]. However, even though the exact mechanisms are not fully elucidated, the bulk of evidence suggests that inhibition of the NHE-1 has therapeutic potential in RV protection in PH.

Cardiac resynchronization therapy

Increased RV afterload prolongs RV contraction which eventually leads to uncoordinated contraction and RV systolic dysfunction [208]. Cardiac resynchronization therapy is a well-established treatment for LV dyssynchrony related to LV failure in patients [209]. Follow-up examinations in patients with LV remodelling/systolic dysfunction revealed that cardiac resynchronization therapy reduced morbidity and mortality [209]. Handoko et al, investigated whether RV pacing would resynchronize the generation of pressure across the septum and thereby improve RV systolic function in the MCT model [208]. Rats were injected with MCT and subjected to RV pacing that was achieved by direct stimulation of the RV free wall with an electrode [208]. Pacing improved RV systolic function and resynchronized the time of RV and LV peak pressure. It had no detrimental effects on LV function or coronary perfusion. Although this technique is not of pharmacological nature, it can also be seen as a candidate treatment for the adverse effects of PH on the RV. However, further experimental testing is required.

In retrospect, it is clear that taking advantage of the similarities between the LV and RV may help to elucidate the mechanisms involved in PH induced RV remodelling and to develop RV protective treatments. A number of studies have shown benefit by targeting various aspects of the ventricular remodelling process. However, in the few available experimental studies, the benefit seen with some RV protective treatments may be due to a dual effect on both the heart and pulmonary vasculature and possibly other organs.

3.4.3. Targeting oxidative stress as a potential treatment for pulmonary hypertension

Another trend in PH treatment research is targeting oxidative stress, which is a very important factor in the pathogenesis of PH [30,210,211]. Oxidative stress is characterized by the increased production of oxidants such as ROS (including superoxide anion radical, hydrogen peroxide and hydroxyl radical) and/or decreased levels of antioxidants or antioxidant enzymes (including superoxide dismutase, catalase and glutathione peroxidase) [210]. It is well known that diseases such as PH enhance the production of ROS and diminish antioxidant activity which results in oxidative damage to crucial cell components such as DNA, proteins and lipids [211]. Oxidative damage elevates the breakdown products of these oxidative processes which can be detected in plasma, serum or urine samples of patients with PH [211]. Breakdown product includes malonic dialdehyde, F2-isoprostane, 8-hydroxyguanosine, protein carbonyl content and nitrotyrosine (Figure 10).

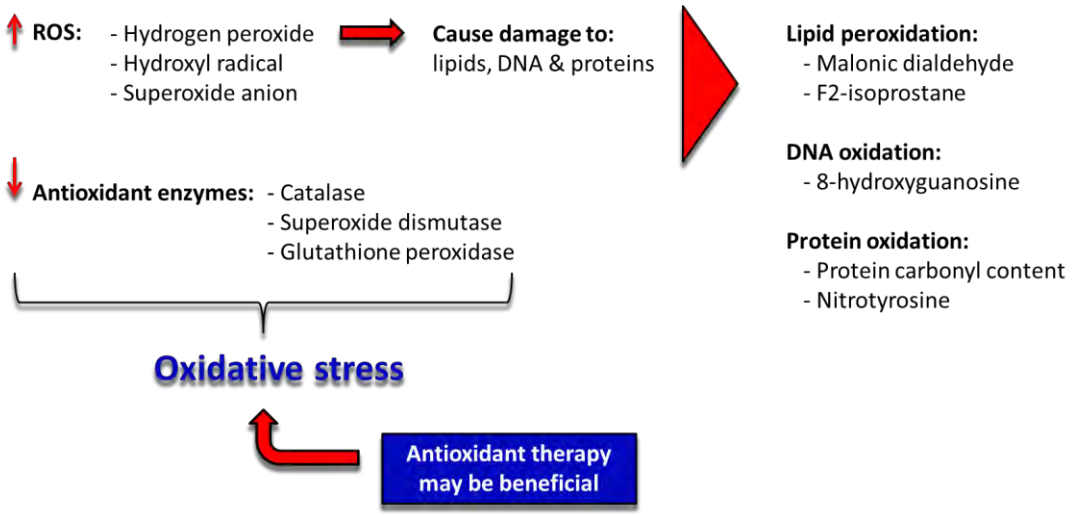


Figure 10. Reactive oxygen species, oxidative stress and a potential role for antioxidants. Reactive oxygen species and their damage they do to cellular lipids, DNA and proteins. A reduced expression and activity of antioxidant enzymes and increase ROS production leads to oxidative stress. Therefore a potential role for antioxidant therapy in PH induced oxidative stress. **ROS**: reactive oxygen species, **DNA**: deoxyribonucleic acid.

In PH, oxidative stress is believed to be involved in the initial "trigger" stages of the disease development where it causes endothelial injury in the pulmonary vasculature [30,210,211]. It has also been reported that all cells in the lung can generate ROS and this production is directly mediated by alveolar macrophages via nicotinamide adenine dinucleotide phosphate-(NADPH)-oxidase [212]. Therefore, oxidative stress is an integral part of the development of PH, as evident in both animal models of PH and patients with PH [211,213-216].

Oxidative stress in animal studies

Rats with MCT-induced PH and left pneumonectomy have increased NADPH-oxidase-4 and oxidative stress-induced lipid peroxidation as seen in the elevated plasma levels of malonic dialdehyde and nitrotyrosine (which are breakdown products of lipid peroxidation) [217]. Interestingly, the levels of the antioxidant enzyme, catalase, were decreased while superoxide dismutase and glutathione peroxidase-1 were elevated [217]. Authors speculated that these findings suggested possible accentuated attenuation of the biological antioxidant defence system [217]. In another study, Mohammadi, showed that MCT rats have decreased plasma levels of catalase, superoxide dismutase and glutathione peroxidase and increased malonic dialdehyde levels [218].

Furthermore, MCT rats also have increased levels of other markers of oxidative stress (such as hydrogen peroxide and thioredoxin reductase activity) in the RV, but not the LV [219]. These results were challenged when Leichsenring-Silva and colleagues showed that oxidative stress was increased in the LV of rats injected with MCT [71]. It is possible that the contradictive results of these studies may be due to the doses of MCT (80mg/kg versus 50 mg/kg) or the site of injection (subcutaneous versus intraperitoneal). It is known that pathological remodelling of the RV also affects the LV with regards to contractility and ejection fraction [220]. Furthermore, in both the LV and RV it has been shown that during ventricular remodelling, ROS production was increased, which led to the generation of nitrotyrosine residues in tissue inhibitor of metalloproteinases and activation of matrix metalloproteinases [221]. All these studies suggest that oxidative stress is increased in animal models of PH. Demarco

Mitochondria are major sites for the production of ROS and particularly mitochondria from endothelial, smooth muscle, and adventitial cells. [211, 222] They modulate the oxygen demand of cells because they consume approximately 90% of cellular oxygen [222]. In brief, mitochondrial energy substrates (such as fatty acids) are catalysed to acetyl coenzyme-A, which is further reduced to reducing equivalents nicotinamide adenine dinucleotide and

flavin adenine dinucleotide-2. These reducing equivalents function as carriers of electrons while oxygen serves as a terminal electron acceptor in the electron transport chain. The electron transport chain is situated in the inner mitochondrial membrane and consists of four protein complexes and is a major means to producing energy in the cell. During the process, ROS are generated and this contributes to oxidative stress [222]. Iqbal et al, isolated and assessed mitochondria from the lungs of male broiler chicks with PH [214]. These mitochondria showed increased oxidative stress, decreased levels of glutathione and impaired respiratory function. These data support the role and involvement of mitochondria in the production of highly toxic and ROS.

Some patients with hereditary haemorrhagic telangiectasia have mutations in the endoglin gene which is an underlying cause in this type of slow onset PH. Consequently, in a mouse model of slow onset PH, lung tissue levels of superoxide were increased [223]. The study supports data from a study by Grobe et al, who established a lamb model that mimics a congenital heart defect with increased pulmonary blood flow using *in utero* placement of an aorta-to-pulmonary artery vascular graft [215]. These lambs had increased mPAP with clinical and pathologic sequelae similar to children with congenital heart defects associated with PH. Although congenital heart disease is not considered hereditary, it is, in fact, associated with a genetic component. In this lamb model analysis of lung tissue revealed increased oxidative stress, as reflected by increased nicotinamide adenine dinucleotide phosphate-oxidase expression and superoxide production [215]. These studies highlight the involvement of oxidative stress in the development and progression of PH associated with hereditary or congenital causes.

Oxidative stress in human studies

There are currently only 2 studies that examined oxidative stress in tissue samples from patients with PH [210,224]. In a study done by Bowers et al, lung tissue from patients with both primary and secondary PH were analysed by microarray gene expression profiling [210]. These tissue samples had increased oxidative stress as displayed by elevated levels of breakdown products from oxidative damage to deoxyribonucleic acid and proteins. Breakdown products included nitrotyrosine, 8-hydroxy guanosine and 5-oxo-eicosatetraenoic acid. The tissue samples also had reduced activity of superoxide dismutase [210]. Furthermore, Masri et al, showed that lung tissue samples from patients with idiopathic PH had deficient superoxide dismutase and glutathione peroxidase activity [224].

A number of studies assessed oxidative stress in plasma and serum samples of patients with PH [213,225-229]. In one such study, electron spin resonance spectroscopy revealed that plasma samples from patients with idiopathic PH have increased xanthine oxidase activity [225,226]. In 16 patients with primary PH, erythrocyte glutathione peroxidase levels were decreased and plasma malonic dialdehyde levels were increased [213]. These findings support data published in another study where serum samples of COPD patients with PH displayed increased malonic dialdehyde levels and decreased erythrocyte glutathione peroxidase activity [230].

Urine samples from patients with PH had increased levels of isoprostaglandin F2-type III (which is an F2-isoprostane and marker of oxidative stress) [227]. Collectively, these studies suggest that PH is associated with elevated oxidative stress in lung tissue which is also reflected in plasma, serum and urine. With this set of evidence, it is clear that oxidative stress is an intrinsic part of PH and RV remodelling, which builds a strong case for the potential use of an antioxidant as a RV protective treatment in PH. A number of studies investigated the effects of various antioxidants in animal models of PH.

3.4.4. Antioxidant treatments in animal models of pulmonary hypertension

EUK-134

EUK-134 is a superoxide dismutase and catalase mimetic [219]. In the MCT model of PH, EUK-134 treatment was commenced at a dose of 25mg/kg (once every two days), ten days after MCT injection (80mg/kg for an additional 22 days) [219]. EUK-134 attenuated RV hypertrophy, oxidative stress and prevented interstitial fibrosis. The fact that antioxidant treatment was commenced on day ten of the MCT model suggests that antioxidant treatment administered fairly late in the disease progression may still be effective. These results were corroborated by Ahmed et al, who showed that mice overexpressing extracellular superoxide dismutase and subsequently exposed to hypoxia for ten days (10% fraction of inspired oxygen) lower lung tissue levels of xanthine oxidase and ROS as well as improved RV hypertrophy [231].

Resveratrol

Resveratrol is a polyphenol compound with strong antioxidant, anti-inflammatory and endothelial protective properties [232]. Resveratrol pre-treatment (for one day; 25 mg/kg/day intraperitoneal) in MCT rats attenuated RV remodelling as well as oxidative stress levels in lung tissue, after 21 days [232]. In a study done by Yang et al, intragastric resveratrol treatment (10 and 30mg/kg twice daily for 21 days) improved PH and RV remodelling in MCT-induced PH (50mg/kg intraperitoneal) [233]. This was accompanied by a reduction in cardiomyocyte apoptosis. Oral resveratrol treatment (3mg/kg) also attenuated MCT-induced PH and RV remodelling [234]. What makes the latter study so crucial is the fact that resveratrol treatment was commenced on day 28 after the MCT injection (50mg/kg intraperitoneal). This is important because at this time point the disease is fully progressed and supports the notion that antioxidant treatment in already progressed PH and RV remodelling may be effective. The authors of both these last two studies did not directly measure markers of oxidative stress but it is appropriate to speculate that the beneficial effects of resveratrol may be largely due to its antioxidant properties [75,76].

Allopurinol

Allopurinol is an inhibitor of the enzyme xanthine oxidase and inhibits ROS production [235]. In heart failure, allopurinol treatment effectively increases myocardial contractile efficiency but also generated some concern of possible renotoxicity [236]. Nevertheless, allopurinol has been shown to attenuate pulmonary vascular remodelling in rats that were exposed to chronic hypoxia (13% fraction of inspired oxygen, for 14 days). Allopurinol treatment (50mg/kg/day intraperitoneal) significantly reduced xanthine oxidase derived superoxide anion production [235]. Thus, allopurinol proves to have merit as a potential antioxidant treatment in PH.

It is worth mentioning that even though oxidative stress is detrimental in the pulmonary vasculature and cardiac remodelling, its actions in the heart are somewhat complex [75,76]. Literature indicates that ROS play two major roles in the heart including its ability to cause injury to cell components and involvement in cell signalling pathways and antioxidant defence [75,76]. Piantadosi et al, showed that ROS plays a crucial role in antioxidant-induced cardiac mitochondrial biogenesis by liberating, stabilizing and translocation of nuclear respiratory factor-2 to the nucleus [237]. Upon activation by ROS, nuclear respiratory factor-2 subsequently binds to multiple antioxidant-response-element motifs which regulate

mitochondrial biogenesis, in conjunction with peroxisome proliferator-activated receptor-gamma coactivator-1-alpha [237].

This suggests that ROS enable cardiomyocytes to "sense" ROS-induced damage, employ its inherent antioxidant systems and counteract the damage as an adaptive response. [76, 237] Furthermore, ROS have been shown to trigger the activation of rat sarcoma protein or Ras, which induces the recruitment of phosphatidylinositol-3'-kinase to Ras [238]. This ROS triggered activation of Ras is required for the activation of cardioprotective prosurvival, downstream signals such as Akt and mitogen activated kinases [238]. Therefore, experts believe that although antioxidant therapy in experimental PH and RV remodelling is strikingly beneficial, this presents a challenge, as ROS also play an important physiological role in health and disease [75,76]. This physiological balance or ROS related functions should be kept in mind when ROS scavenging or antioxidant treatments are administered, as complete depletion of ROS may be detrimental.

Clinical trials employing antioxidant therapy in humans with cardiovascular disease were disappointing and this may be due to the fact that the biological function of ROS is not completely understood [75,76,239]. In the MRC/BHF Heart Protection study (Heart Protection Study Collaborative Group, 2002), 20,536 adults were studied for a period of five years [240]. These patients had coronary artery disease, other occlusive arterial disease or diabetes and were randomly divided in three groups that received daily oral supplementation with antioxidant vitamins (600mg vitamin-E, 250mg vitamin-C, and 20mg beta-carotene) or matching placebo. Primary outcomes were, major coronary events and fatal or nonfatal vascular events with assessments of cancer and other major morbidities. In this patient population, antioxidant treatment was shown to be safe but did not cause any significant reduction in the five year mortality from any type of vascular disease, cancer or any other major disease [240].

Palumbo et al, conducted a randomized controlled, open trial and investigated the effects of vitamin-E supplementation (300mg/day for twelve weeks) on clinic and 24 hours ambulatory blood pressure in 142 treated hypertensive patients [241]. After the treatment period, ambulatory blood pressure measurements showed no change in either patients treated with vitamin-E or controls. Clinic blood pressure measurements were lower whether or not the patients received the vitamin-E supplementation. The authors concluded that chronic vitamin-E supplementation has no clinically relevant effect on blood pressure in hypertensive patients [241]. Similar outcomes were seen in a study by Kim et al, who investigated the effect of chronic vitamin-C treatment (50 and 500mg daily for five years) on blood pressure of 439

cancer and stroke patients [242]. In this study, long term vitamin-C supplementation had no effect on either blood pressure or mortality [242].

Despite the overwhelming success of antioxidant therapy in animal studies, the dismal failure of antioxidant therapy to impact human disease left a great sense of disappointment [75,76,239]. However, this obstacle may be overcome by treatments that specifically targets major cellular producers of oxidative stress in particular mitochondria [239,243]. The doses of pharmacologically administered antioxidants should be carefully controlled to match physiological levels of ROS. Another antioxidant that has been tested and shown to be effective against oxidative stress and cardiovascular disease in both animal studies and clinical trials is melatonin.

4. Melatonin and its role as an antioxidant

4.1. Biosynthesis of melatonin, circulating levels and its effect on sleep

Melatonin is an idoleamine hormone synthesized by the pineal gland in the brain, is lipophilic and able to cross cell membranes [244-247]. Besides being produced in the pineal gland it is also secreted by entero-endocrine cells in the upper gastrointestinal tract [248]. Melatonin is not only produced in the body but also in plants, fruits, during the process of milk production and the fermentation of wine [249] [250] . It is predominantly synthesized at night time, as day light has an inhibitory effect on its production and secretion [251,252]. The secretion of melatonin is synchronized with a day and night or circadian cycle, during which, night time or darkness is sensed by the retina. This light pathway, modulates the activity of the suprachiasmatic nucleus which leads to the activation of neurons, from the suprachiasmatic nucleus to the paraventricular nucleus in the pineal gland. Here, rate limiting enzymes control the synthesis of melatonin from its precursor, the amino acid tryptophan [251,252]. During the biosynthesis of melatonin, the first rate-limiting enzyme, arylakylamine-N-acetyltransferase convert serotonin to N-acetylserotonin, while the second rate-limiting enzyme, hydroxyindole-methyltransferase, transform acetyl-serotonin into melatonin [248]. After melatonin is synthesized, it is released into the blood and can reach peak, night time serum levels of approximately 10^{-10} to 10^{-9} mol/L or 50-140pg/L, while during the day levels can be as low as 20pg/L [253,254]. This pattern of melatonin synthesis and release, led to many studies showing the ability of melatonin to reset the biological clock and increase time to sleep onset in people with jet-lag, shift workers or children with neurodevelopmental disorders [255-257]. In 2012, Appleton et al, performed a study that included 146 children (3 years to 15 years), with

a whole range of neurological and developmental disorders and a severe sleep problems [258]. Immediate release melatonin or placebo capsules were administered 45 minutes before bed time for a period of 12 weeks. All children started with a 0.5mg capsule, which was increased to 2mg, 6mg, and 12mg depending on their response to treatment. In this study, melatonin increased the total sleep time by 22.4 minutes and reduced sleep onset latency measured by sleep diaries. Melatonin treatment was also associated with earlier waking times than placebo [256]. These data suggest that melatonin is very efficient in resetting the biological clock and to regulate the sleeping pattern. However, despite its efficacy in regulating the sleeping pattern, its bioavailability has been drawn into question [259].

4.2. Bioavailability of melatonin

Although melatonin is a highly effective antioxidant, is has a relatively short half-life and undergo extensive first-pass metabolism [260,261]. Circulating melatonin is metabolised in the liver where the first step is hydroxylation at the C-6 position by cytochrome-P450-mono-oxygenases (isoenzymes CYP1A2, CYP1A1 and to a lesser extent CYP1B1) [262-264]. These isoenzymes catalyse the formation of 6-hydroxymelatonin, which undergoes further conjugation with either sulphate or glucoronic acid. During these steps, melatonin's highly antioxidant by-products, N-acetyl-N-formyl-5-methoxy-kynuramine and N-acetyl-5-methoxy-kynuramine are formed [262,263,265]. However, the majority of orally administered melatonin is lost due to the first-pass effect and is excreted in the urine as unchanged melatonin [262-264,266].

A number of studies, investigated the bioavailability of melatonin after oral administration in humans [259,260,267,268]. In a study by DeMuro et al, oral melatonin at doses 2-4mg had a bioavailability of approximately 15%, as only so much of the administered dose, managed to reach systemic circulation [259]. Due to this low bioavailability, Bartoli et al, developed an oral melatonin emulsion spray, administered it sublingually to healthy individuals and compared this to the bioavailability after drinking a melatonin pill [260]. This spray is absorbed via the oral mucosa and avoids the first-pass effect associated with metabolism of melatonin in the liver. In this study, the amount of melatonin that reached systemic circulation was twice as high as with the pill form, even though the dose of melatonin was the same for both the spray and the pills (5mg). Similar work was done by Mao et al, who prepared starch microspheres (particle size of 30-60μm) for intranasal administration of melatonin, by an emulsification-crosslinking technique [269]. In this study, the entrapment ratio of melatonin in the microspheres was 11.0% and melatonin was released from the microspheres in a sustained

manner *in vitro*. The absorption rate was rapid (7.8 min) and the absolute bioavailability was as high as 84%. These studies, are important as they develop new ways to increase the bioavailability of melatonin in order to increase its efficacy in long-term treatment [270].

Not undermining the importance of these studies, one need to carefully evaluate the data. In the study by DeMuro et al, after oral melatonin administration (pills of 2-4mg), 85% of melatonin was lost due to first-pass metabolism [259]. However, despite the low bioavailability of 15%, the mean concentration of melatonin in the serum of these individuals, reached peak values of approximately 4000-5000pg/mL after melatonin administration. These serum concentrations are much higher than physiological peak levels and could therefore be the reason why, in most studies, the melatonin is still highly effective in scavenging free radicals and modulating pathology [250,271-273]. Furthermore, apart from its well-known effects, such as scavenging free radicals and regulation of the sleeping pattern, melatonin has been shown to have many others effects including anti-inflammatory, anti-thrombotic, anti-lipidemic, and anti-cancer [244-246,253,254,274].

4.3. Melatonin as a multifunctional molecule

In 2010, Borazan et al, assessed the efficacy of melatonin as a preoperative medication to improve postoperative analgesia in patients undergoing elective prostatectomy [275]. In this study, 6mg melatonin given orally the night before surgery and one hour post-surgery, reduced pain scores in addition to improving quality of sleep of these patients.

In work by Rosales-Corral et al, melatonin treatment reversed the adverse effects of an intracerebral injection of amyloid-Aβ, in an animal model of Alzheimer's disease [276]. Here, melatonin reversed the mitochondrial dysfunction and reduced oxidative stress in the brains of these animals.

Furthermore, the anti-cancer effect of melatonin was demonstrated when Ehrlich ascites carcinoma cells, were exposed to melatonin [277]. Melatonin, reduced the vitality and cell volume of these cancer cells and induced apoptosis. These anti-cancer effects of melatonin was believed to be mediated via the inhibition of the cell cycle in cancer cells. Also, melatonin has been shown to reduce plasma levels of total cholesterol, very low density lipoproteins and low density lipoproteins in hypercholesterolemic rats [278]. These findings were corroborated by Dominguez-Rodriguez et al, who showed that in patients with myocardial infarction, nocturnal elevated serum levels of oxidised low density lipoproteins are associated with reduced circulating melatonin levels [279]. Taken together these studies

strongly suggest melatonin as multifunctional molecule with great potential to improve various pathologies and their adverse consequences on health. Furthermore, this wide range of physiological functions include its potent antioxidant and anti-hypertensive properties.

4.4. Melatonin as an antioxidant and anti-hypertensive agent

There is a plethora of research proving melatonin to be up to a hundred times more effective in scavenging free radicals compared to vitamin E [280,281]. Melatonin has its antioxidant effects by either directly scavenging ROS or by indirectly stimulating the synthesis/activity of antioxidant enzymes [282]. These direct antioxidant effects are believed to be receptor independent [247,278]. Almost more than twenty one years ago, the first report was published that melatonin neutralises the highly toxic hydroxyl radical [283]. These was corroborated by others who by means of a computational study showed that this interaction of melatonin with hydroxyl radical is when melatonin chemically binds hydroxyl radical to the C-2 position of its indole ring [284]. Similarly, melatonin can also interact with hydrogen peroxide and form by-products such as water and N-acetyl-n-formyl-5-methoxy-kynuramine (AFMK) [285]. An interesting fact of melatonin is that during most of these oxygen free radical scavenging reactions, melatonin's by-products including AFMK or 3-hydroxymelatonin also have antioxidant abilities [286] [282]. This was shown by Tan et al, that AFMK reduces oxidative damage to lipids and DNA in cells exposed to hydrogen peroxide, glutamate or amyloid-beta [285,287]. Therefore, the potent antioxidant effects of melatonin are not just due to its own actions but also its daughter molecules.

The indirect effects of melatonin are usually achieved via its membrane bound G-protein coupled receptors, melatonin receptors (MT-1, 2 and 3) [282]. These receptors of melatonin have been shown to be present throughout the cardiovascular and the pulmonary vascular systems [247]. In particular, the melatonin receptors 1 and 2 have been found to be present on cardiomyocytes of the human heart and coronary arteries [253,254]. Upon binding to its receptors (MT-1 and MT-2), melatonin can activate various signalling pathways including the cAMP/PKA pathway which could inhibit the cAMP-response element binding protein or activation transcription factor (CREB-ATF) [288,289]. This could modulate immediate early gene transcription and gene transcription regulation of antioxidant enzymes. Other pathways regulated by melatonin include the ERK/JNK pathway and the phospholipase-C/PKC/NFkB pathway, which could also modulate antioxidant enzyme gene expression (Figure 11).

Melatonin also has antihypertensive properties either by increasing plasma levels of L-arginine, which is a substrate used by endothelial nitric oxide synthase to produce nitric oxide [251,252]. Furthermore, melatonin also reduces circulating levels of the natural inhibitor of nitric oxide, asymmetric dimethyl arginine, or intracellular calcium levels, and elevate cyclic-GMP in order to cause vasodilation [290] (Figure 11). Melatonin has been shown to be beneficial in various pathologies including atherosclerosis, ischemia/reperfusion injury, cardiac hypertrophy as well as hypertension [244-246,273,291]. Evidently, both the antihypertensive and antioxidant effects of melatonin have been well substantiated in animal models of cardiovascular pathology.

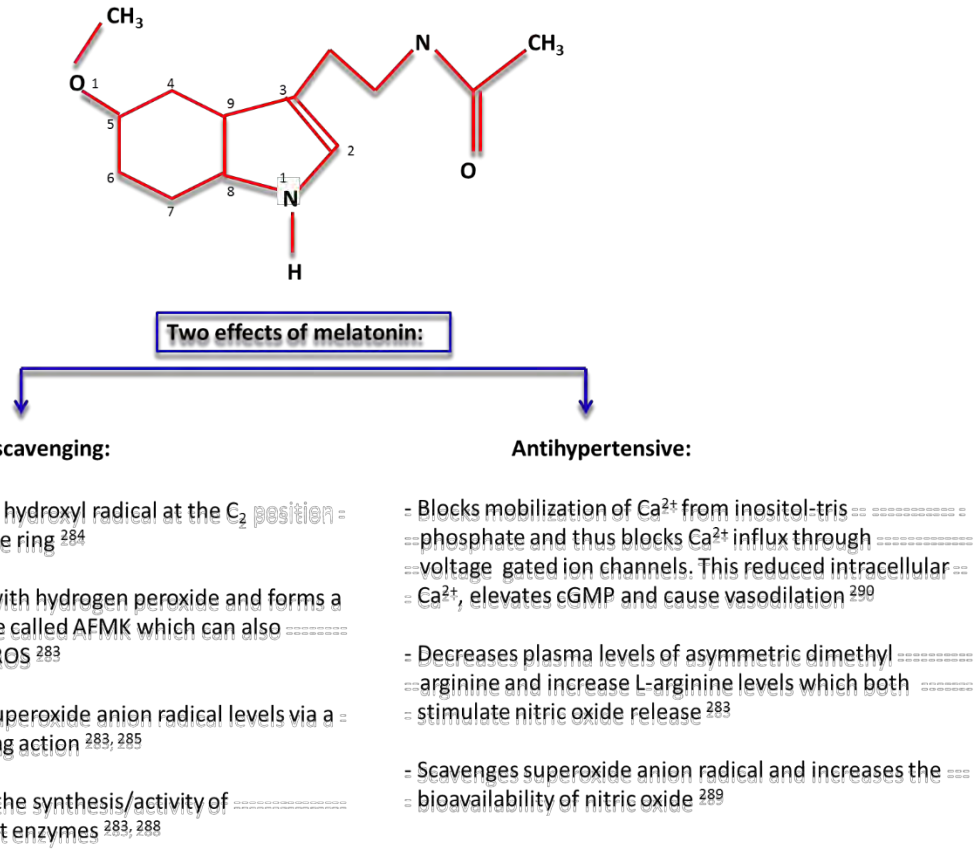


Figure 11. Melatonin and its antioxidant and anti-hypertensive properties. A schematic showing the chemical structure of melatonin and two main effects which include scavenging of reactive oxygen species and antihypertensive effects. **CH_3**: methyl group, **O**: oxygen, **N**: nitrogen, **AFMK**: N-acetyl-N-formyl-5-methoxykenuramine, **Ca^{2+}**: calcium ion, **cGMP**: cyclic guanosine monophosphate.

4.5. Indications that melatonin could be a potential treatment for pulmonary hypertension

According to our knowledge, there are currently no studies that tested melatonin as a possible treatment against PH. However, both its antioxidant and antihypertensive properties makes it a likely option for a cardioprotective treatment that can be given as a complimentary treatment in PH. The cardioprotective effects of melatonin have been confirmed in the literature where melatonin treatment, at the concentration found in red wine, could protect against ischemic/reperfusion injury [250]. Similarly, chronic melatonin treatment, in a rat model of diet induced obesity, was found to be cardioprotective [273]. There is no scientific evidence that melatonin has been tested as a treatment in PH but there are some indications that melatonin could be a potential cardioprotective treatment in PH. These indications are found in studies performed in both animal models that display oxidative stress or hypertensive cardiomyopathy in humans with systemic hypertension.

4.5.1. Melatonin in animal models: Antioxidant and antihypertensive effects

Gosh and colleagues investigated the effects of melatonin in a rat model of triiodo-L-thyronine-induced cardiac hypertrophy [292]. Melatonin was administered intraperitoneally (2mg/100g body weight) one hour before triiodo-L-thyronine treatment and after 15 days the rats were sacrificed. Triiodo-L-thyronine resulted in cardiac hypertrophy, which was reduced by the melatonin pre-treatment. Melatonin also suppressed lipid peroxidation and hydroxyl radical levels and prevented the inhibition of superoxide dismutase in the hypertrophic hearts [292]. This was a striking finding, as it showed melatonin's ability to have beneficial effects even in such a short period of pre-treatment. In rats injected with melatonin (10mg/kg intraperitoneally) and subsequent exposure to intermittent hypoxia, cardiac, kidney and lung lipid peroxidation was decreased, with no effect on liver peroxidation. It is important to note that in the first study, the heart was the organ that had the pathology (triiodo-L-thyronine-induced cardiac hypertrophy) while in the second study it was the lung that was directly exposed to the pathological insult by means of hypoxia. These studies suggest that melatonin, has the ability to protect the biological site that is directly under "attack" as well as to those affected indirectly. This is an important point as, in the case of PH, the lung is the main site of pathology but the heart is indirectly affected. Thus, the antioxidant effect of melatonin may be of real benefit in PH with regards to both the lung and the heart.

Tain et al, assessed the effect of melatonin on blood pressure in spontaneous hypertensive rats [293]. The rats were caged for 4 weeks to allow hypertension to fully develop and in the following 8 weeks, they received melatonin treatment (0.01% in the drinking water). Both the systolic and diastolic blood pressures were decreased in the rats treated with melatonin. Melatonin also reduced the plasma levels of asymmetric dimethyl arginine, which is a natural inhibitor of nitric oxide. Furthermore, it also restored plasma levels of L-arginine (a substrate for the production of nitric oxide) and reduced oxidative stress as displayed by the levels of 8-hydroxydeoxyguanosine.

Here, melatonin attenuated hypertension by restoring the nitric oxide pathway and attenuating oxidative stress [293]. This study corroborates data by Girouard, who also investigated the effects of melatonin treatment (30mg/kg for four weeks) on blood pressure in spontaneous hypertensive rats [294]. Melatonin treatment reduced mPAP, systolic and diastolic pressures. In this study, vasorelaxation was induced with a calcium ionophore, in mesenteric artery beds isolated from spontaneous hypertensive rats. Melatonin improved vasorelaxation as well as sensitivity of the nitric oxide pathway. It is suggested by others that this melatonin-induced sensitivity of the nitric oxide, may occur via the increase in cyclic guanosine monophosphate levels and decreased cytosolic calcium in smooth muscle cells [251,252]. Another possible mechanism for the effect of melatonin on blood pressure may be nitric oxide-induced vasodilation and antioxidative properties (See figure 10). Overall, melatonin proved to be a molecule with surprising ability and its striking efficacy in attenuating cardiovascular disease in animal models created great hope for similar therapeutic success in humans. There were in fact some studies that investigated the antioxidant and antihypertensive effects of melatonin.

4.5.2. Melatonin in humans: Antioxidant and antihypertensive effects

Healthy volunteers were randomly divided into two groups: one group that received oral administration of melatonin (6mg) for two weeks and the other group that received no melatonin treatment [295]. Blood samples were collected before melatonin administration, on the seventh and fourteenth day of melatonin treatment and on the tenth day after the last dose of melatonin. Blood samples were assessed and plasma antioxidant ferric reducing ability was increased and lipid/deoxyribonucleic acid peroxidation was decreased in the melatonin treated group.

Furthermore, investigators noticed that these beneficial effects of melatonin were maintained even 10 days after discontinuation of melatonin treatment [295]. This study is a clear indication that melatonin can increase the antioxidant ability of the plasma even in the absence of pathology. In another study, melatonin treatment before a major event such as surgery has been shown to decrease pain scores, the use of pain medication and subjective analgesic efficacy during the postoperative period [275]. Umbilical arteries from healthy pregnant women who delivered between 37 and 39 weeks of gestation were cut into small sections [296]. These artery sections were pre-treated with melatonin (10^{-8}M to 10^{-6}M) and the effect of hydrogen peroxide on the response of the segments to external calcium was determined. Melatonin pre-treatment significantly suppressed the contractile response to external calcium and thus the vaso-spastic effect of hydrogen peroxide on umbilical arteries. This effect of melatonin was attributed to the scavenging of the hydroxyl radical [296]. All these studies suggest that pre-treatment with melatonin may have beneficial effects but one should keep in mind that excessive use of antioxidants may be detrimental.

In a randomized, double blinded, placebo controlled, crossover trial in sixteen men with untreated hypertension, repeated oral doses of melatonin (2.5mg/kg for three weeks) reduced systolic and diastolic blood pressure [297]. In another study where normotensive and hypertensive women were treated with melatonin (3mg/kg) for three weeks, investigators observed a significant reduction in the nocturnal systolic diastolic and mean arterial pressure [298]. It is known that systolic and diastolic blood pressures fluctuates in humans with hypertension and is at its highest during the day and lowest at night [244-246]. Patients who do not display a lower blood pressure at night are at a higher risk for cardiovascular mortality [299]. Therefore, these two studies are very crucial as they show that melatonin has the ability to reduce nocturnal blood pressure in hypertensive patients which in theory should reduce their risk of cardiovascular mortality.

Lastly, in a randomized, double blinded, placebo controlled study on 36 patients with clinically stable moderate to severe COPD, melatonin treatment (3mg capsule, once a day before bedtime for 3 months) reduced oxidative stress and improved dyspnoea but had no significant effect on exercise capacity or lung function [300]. It appears that melatonin has the ability to reduce oxidative stress in COPD which is also a well-known risk factor for PH [300]. The latter study elicited my interest into whether anyone investigated the ability of melatonin to affect oxidative lung damage or lung injury.

4.5.3. Melatonin in animal models: Its effect on oxidative lung damage and lung injury

In rats subjected to aortic occlusion for 30 minutes and reperfusion for 12 hours, ischemia/reperfusion injury was present in the lungs with concomitant low survival [301]. Melatonin pre-treatment (2mg/kg intraperitoneally) one hour before surgery, reduced oxidative stress (in particular, malonic dialdehyde levels). In another study, rats received a single injection of melatonin (10mg/kg intraperitoneally) and were exposed to hyperbaric oxygen (100% oxygen at four atmospheres) for 90 minutes [302]. Here, melatonin prevented the increase in lipid peroxidation (malonic dialdehyde levels) as observed in the control group and maintained reduced oxidative stress at levels similar to that of the control rats.

In rabbits exposed to cigarette smoke in a glass chamber for one hour daily (for one month), melatonin (1mg/kg intraperitoneal) reduced oxidative stress and damage in the bronchioles [303]. These data are supported by the work of Pedreira and colleagues who showed that pre-treated with melatonin (10mg/kg intraperitoneally), decreased lung injury oedema, haemorrhage and infiltration of inflammatory cells, in a mouse model of ventilator-induced lung injury [304]. These studies collectively suggest that melatonin is effective in improving lung damage associated with either oxidative stress or damage of a mechanical cause. There are currently no reports of the effects of melatonin in animal models of PH. However, the studies which are available highlight important aspects of melatonin and support it as a potential treatment for PH. These available studies mostly investigate the effects of melatonin on isolated pulmonary arteries.

5. Concluding remarks

The evidence that melatonin may be of benefit in PH is copious and there is a whole range of reasons to support this notion: (i) other antioxidants have failed in altering cardiovascular pathology when tested in clinical trials, while melatonin has been successful in doing this, (ii) melatonin is strikingly effective in attenuating hypertension in both animals and humans. In brief, various mechanisms which underlie these effects have been proposed and include suppression of circulating catecholamines, relaxation of smooth muscle in the peripheral blood vessels due to its antioxidant activity or receptors present on peripheral blood vessels [244-246]. More reasons in support of the notion that melatonin may be of benefit in PH, are that (iii) melatonin treatment is not associated with toxic or adverse effects in either animals or humans and (iv) circulating melatonin levels has been shown to be decreased in patients with hypoxic/destructive lung disease as well as cardiovascular disease which are risk factors for

PH [305-308]. Therefore, based on the bulk of the evidence presented here, the antioxidant and antihypertensive effects of melatonin makes it an attractive therapeutic target for the protection of the heart and possible treatment of PH. Melatonin is investigated in this PhD study for its potential as a possible complementary cardioprotective treatment for PH.

SECTION B

HYPOTHESIS AND OBJECTIVES

PH is a fatal disorder that currently has no cure and only palliative therapy with limited health benefits. It is a global health threat with increasing disease burden and patients with PH have a poor prognosis and quality of life. In Sub-Saharan Africa, in particular South Africa, PH has become a depressing reality as a considerable number of patients with HIV/AIDS, chronic obstructive lung disease and congenital heart defects often develop PH [145]. There is a wide range of drugs available to limit the negative outcomes of patients with PH including endothelin-1 receptor antagonists, prostacyclin analogues, and phosphodiesterase type-5 inhibitors (e.g. sildenafil). Sadly, sildenafil is the only drug currently available in South Africa and is unfortunately very expensive [145]. All of this paints a grim picture for South African patients living with this dreadful disease, but it also highlights the need for new treatments. There are various mechanisms involved in the development of PH, in particular oxidative stress caused by highly ROS [29,30,210,211]. A very promising novel therapeutic drug target, melatonin, has been shown to provide protection against cardiovascular disease. Melatonin is a powerful antioxidant, shown to be up to a hundred times more potent than vitamin E and it also presents anti-hypertensive properties [281,285]. The use of melatonin in humans has no reported side effects, it is relatively inexpensive and available in many countries. Melatonin levels are disrupted in certain cardiovascular pathologies such as myocardial infarction, and exogenous melatonin has been proven to be beneficial against ischaemic and hypertensive heart disease, most probably due to its antihypertensive properties [250,273].

1. Hypothesis

We therefore hypothesise that melatonin may be used as an effective treatment in pulmonary vascular diseases, providing beneficial effects on the pathophysiological outcome of experimental PH.

2. Objectives:

To test this hypothesis the following objectives will be pursued:

a) Assess oxidative stress in South African patients with PH by measuring blood plasma levels of lipid peroxidation (a marker of oxidative stress), plasma antioxidant capacity (oxygen radical absorbance capacity), antioxidant enzymes catalase and superoxide dismutase activities (Section C).

b) Validate an experimental model of PH by injection in Long Evans rats a toxin called monocrotaline (MCT) (80mg/kg subcutaneous). This model has never been induced in this particular rat strain and therefore we need to first validate whether the Long Evans rat strain will recapitulate the characteristics of PH, 28 days after the injection. We will also establish whether oxidative stress is present in the blood plasma of these rats, with assays that were performed in the blood plasma of patients. We will also collect heart samples for histological analyses of interstitial fibrosis (Section D).

c) Test various doses of melatonin, to see whether melatonin can confer cardioprotection and to ascertain the most efficacious dose against PH. To assess this hypothesis we will measure cardiac function with echocardiography and the isolated heart perfusion apparatus in rats with/without PH and treated daily with melatonin from the day of the injection with MCT. In addition we will measure biometric parameters, collect blood plasma samples and perform blood plasma assays to assess lipid peroxidation, plasma antioxidant capacity and activities of catalase and superoxide dismutase. We will also collect and prepare heart samples from all treatment groups to do histological staining in order to assess cardiac interstitial fibrosis. The first concentration of melatonin will be 75ng/L (oral) which corresponds to the concentration of melatonin absorbed via nutrition [250]. The other doses of melatonin (4mg/kg and 6mg/kg, oral) are comparable to the pharmacological doses of melatonin absorbed by taking tablets [273,309] (Section E).

d) Administer melatonin treatment either as a therapeutic intervention given daily (given fourteen days after the induction of experimental PH) or as a preventive intervention (given five days before the induction of experimental PH). Hereafter, we will assess cardiac function and blood plasma oxidative stress and antioxidant capacity as described above. Heart samples will also be collected and used for histological staining for cardiac interstitial fibrosis (Section F).

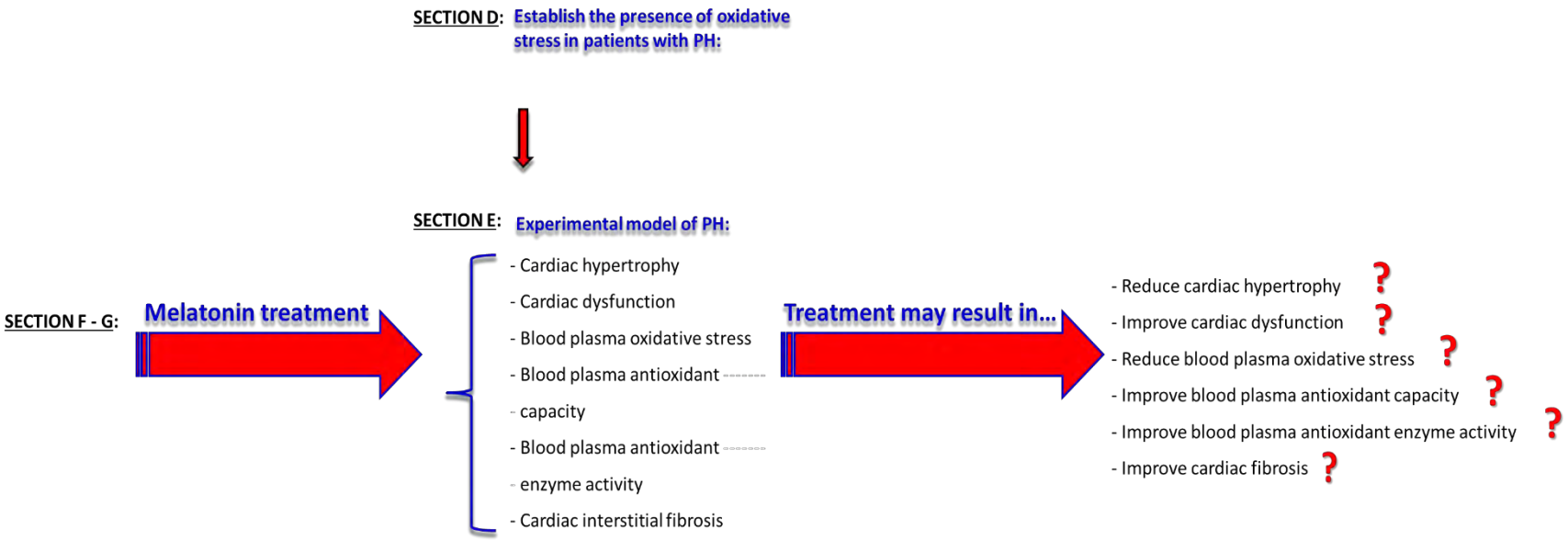


Figure 12. Potential role for melatonin in PH and RV remodelling. A graphical representation of our hypothesis that melatonin may be cardioprotective in PH, **PH:** pulmonary hypertension.

SECTION C

MATERIALS AND METHODS

1. Evaluation of oxidative stress in a patient population with pulmonary hypertension

1.1. Patient recruitment and clinical assessments

Patients were referred from a primary to a secondary cardiac clinic at GF Jooste hospital (Cape Town, South Africa) if they had symptoms including shortness of breath, fatigue, peripheral oedema, and persistent cough at night (tuberculosis was excluded at primary care level). Ten of these patients underwent clinical investigation which comprised medical history, clinical examination, chest X-ray, electrocardiogram and echocardiography (where a RV systolic pressure of above 35 mmHg confirmed the diagnosis of idiopathic-PH). The patients were also required to do a six minute walk test, to determine their capacity to do exercise and then blood samples were collected and stored for later experimentation. Furthermore, blood samples were collected from ten healthy individuals and used as controls. All the blood samples were used for the determination of the overall antioxidant capacity of the blood plasma, as well as the activity of antioxidant enzymes; a marker of oxidative stress, catalase and superoxide dismutase. All experimental protocols were approved by the faculty of health sciences human research ethics committee, university of Cape Town (241/2011).

Systolic and diastolic blood pressure was measured using conventional Korotkof sounds. Blood pressure was measured at the right arm three times, with a five minute rest between each measurement. Patients were weighed without shoes and in light clothing on a standard beam balance. Height was measured to the nearest centimetre using an anthropometric plane, with patients not wearing shoes or headgear. Body mass index was calculated as kg/m^2. All subjects also had 12-lead electrocardiogram performed by a trained technician [310]. Echocardiography was performed using a commercially available ultrasound system (IVIS-60). Subjects were examined in the left lateral decubitus position using standard parasternal, short-axis, and apical views. Measurements were performed according to the guidelines of the American Society of Echocardiography [311]. The LV measurements included interventricular septal thickness at end-diastole (IVSd), the posterior wall thickness at end-diastole (PWTd), and the LV internal dimensions at end-diastole (LVIDd) and end-systole (LVIDs). The LV systolic function was calculated by Teichholz's formula and by volumetric analysis when a regional wall motion abnormality was detected. The LV flow velocities were measured using pulsed-wave Doppler from the apical four-chamber view with sample volume located between the tips of the mitral valve leaflet during ventricular diastole. Peak velocity of early rapid filling (E), peak velocity of late filling caused by atrial contraction (A), and the interval from peak E wave to its extrapolation to the baseline or deaccelaration time

(DT) were measured. LV filling was classified into normal filling (E/A ¼ 1–2 and eDT ¼ 130–220 ms), impaired relaxation (E/A .1 and DT 220 ms), restrictive filling (E/A .2 and DT, 130 ms) and pseudonormal filling (E/A ¼ 1–2 and DT 220 ms). The right heart function was measured according to standard criteria [311]. Right-sided heart failure or PH was defined as heart failure secondary to right-sided pathology with increased jugular venous pressure and liver size, tricuspid regurgitation, and/or elevated RV systolic pressure ≥ 35 mmHg [312].

1.2. Blood sample collection

Blood samples were collected from the median cubital vein and the nurse or physician used a scalp vain infusion set (23Gx3/4") (Lasec, South Africa) that was connected to a blood collecting tube (BD-vacutainer, containing EDTA as anticoagulant) (Lasec, South Africa). The tube was gently tilted twice and immediately placed on ice. The tubes were transferred to a precooled (4°C) bench centrifuge (Eppendorf Centrifuge 5804R) and centrifuged for ten minutes at 2500rpm (revolutions per minute). Centrifugation separated the blood into two phases with the plasma phase on top. The plasma was transferred with a pipette (Lasec, South Africa) into 2mL Eppendorf tubes in aliquots of 300µL each. These tubes were marked with the number of the patient, the date and whether the patient was part of the control or PH group. Plasma aliquots were stored at -80°C until used for the various assay analyses.

1.3. Thiobarbituric reactive substance assay

The thiobarbituric acid reactive substance (TBARS) assay measures malonic dialdehyde, which is a reactive compound formed during lipid peroxidation caused by ROS (also known as oxidative stress) [313]. Lipid peroxidation occurs during various diseases, including cardiopulmonary vascular disease. During this assay, blood plasma samples are mixed with thiobarbituric acid and malonic dialdehyde, formed during lipid peroxidation within the sample, forms a pink coloured adduct with thiobarbituric acid. This pink colour is measured at a certain wave length and used as an indication of the level of TBARS produced. [313] The darker the pink colour, the higher the level of TBARS. The levels or concentrations of TBARS were measured by a spectrophotometric method (SPECTRAmaxPLUS-384).

Concentrations were calculated using the appropriate molar extinction coefficients and standards. Fifty microlitres of the blood plasma (sample) was mixed with 6.25µL butylated hydroxytoluene (4mM, in ethanol) (Sigma, South Africa) and 50µL of ortho-phosphoric acid (0.2M) (Sigma, South Africa) in an Eppendorf tube. The tube was vortexed for ten seconds

after which, 6.25µL of TBA or thiobarbituric acid reagent (Sigma, South Africa) was added and the tube was vortexed for another ten seconds.

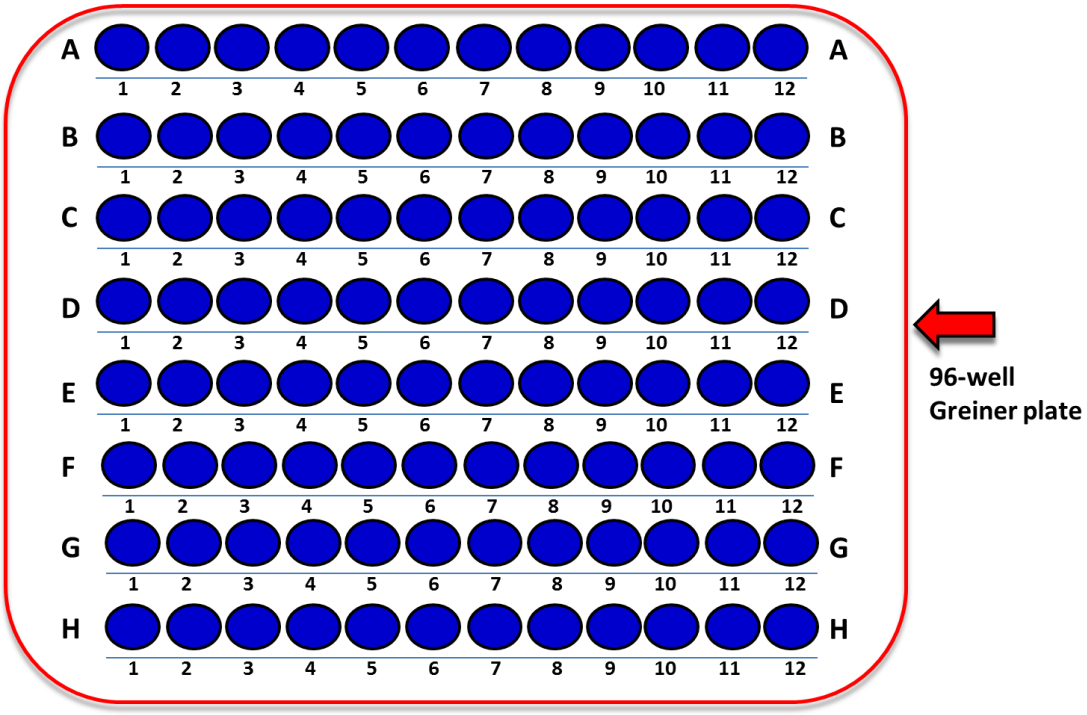
The samples were microfuged (Spectrafuge 16M, Labnet) for two minutes at a speed of 3000rpm, at 4°C to collect the volumes at the bottom of the tubes. The samples were placed on a heating block, pre-set at 90°C for 45 minutes. Subsequently, the samples were placed on ice for two minutes and left at room temperature for five minutes. Five hundred microlitres of n-butanol (Merck Chemical, South Africa) was added to each sample as well as, 50µL of saturated sodium chloride and vortexed for ten seconds.

Hereafter, the samples were microfuged at 12000rpm for two minutes at 4°C. This separated the samples into a transparent, top-butanol phase and a bottom-phase containing the white protein pellets. Three hundred microliters of the butanol phase was pipetted into the wells of a UV readable, Greiner-96 well plate (Lasec, South Africa). The absorbance was read at 532nm, with a micro plate data acquisition program (SoftMax® Pro 4.8) and the Beer-Lamberts law was used to calculate the concentration of malonic dialdehyde in the samples with an extinction coefficient of 1.54x105 M-1/cm^{-1} [313]. The final values were expressed in µmol malonic dialdehyde/mL plasma (Figure 13).

- 50µL blood plasma + 6.25µL BHT/EtoH (4nM) + 50µL ortho-phosphoric acid (0.2M) in tubes
- Vortexed for 10 seconds
- Added 6.25µL TBA reagent (0.793g in 50mL 0.1M NaOH)
- Vortexed for 10 seconds
- Tubes microfuged for 2 minutes at 3000rpm, 4°C (to collect volumes at bottom of tube)
- Tubes placed in a heating block, pre-heated to 90°C for 45 minutes (colour changed to pink)
- Tubes placed on ice, for 5 minutes
- Added 500µL of n-butanol + 50µL saturated NaCl (latter enhanced phase separation)
- Vortexed for 10 seconds
- Tubes microfuged for 2 minutes at 12000rpm, at 4°C

*two phases formed: top butanol phase and bottom protein phase

- Pipetted 300µL of top butanol phase from each tube into different wells of the plate



- Inserted 96-well plate, into spectrophotometer and read, at 532nm, at room temperature

Figure 13. Thiobarbituric acid reactive substance – assay protocol. The protocol for the determination of oxidative stress (in the form of lipid peroxidation) of blood plasma samples. The lipid peroxidation was measured by the reaction between a by-product of peroxidation and thiobarbituric acid. **TBARS**: thiobarbituric acid reactive substance, **BHT**: butylated hydroxytoluene, **EtoH**: ethanol, **NaOH**: sodium hydroxide, **NaCl:** sodium chloride

1.4. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay is a method to assess the antioxidant capacity of biological samples [314,315]. The assay determines the ability of plasma samples, to scavenge free radicals. With this assay, free radicals are generated from the thermal decomposition of 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (Sigma Aldrich, South Africa), on the signal intensity from the fluorescent probe (fluorescein, 3', 6'-dihydroxyspiro [isobenzofuran-1[3H], 9' [9H]-xanthen-3]) (Sigma Aldrich, South Africa), in the presence of an oxygen radical absorbing substance. The stronger the absorbing capacity, the more the free radicals are scavenged, thus maintaining the intensity of the fluorescent signal observed. In order to obtain measurements, the area under the curve of the fluorescence intensity versus time is subtracted from that of the blank, to determine the antioxidant capacity of the blood plasma, in trolox equivalents. Trolox is a vitamin-E analogue that is used as the standard measure of antioxidant capacity.

A trolox (6-OH-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (Sigma Aldrich, South Africa) standard curve was prepared by doing a range of dilutions. Firstly, 0.0050g of trolox was diluted in 200µL ethanol (100M) of which 100µL was added to 9.9mL of phosphate buffer (1000µM). Then, 1mL of this solution was added to 9mL of phosphate buffer (5nmol/L). We then took, 300µL of this solution and added it to 300µL of phosphate buffer (2.5nmol). Hereafter, 300µL was added to 300µL of phosphate buffer (1.25nmol/L). Similarly, further dilutions (0.625nmol/L, 0.313nmol/L, 0.156nmol/L and 0.078nmol/L) were prepared. The phosphate buffer contained $K_2HPO_4 \cdot 3H_2O$ (0.75M) and $NaH_2PO_4 \cdot H_2O$ (0.75M) mixed to a final concentration of 0.75M, pH 7.4 (Figure 14). Fluorescein was prepared by making a range of dilutions. The first stock solution was prepared by adding 0.0225g fluorescein to 50mL of phosphate buffer (0.0012 mol/L), the second stock solution, by adding 50µL of stock number one to 10mL of phosphate buffer (5.98µmol/L) and the working solution was made by adding 320µL of stock solution two to 20mL of buffer (95.7nmol/L)(Figure 15).

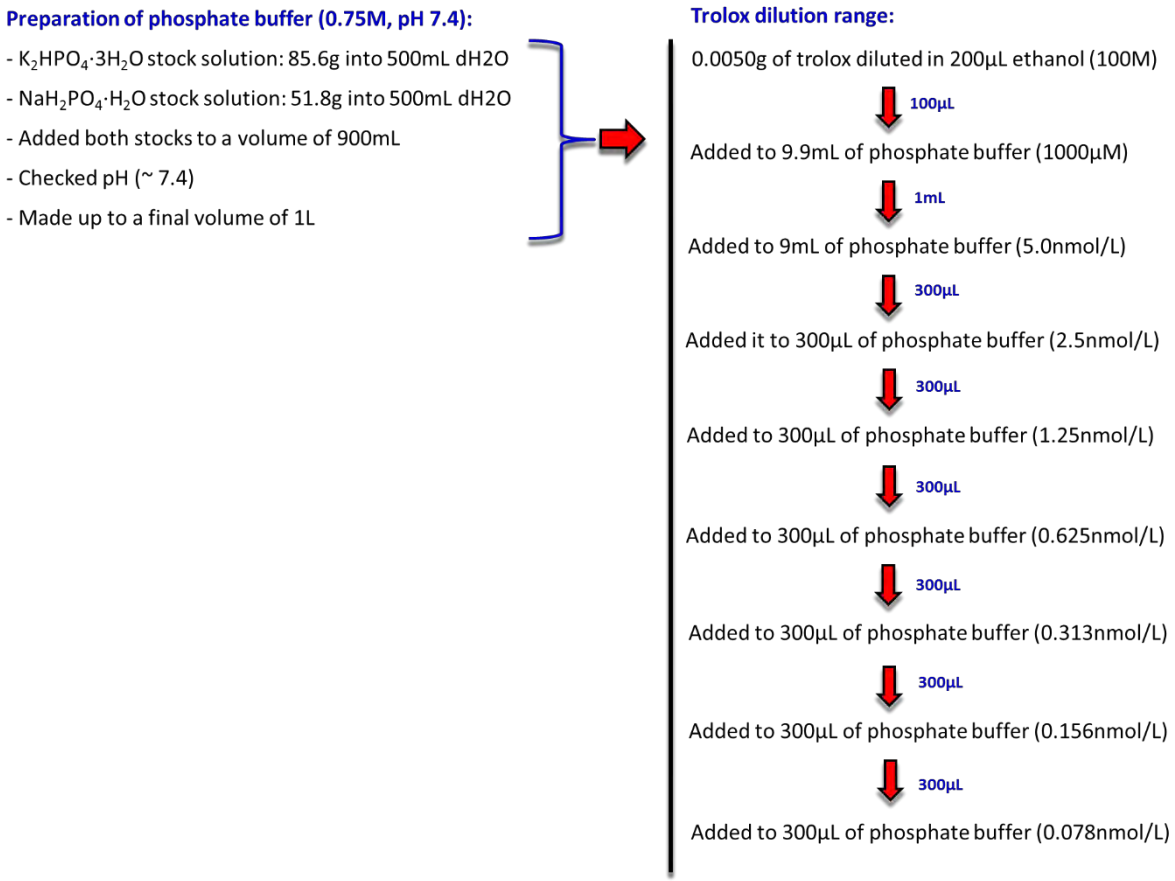


Figure 14. Preparation of phosphate buffer and range of dilutions used to generate a trolox standard curve. Protocol for the preparation of a dilution range which was used for the trolox standard curve. Also denoted here is the preparation of the phosphate buffer, which was used to dilute the trolox. **dH_2O:** distilled water, **Trolox**: 6-OH-2,5,7,8-tetramethylchroman-2-carboxylic acid

Stock solution 1:

- 0.0225g into 50mL of phosphate buffer (0.0011959mol/L)

Stock solution 2:

- Add 50µL of stock solution 1 into 10mL of phosphate buffer (5.98µmol/L)

Working solution of fluorescein:

- 320µL of stock solution 2 into 20mL of phosphate buffer (95.7nmol/L)

Figure 15. Preparation of the fluorescein dilution range. Preparation of the fluorescein dilutions which were used for the rest of the ORAC assay. For these dilutions the same phosphate buffer was used as described in the previous figure. **Fluorescein**: 3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3, **ORAC:** oxygen radical absorbance capacity.

The AAPH solution was prepared by adding 0.087g of AAPH to 980µL of warm (37°C) phosphate buffer, to a final concentration of 0.33M. Fifty microlitres of AAPH was quickly added from wells A5 to well H12 and the timer was started. The amount of AAPH was dependent on the amount of samples analysed plus the number of wells used for the blanks and the trolox standard curve (Figure 16).

The amount of AAP was weighed out:

Enough for the wells with the standards, blanks & each sample

0.0879g AAPH added into 980µL phosphate buffer

or

0.348g AAPH added into 3920µL phosphate buffer

or

0.696g AAP added into 7840µL phosphate buffer

or

1.392g AAPH added into 8800µL phosphate buffer

NOTE: When the correct amount of buffer needed was calculated, it was placed in a separate tube and placed in an incubator to warm to 37°C for later use.

Figure 16. Preparation of AAPH. Preparation of the AAPH that was used for the ORAC assay. **AAPH**: 2,2'-azobis (2-amidinopropane) di-hydrochloride, **ORAC:** oxygen radical absorbance capacity.

The plasma samples were deproteinized by adding 200µL of ice cold (4°C) ethanol (Merck Chemicals, South Africa) to 100µL of plasma. This was vortexed for ten seconds and microfuged at 12000rpm, for five minutes and the proteins precipitated at the bottom of the tube. The supernatant was pipetted into a new tube, to which 500µL of phosphate buffer (0.075M, pH 7.4) was added. Then, the supernatant (sample) was further diluted by first taking 160µL and adding it to 840µL of phosphate buffer. This was vortexed for 10 seconds and 100µL was added to 700µL of phosphate buffer and the final dilution was achieved by taking 200µL of the previous dilution and adding 200µL of buffer. This formed the final sample which was used for further experimentation (Figure 17 and 18).

- 100µL blood plasma + 200µL of ice cold (4°C) ethanol in tubes
- Vortexed for 10 seconds
- Tubes microfuge at 12000rpm, for 5 minutes
- Supernatant transferred into new tubes with pipette
- Supernatant + 500µL of phosphate buffer (0.075M, pH 7.4) - **Dilution A**

Samples were further diluted in the following manner:

- 160µL of dilution A + 840µL of phosphate buffer
- Vortexed for 10 seconds – **Dilution B (50x dilution)**
- 100µL of dilution B + 700µL of phosphate buffer
- Vortexed for 10 seconds – **Dilution C (363x dilution)**
- 200µL of dilution C + 200µL of phosphate buffer
- Vortexed for 10 seconds – **Dilution D (726x dilution)**

***Dilution D, formed the final diluted sample used for further experimentation**

Figure 17. Oxygen radical absorbance capacity – Assay protocol. Range of dilutions made of the samples which were used for the ORAC assay. **ORAC:** oxygen radical absorbance capacity.

- Trolox dilutions, fluorescein, phosphate buffer, diluted samples and AAPH were pipetted into the wells of the 96-well PolySorp-Nunc plate
- Once all the additions were made, the AAPH was added to the warm (37°C) phosphate buffer
- 50µL AAPH was quickly added from wells A5 to well H12 (here, the timer was started)
- Plate was inserted into the plate reader
- Readings were done at excitation wavelength of 485nm and emission wavelength of 520nm

*A micro plate data acquisition program (Advanced Reads®) was used

Figure 18. Oxygen radical absorbance capacity – Assay protocol. Detailed and step-wise protocol of how the trolox standards, fluorescein, phosphate buffer and diluted samples were pipetted into each designated well for the ORAC assay. **Fluorescein**: 3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3, **ORAC:** oxygen radical absorbance capacity, **Trolox**: 6-OH-2,5,7,8-tetramethylchroman-2-carboxylic acid, **AAPH**: 2,2'-azobis (2-amidinopropane) dihydrochloride.

Three hundred microliters of phosphate buffer was added to well A1 and A2 (blank), 200µL of phosphate buffer in A3 and A4 plus 100µL of fluorescein. To wells A5 and A6, 100µL of phosphate buffer was added and 100µL of fluorescein, into wells A7 and in A8 50µL of phosphate buffer and 50µL of fluorescein. In wells A7 to B8, 50µL of phosphate buffer was added as well as 50µL of fluorescein and 50µL of trolox (0.078nmol/L, 0.156nmol/L, 0.313nmol/L, 0.625nmol/L, 1.25nmol/L, 2.5nmol/L and 0.078nmol/L respectively) (table 1). All the samples were then added into wells B9 until H12 (dependent on the amount of samples). Every component of this assay including the samples was added into the wells in a duplicated fashion. For the ORAC assay, the samples were loaded into a 96-well, white flat bottomed, micro titre plate Polysorp®-Nunc (Lasec, South Africa) and readings were recorded at an excitation wavelength of 485nm and emission wavelength of 520nm, with a micro plate data acquisition program (Advanced Reads®).

Table 1. A representation of the amount of phosphate buffer, fluorescein, AAPH and trolox that was added to the wells, from well A1 to B8.

Well number: µL	Buffer: µL	Fluorescein: µL	AAPH: µL	Trolox: µL
A1	300µL	-	-	-
A2	300µL	-	-	-
A3	200µL	100µL	-	-
A4	200µL	100µL	-	-
A5	100µL	100µL	100µL	-
A6	100µL	100µL	100µL	-
A7	50µL	100µL	100µL	50µL (0.078nmol)
A8	50µL	100µL	100µL	50µL (0.078nmol)
A9	50µL	100µL	100µL	50µL (0.156nmol)
A10	50µL	100µL	100µL	50µL (0.156nmol)
A11	50µL	100µL	100µL	50µL (0.313nmol)
A12	50µL	100µL	100µL	50µL (0.313nmol)
B1	50µL	100µL	100µL	50µL (0.625nmol)
B2	50µL	100µL	100µL	50µL (0.625nmol)
B3	50µL	100µL	100µL	50µL (1.25nmol)
B4	50µL	100µL	100µL	50µL (1.25nmol)
B5	50µL	100µL	100µL	50µL (2.5nmol)
B6	50µL	100µL	100µL	50µL (2.5nmol)
B7	50µL	100µL	100µL	50µL (5.0nmol)
B8	50µL	100µL	100µL	50µL (5.0nmol)

1.5. Catalase activity assay

The catalase activity assay is based on the principle that catalase, an antioxidant enzyme, catalyses the reduction of hydrogen peroxide to water [316]. For this assay, 30% hydrogen peroxide (Sigma Aldrich, South Africa) was diluted to a concentration of 12mM by adding 150µL of hydrogen peroxide to 100mL of phosphate buffer. The phosphate buffer was prepared by adding KH_2PO_4 (50mM) to Na_2HPO_4 (50mM) to a final volume of one litre and a final concentration of 50mM (pH 7.0). All the samples were diluted to a ratio of one-in-ten. Into the first two wells (A1 and A2), 241µL of phosphate buffer was added and 241µL of hydrogen peroxide into wells A3 and A4. In the remainder of wells, 10µL of the diluted samples was added as well as 10µL of phosphate buffer.

The Costar 96-well plate (Lasec, South Africa) was then inserted into the spectrophotometer (SPECTRAmaxPLUS-384) and a reading was recorded at 240nm to ascertain that the absorbance of the diluted hydrogen peroxide was approximately 0.523 ± 0.025. Hereafter, 221µL of the diluted hydrogen peroxide was added to each well and the timer was started. A reading was recorded (at time point zero) and exactly one minute later another reading was recorded (Figure 19). The values were typed into a Microsoft Excel sheet, and the one minute reading was subtracted from the reading at time point zero. The difference between these two values was used to calculate the final catalase activity which was expressed as international units per milligram protein.

- Phosphate buffer was prepared by adding KH_2PO_4 (50mM) to Na_2HPO_4 (50mM) = 1 litre (pH 7.0)
- 150µL of hydrogen peroxide (30%) was added into 100mL of phosphate buffer (12mM, pH 7.0)
- 10µL of blood plasma + 90µL phosphate buffer (a one-in-ten dilution)
- 241µL of phosphate buffer was added into the first two wells (A1 and A2)
- 241µL of the hydrogen peroxide was added into wells A3 and A4
- 10µL of the diluted samples + 10µL of phosphate buffer was added into all wells
- The 96-well plate was inserted into the spectrophotometer
- A reading was recorded at 240nm
- 221µL of the diluted hydrogen peroxide was added to each well & the timer was started
- Immediately after, another reading was recorded (time point zero)
- Exactly one minute later the final reading was recorded

Figure 19. Catalase activity – Assay protocol. A detailed and step-wise protocol of how the phosphate buffer, diluted samples and diluted hydrogen peroxide were pipetted into each designated well, for the catalase activity assay. **KH_2PO_4**: dipotassium phosphate, **Na_2HPO_4:** sodium diphosphate.

1.6. Superoxide dismutase assay

Plasma superoxide dismutase (SOD) activity was measured by using a method adapted from Dalloz et al. [317]. This assay is based on the principle that xanthine oxidase catalyses the oxidation of xanthine to uric acid and in the process ROS is produced. During this chemical reaction, cytochrome-C is reduced and this reduction rate as measured at 550nm wavelength[317]. The first step was to prepare all constituents of the assay including phosphate buffer, sodium azide, xanthine, ammonium sulphate, superoxide dismutase (stock solutions for the purpose of the standard curve), xanthine oxidase solution and the reaction solution (Figure 20). The second step was to determine the reaction slope (absorbance) of the xanthine oxidase solution, during which more or less xanthine oxidase was added to adjust the reaction slope (absorbance) to approximately 0.025±0.027. To do this, 290µL of the reaction solution was added to wells A1 and A2 of the Greiner 96-well plate, together with 6µL of phosphate buffer. In wells A3 and A4 was added 290µL of reaction solution and 9.5µL of phosphate buffer. Then, 3.5µL of xanthine oxidase was only added to wells A1 and A2 and the timer was started for 3 minutes. The plate was inserted into the spectrophotometer (pre-heated to 37°C) and the absorbance was read at 550nm, with a micro plate data acquisition program (SoftMax® Pro 4.8). This was considered the reading at time point zero and after exactly three minutes, another reading was recorded. The reaction slope of the xanthine oxidase was then calculated by subtracting the last reading from the first. Once, this slope (absorbance) was adjusted to approximately 0.025±0.027, the rest of the assay was performed (Figure 21).

The third step was to prepare the range of SOD dilutions in order to generate the SOD standard curve. Here, the SOD stock solution was used to prepare five standards: zero IU/mL (0µL SOD in 5mL phosphate buffer), 10 IU/mL (10µL SOD in 4990µL phosphate buffer), 20 IU/mL (20µL SOD in 4980µL phosphate buffer), 40I U/mL (40µL SOD in 4960µL phosphate buffer), 80 IU/mL (80µL SOD in 4920µL phosphate buffer). In a clean 96 well plate, 3.5µL of each standard was pipetted into wells A1 to A10 in a duplicate manner. In these wells were also added 290µL of reaction solution and 6µL of phosphate buffer. During the fourth step of the assay, 3.8µL of each blood plasma sample was added to the separate wells (dependant on the amount of samples assayed). After all the additions were made, 3.5µL of xanthine oxidase was added to all the wells containing the SOD and the plate was inserted into the spectrophotometer. A reading was recorded at time point zero with a micro plate data acquisition program (SoftMax® Pro 4.8) and exactly three minutes later another reading was

recorded (Figure 22).The final reaction slope was determined and the final plasma SOD activity was expressed in international units per milligram protein.

Phosphate buffer (50mM, pH 7.7, EDTA 0.1mM):

Part 1: KH_2PO_4 solution
- 1.7g of KH_2PO_4 in 250mL distilled water

Part 2: Na_2HPO_4 solution
- 7.1g of Na_2HPO_4 in 1L distilled water

Part 3:
- Weighed out 34.2mg EDTA

*In a 1L beaker, the EDTA was added to approximately 100mL of the KH_2PO_4 solution and 800mL of the Na_2HPO_4 solution. The pH was adjusted to 7.7 with either KH_2PO_4 or Na_2HPO_4.

Sodium azide solution (10mM):

- 6.5mg of sodium azide was dissolved in 10mL distilled water

Ammonium sulphate solution [$(NH_4)_2SO_4$ 3mM, pH 7.00]:

- 39.63mg of $(NH_4)_2SO_4$ was dissolved in 100mL distilled water and the pH was adjusted to 7.00.

Xanthine solution (2mM, pH 7.7):

- 15.2mg of xanthine was dissolved by added a few drops of NaOH (0.1M). 40mL of distilled water was added, the pH adjusted to 7.00 with NaOH or HCl and made up to a final volume of 50mL.

SOD solution (5000IU/mL):

- 10mg SOD (300 000IU/58mg vile) was dissolved into 10.5mL $(NH_4)_2SO_4$. Aliquots of 150µL each was prepared and stored in a -20°C freezer for later use.

Reaction solution:

- 22.4mg cytochrome-C, 4.544mL xanthine solution, 0.1mL sodium azide and 95mL phosphate buffer was mixed together. This reaction solution was incubated at 25-26°C and protected from light.

Figure 20. Superoxide dismutase activity – Assay: Preparation of assay solutions/buffers. A detailed protocol of how the phosphate buffer, was prepared for the SOD activity assay. **KH_2PO_4**: di-potassium phosphate, **Na_2HPO_4:** sodium diphosphate, **EDTA:** Ethylene-di-amine-tetra-acetic acid, **SOD:** superoxide dismutase, **$(NH_4)_2SO_4$:** ammonium sulphate, **NaOH:** sodium hydroxide, **HCl:** hydrogen chloride).

- 290µL of the reaction solution added to wells A1 and A2 of a 96-well plate (Greiner)
- 290µL of reaction solution added to wells A3 and A4 plus 9.5µL of buffer
- Added 6µL of phosphate buffer and 3.5µL of xanthine oxidase solution
- The plate was inserted into the spectrophotometer (pre-heated to 37°C)
- The absorbance was read at 550nm (this was considered the reading at time point zero)
- Exactly two minutes later, another reading was recorded
- The reaction slope of the xanthine oxidase was then calculated by subtracting the last reading from the first
- Once, this slope was adjusted to approximately 0.025±0.027, the rest of the assay was performed

Figure 21. Superoxide dismutase activity – Assay: Determination of xanthine oxidase reaction slope. A detailed and step-wise protocol of how the xanthine oxidase reaction slope was determined for the SOD activity assay.

One aliquot of SOD stock solution, prepared previously, was thawed and kept on ice and used to prepare the five SOD standards as shown below:

- Standard 1: zero IU/mL (0µL SOD in 5mL phosphate buffer), Standard 2: 10 IU/mL (10µL SOD in 4990µL phosphate buffer), Standard 3: 20 IU/mL (20µL SOD in 4980µL phosphate buffer), Standard 4: 40 IU/mL (40µL SOD in 4960µL phosphate buffer) and Standard 5: 80 IU/mL (80µL SOD in 4920µL phosphate buffer)
- In a clean 96 well (Greiner) plate, 5µL of each standard was pipetted into wells A1 to A10 in a duplicate manner
- In these wells were also added 290µL of reaction solution and 6µL of phosphate buffer

- Then, 3.8µL of each blood plasma sample was added to the designated wells (which depended on the amount of samples assayed)
- After all the additions were made, 3.5µL of xanthine oxidase was added to all the wells
- The plate was inserted into the spectrophotometer
- A reading was recorded at time point zero and exactly 3 minutes later another reading was recorded. Final reaction slope was then calculated (OD reading at time point two minutes subtracted from the OD reading at time point zero)

Figure 22. Superoxide dismutase activity – Assay: Preparation of the superoxide dismutase standards and protocol of addition of samples for final assay. A detailed and step-wise protocol of how the SOD standards were prepared and the blood plasma samples, phosphate buffer and xanthine oxidase solution were added to the designated wells for the determination of SOD activity. **SOD:** superoxide dismutase, **OD:** optical density.

1.7. Protein determination of blood plasma samples

Blood plasma protein concentrations were determined by using the Quant-iT fluorometer (Invitrogen, South Africa). The kit was very selective for proteins in a range of 0.25-5.0µg/mL and when purchased, it included protein buffer, three standards and a protein reagent (fluorophore). The fluorometer was calibrated with three standards and all dilutions were made as described by the manual provided by the manufacturer. One micro litre of plasma was diluted by adding 199µL working solution (protein reagent plus protein buffer) and left to incubate for 15 minutes at room temperature. The tubes containing the diluted plasma samples were inserted into the fluorometer and a reading was recorded at 470nm excitation/590nm emission. If the protein concentration of the samples were too high or out of range, the samples were further diluted by adding 100µL of the previous sample dilution to 100µL of protein buffer.

2. Validation of the experimental model of pulmonary hypertension

2.1. Housing of rats

All experiments were conducted in male Long Evans rats, as housed by the University of Cape Town's animal unit, in a 12 hour dark/light cycle. Room temperature was kept at 22°C and humidity at 40% and the rats had *ad libitum* access to food and drinking water. All protocols were carried out in compliance with the guide for the care and use of laboratory animals published by the United States National Institute for Health in 1996. All experimental protocols were approved by the Faculty of Health Sciences animal Ethics Committee, University of Cape Town (ethical number 011/033). A maximum of three rats were housed in a cage and their tails were marked with a permanent marker which ensured that we knew exactly which data was generated from which rat. This was important as rats may respond differently to treatment and allowed us to retrospectively analysed data of every individual rat.

2.2. Experimental model of monocrotaline-induced pulmonary hypertension

Monocrotaline (MCT) an alkaloid that originates from the plant *Crotalaria spectabilis,* was found to be a constituent of herbal tea used by natives in the West Indies many years ago and has been reported to cause hepatotoxicity as well as PH [318-321]. Initially, a model of PH was established by feeding rats the seeds of *Crotalaria spectabilis* or by injecting non-human primates with a suspension of MCT [322-324].

The mechanism whereby MCT causes PH requires it to be metabolized in the liver by the enzyme cytochrome-P450 into pyrrolic derivatives which initiates endothelial injury in the lung [325]. This leads to pulmonary vasculitis and obstructive pulmonary vascular remodelling characterized by narrowing/obliteration of the vascular lumen features that are seen in various manifestations of PH [324,325]. This pulmonary vascular remodelling precipitates as PH and RV remodelling [324,325]. It is now general practice to inject rats with a single subcutaneous dose of MCT (60-80mg/kg) to induce moderate to severe PH and RV remodelling after 21-28 days following injection of MCT [208,326,327].

In this project, the body weights of all the rats were measured every day during the treatment protocol (from day zero, which represents the day of injection, until day 28 or day minus five until day 28) when appropriate. The weights of the animals were recorded in monitoring sheets tailored specifically for this project (Table 2). In the monitoring sheet, a whole range of factors were assessed including abnormal feeding, lethargy, piloerection, cyanosis of lower extremities, dyspnoea, discomfort and stress and physical deterioration as well as injection with MCT or the treatment with melatonin. If any rat lost more than ten percent of its initial body weight (as documented on day zero) it was euthanized with an intraperitoneal injection of sodium pentobarbitone (160mg/kg). The carcase was concealed in a plastic bag; placed in a -20°C refrigerator and incinerated by a bio waste removal company.

Table 2. A representation of the animal monitoring sheet that was used for the daily monitoring of animals in this project

Animal Identification/Animal number/Animal treatment group:										
Date:										
Time of day:	Am	Pm	Am	Pm	Am	Pm	Am	Pm	Am	Pm
Monocrotaline:										
Melatonin:										
Bodyweight:										
Abnormal feeding:										
Low physical activity:										
Piloerection:										
Cyanosis of lower extremities:										
Fast shallow breathing:										
Discomfort/Stress: Score = 1 (mild) Score =2 (moderate) Score =3 (severe)										
Physical deterioration or distress:										
Signature of investigator:										

The dose of MCT (Sigma-Aldrich, South Africa) consisted of a single subcutaneous injection of MCT at 80mg/kg and this dose was chosen based on a study done by Handoko et al. (2009) as this was the only study that used this particular dose of MCT and measured cardiac function with the isolated rat heart perfusion apparatus. The MCT injection resulted in RV remodelling in the third to fourth week after injection (approximately 28 days) [208]. All control rats received one single subcutaneous injection of sterile saline (0.9% NaCl). The calculation and preparation of MCT, for example three rats (per cage), was done by weighing all three of the rats on day zero. On day zero, rats were dispatched by the animal unit staff according to the required range of body weight. As an example, for one set of experiments, a total of 24 rats were received and randomly divided into six rats per group. Then for each group, three rats were kept in one cage which meant that each group had two cages with three rats per cage. In total, there were eight cages for such an experiment. The three rats in each cage were marked on the tail with a black permanent marker by means of black stripes (one to three). All the rats were weighed and the initial body weights were recorded in rat monitoring sheets (Table 2).

2.3. Preparation of the monocrotaline solution

The amount of 140mg MCT was then dissolved in approximately 500µL of hydrochloric acid (1N) and neutralized with 500µL of sodium hydroxide (10N) [328]. This yielded a total of 1mL MCT, which was made up to 5mL with sterile saline (0.9%). The pH of this solution was then measured and set to a pH of 7.4-7.5 with either hydrochloric acid (1N) or sodium hydroxide (10N) (Jenway 3510 pH meter, Lasec, South Africa). After the pH was adjusted, the solution was made up to a volume of 8mL and the pH was checked again just to confirm the previous measurement. If the pH was correct, the solution was brought to final volume of 10mL and the final concentration of MCT was 14mg/mL.

2.4. Echocardiography: Cardiac function

Anaesthesia was induced with 4% isoflurane gas (SAFELINE Pharmaceuticals (pty) ltd.) and maintained at 2% isoflurane in room air supplemented with 100% O_2 (Afrox, South Africa). Rats were laid in a supine position, with all legs taped to a heated pad. Body temperature was maintained at 36.5°C to 37.5°C using the heated pad and infrared lamp. The rat's chest was shaved with an electrical hear removal machine to reduce ultrasound interference. To provide a coupling medium for the transducer, a warm ultrasound gel was spread over the

chest wall. Transthoracic 2-dimensional, B and M-mode imaging was performed with a high-resolution imaging system equipped with a 10-MHz transducer (Vivid-E, GE Healthcare). A number of parameters were measured including LV-end diastolic volume, LV-ejection fraction; LV-fractional shortening and LV end diastolic volume. After the echocardiography procedure was performed on day zero, the rat was placed in a cage and allowed to regain consciousness. When the procedure was performed on the day of sacrifice (day 28), the rat was kept on isoflurane (4%) inhalation, to induce a deep unconscious state.

2.5. Isolated rat heart perfusions: Cardiac function

Directly after echocardiography, the isoflurane was kept at 4% and maintained for approximately 15 minutes which allowed the rat to become completely unconscious. The chest cavity was cut open with scissors; the heart was rapidly excised and placed in ice cold Krebs-Henseleit buffer. The final composition of the buffer was as follow: 118.5mM NaCl, 25mM $NaHCO_3$, 4.75mM KCl, 1.18mM $MgSO_4.7H_2O$, 1.2mM KH_2PO4, 1.36mM $CaCl_2.2H_20$ and 11.1mM Glucose. Within a few minutes, the heart was cannulated via the aorta onto the Langendorff isolated rat heart perfusion apparatus and perfused retrogradely.

Perfusion was performed at a constant pressure of 100cm H_20 at 37°C, with Krebs-Henseleit buffer (pH 7.4) equilibrated with oxygen/carbon dioxide ratio of 95:5% (Afrox, SA). A water-filled, latex balloon which (was custom made) was connected to a pressure transducer and the data were recorded on a Powerlab, Lab chart computerized data acquisition program (ADI Instruments, South Africa). The balloon was inserted into the left ventricle via the left atrium. The temperature of the heart was measured with a fine thermocouple wire (Physitemp, NJ, USA) and monitored with a Digitron 2600T temperature sensor (Torquay, UK). The LV-end diastolic pressure was adjusted and maintained at 8-9mmHg and the heart was perfused for 30 minutes (with the latex balloon still in the LV). This served as stabilization period. After this, the heart was perfused for an additional 30 minutes upon which the balloon was removed and inserted into the RV. Once the balloon was in the RV, the heart was allowed to stabilize for 30 minutes and perfused for an additional 30 minutes (to obtain RV measurements) (Figure 23).

The cardiac parameters that were measured in both ventricles included heart rate, coronary flow rate (mL/min) and ventricular developed pressure (LV and RV) [LV-developed pressure (LVDP) and RV-developed pressure (RVDP)]. The LVDP and RVDP were calculated by subtracting the systolic pressure from the diastolic pressure. Our exclusion criteria were (i) a

minimum LVDP of 80mmHg, (ii) a minimum coronary flow rate of 8mL/min and a maximum of 16mL/min, (iii) a minimum heart rate of 250 beats per minute and a maximum of 400 beats per minute.

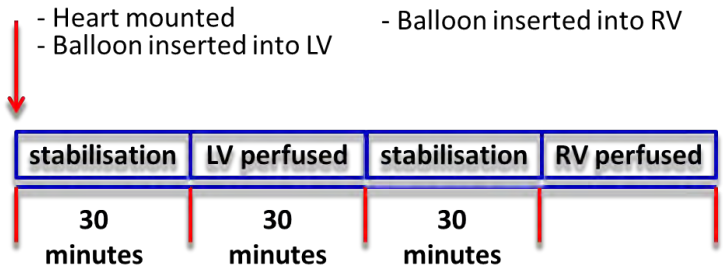


Figure 23. Isolated rat heart perfusion protocol. Rat heart was mounted onto the cannula of the Langendorff isolated rat heart apparatus via the aorta and perfused retrogradely. The heart was perfused at a constant pressure of 100cm H_20 at 37°C with Krebs-Henseleit buffer. A water filled latex balloon was inserted into the LV and perfused with the balloon in the LV for a stabilisation period of 30 minutes. This was followed by an additional 30 minutes perfusion in the LV. Then the balloon was removed and inserted into the RV, stabilized for 30 minute and perfused for another 30 minutes. **RV**: right ventricle/ventricular, **LV**: left ventricle/ventricular, **H_20**: water, **°C**: degrees Celsius

2.6. Blood sample collection

Blood samples were collected from the thoracic cavity immediately after the heart was removed for perfusion. A squeezable plastic Pasteur pipette (Lasec, South Africa) was used to pipette the blood from the thoracic cavity into a BD vacutainer blood collection tube (containing EDTA) (Lasec, South Africa). The tube was gently tilted twice and immediately placed on ice. The tubes were then transferred to a precooled (4°C) bench centrifuge and centrifuged for 10 minutes at a rotor speed of 2500rpm. After centrifugation, the blood samples separated into two phases with the plasma phase on top. Aliquots of 300µL plasma were transferred to 2mL Eppendorf tubes (Lasec, South Africa). These tubes were marked with the number of the rat, the treatment group, melatonin dose and date. The aliquots were stored at -80°C until the measurement of the various assays including the protein concentration assay kit were performed.

2.7. Biometrical measurements

After completion of the perfusion protocol, the heart was weighed and the RV separated from the LV plus septum. The weight of the whole heart, RV and LV plus septum was recorded. The right tibia of every rat was excised and its length was also measured and recorded. The weight of the RV was divided by the LV plus septum, or tibial length or the whole heart weight and these ratios were used as different indexes of RV hypertrophy [329]. The liver and lungs were excised from the rat carcase, rinsed with 1% phosphate buffered saline, slightly dried with a paper towel and weighed. Other biometrical indexes included liver weight and lung weights divided by body weight which gave an indication of either loss of liver mass (liver atrophy) or pulmonary oedema [69].

2.8. Blood plasma assays and protein determination

The protocol for the determination of protein concentration of each blood plasma was the same as the protocol for the human blood plasma. The blood samples from each group were designated for the determination of oxidative stress levels with assays including the TBARS, ORAC, and catalase and superoxide dismutase activity (Figures 13 – 21 and Table 1), as previously described.

2.9. Histology of hearts

2.9.1. Cardiac interstitial fibrosis

In a separate set of experiments, the hearts were perfused on the isolated apparatus, the RV was separated from the LV plus septum and washed with 1% phosphate buffered saline. The RV and LV plus septum were fixed with 4% paraformaldehyde (Sigma Aldrich, South Africa) for one day. The following day, the LV plus septum were sliced into four equally sized cross sections and the RV was kept unsliced. This sectioning procedure allowed for the proper fixation of all areas of the heart. The heart sections (RV and LV plus septum) were placed back into the 4% paraformaldehyde for another day. Thus, in total, the heart sections were fixed in 4% paraformaldehyde for two consecutive days.

After the two days, the sections were washed with and placed in 70% ethanol and embedded in paraffin. Subsequently, the slices were sectioned (cross sectional and transverse) at a thickness of 3µm for histological analysis [329]. The purpose of the 70% ethanol step was to stop the fixation process in order to detect collagen deposition. Furthermore, the RV and LV

sections were stained with 0.1% Sirius red in picric acid (Sigma Aldrich, South Africa) for the determination of collagen deposition (Table 3). Sections were examined with a Nikon Eclipse 90i microscope using a 20 times objective and the images were captured with the use of NIS-Elements Basic Research software (Nikon, Instech Co. Ltd, Japan). Further quantification, to determine the interstitial collagen fraction was done with the Visiopharm Integrator System (Leica Microsystem, Wetzlar, Germany).

Table 3. Sirius-red in picric acid staining protocol

Step:	Solution/Incubation:	Remarks:	Time:
1	Incubation at 58°C	Melting of paraffin	60 minutes
2	Xylol	Deparaffinization	10 minutes
3	Xylol	Deparaffinization	10 minutes
4	Xylol	Deparaffinization	10 minutes
5	99.6% Ethanol	Rehydration	5 minutes
6	99.6% Ethanol	Rehydration	5 minutes
7	96% Ethanol	Rehydration	5 minutes
8	70% Ethanol	Rehydration	5 minutes
9	Distilled water	Washing	3 minutes
10	0.1% Sirius red in picric acid	Staining	60 minutes
11	1% Glacial acetic acid	Washing	5 seconds
12	1% Glacial acetic acid	Washing	5 seconds
13	1% Glacial acetic acid	Washing	5 seconds
14	Distilled water	Washing	1 minute
15	70% Ethanol	Dehydration	5 minutes
16	96% Ethanol	Dehydration	5 minutes
17	99.6% Ethanol	Dehydration	5 minutes
18	Xylol	Clearing	10 minutes
19	Xylol	Clearing	10 minutes
20	Mounting & coverslip	-	-
21	Dry at room 25°C	-	24hrs

3. Melatonin as a therapy in PH

3.1. Melatonin dose calculation: Three different doses

Melatonin (Sigma Aldrich, South Africa) was given orally in the drinking water at 75ng/L, 4mg/kg or 6mg/kg. These doses of melatonin were based on studies previously published [250,273,309]. The concentration of 75ng/L is the same as the concentration of melatonin found in red wine and other foods/plants, while the higher doses of melatonin (4mg/kg and 6mg/kg) are comparable to the doses found in pills available at pharmacies. The treatment with melatonin was started on day zero of the treatment protocol i.e. on the same day as that the MCT injection was given. Melatonin treatment continued until day 28.

3.2. Preparation and administration of melatonin

3.2.1. 75ng/L concentration of melatonin

75mg of melatonin was dissolved in absolute ethanol at a final concentration of 0.05% (v/v) and made up to a final volume of 1L of distilled water (this was solution A). Two successive dilutions were prepared by firstly taking 1mL of solution A and adding it to 100mL of distilled water (solution B). Secondly, 100µL of solution B was added to 100mL of distilled water (solution C.) Solution C had a final melatonin concentration of 75ng/L and was used to treat the rats. Other researchers in the laboratory had previously performed preliminary studies to confirm that addition of melatonin in the drinking water does not change the drinking behaviour of the rats.

3.2.2. 4mg/kg and 6mg/kg melatonin

For the calculation of these doses of melatonin, two factors were taken into consideration including average daily water consumption and body weight. As three rats were housed in one cage, they shared a water bottle. The first factor taken into consideration was the average body weight, which was used to calculate the amount (grams) of melatonin necessary for three rats. It is known that a rat with a body weight of approximately 100g drinks approximately 14-15mL water per day. [330] However, during the 28 day treatment period, the rats either gained or lost weight and this was also taken into account. Thus, on average, a rat with a body weight of 100g drinks 15mL of water per day, a 200g rat 30mL and a 300g rat 45mL. If a cage had for example an average body weight of approximately 160g, then the daily water consumption was taken as 15mL. Thus, together, the average body

weight and daily water consumption was used to calculate the amount of melatonin each rat received per total amount of water drank per day.

Usually, when the animal unit staff exchanged the cages and bedding, the water bottles were often moved or shaken and inevitably leaked. With this arose the possibility that some rats may not receive enough melatonin or the bottle may be empty before adequate amounts of melatonin were consumed by the rats. To prevent this, enough melatonin (a maximum of 500mL per bottle) was prepared so that even in the event of accidental loss, the rats still had enough melatonin to last for the night. This was done, by multiplying the amount of melatonin by a certain factor (e.g. 6) in order to achieve a maximum volume of 500mL melatonin per bottle. The total amount of water and melatonin was multiplied with the same factor to ensure that the final concentration ratio remained correct.

After the correct final amount of melatonin was calculated, it was dissolved in absolute ethanol (Sigma Aldrich, South Africa) at a final concentration of 0.05% (v/v) and then added it to the total amount of water for each cage [273]. The water bottles containing the melatonin were covered with aluminium foil, because melatonin is light sensitive and the foil was replaced on a daily basis, if necessary. Melatonin was made up fresh every day, even if there was some melatonin left the next day.

4. Data analyses: Statistical tests

Unless stated otherwise, all data were expressed as mean ± standard error of the mean (SEM). For comparative studies, Student's t-test (unpaired) or two-way ANOVA analyses (with Bonferroni post-test, if $p< 0.05$) were used respectively. All statistical test were performed using Graph Pad Prism-5. The experimental groups used for the fibrosis determination were statistically analysed by one-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. A $p<0.05$ was considered as significant.

SECTION D

EVALUATION OF OXIDATIVE STRESS IN A PATIENT POPULATION OF PULMONARY HYPERTENSION

1. Introduction

Over the past few years, experimental and clinical studies suggest that PH is associated with an increase in oxidative stress in the lung, the heart as well as the plasma/serum [213,225-227,229,331]. Although it is believed that oxidative stress may play a role in the development and progression of PH and RV remodelling, its exact mechanism is not fully understood [30,210,211]. In a recent study, oxidative stress was shown to be associated with increased pulmonary artery systolic pressure in patients with PH, displaying the ability of oxidative stress to be used a possible biomarker of PH progression [332]. Currently, there is no effective treatment available for PH, in part, to the diversity and lack of knowledge of the mechanisms involved in the development of the disease. [145] A better understanding of the mechanisms involved with oxidative stress-induced cardiovascular damage associated with PH, may lead to the development of novel and effective therapies against the disease. In the present section, we aim to evaluate the presence of oxidative stress in a South African population suffering from PH.

2. Materials and Methods

In brief, patients were referred from a primary to a secondary health institution, with symptoms including shortness of breath, fatigue, peripheral oedema, and persistent cough at night. Ten of these patients underwent clinical investigation and examinations in order to confirm diagnosis of PH. Blood samples were collected from the ten PH patients and ten healthy individuals (used as controls). All the blood samples were used for the determination of lipid peroxidation, overall antioxidant capacity as well as the activity of antioxidant enzymes catalase and superoxide dismutase (Figure 24) as described in sections C.

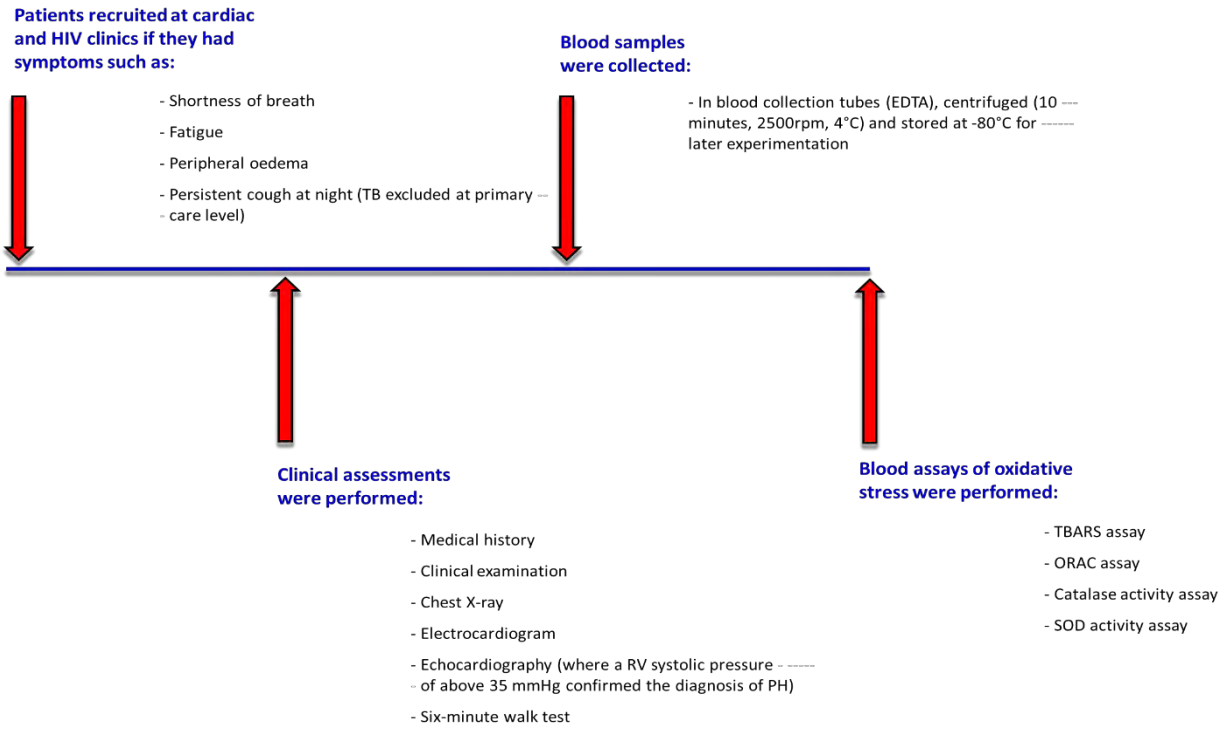


Figure 24. Evaluation of oxidative stress in patients with PH: Experimental design. A summarized layout of the experimental design depicting the recruitment, assessments of patients as well as the collection of blood samples and designated experimental procedures. **HIV:** human immune deficiency virus, **TB:** tuberculosis, **EDTA:** ethylene-diamine-tetra acid, **RV:** right ventricle, **PH:** pulmonary hypertension, **TBARS:** thiobarbituric acid reactive substance, **ORAC:** oxygen radical absorbance capacity, **SOD:** superoxide dismutase.

3. Data analyses: Statistical tests

All data were expressed as mean ± standard error of the mean (SEM). For comparative studies, a Student's t-test (unpaired) was used performed. A $p<0.05$ was considered as statistically significant.

4. Results

4.1. Demographic and clinical profile of patients with pulmonary hypertension

Table 4 summarizes the demographic data and clinical profile of the ten patients with PH who were included in our study. These data included mean age, history of smoking, body mass index (BMI), World Health Organisation functional classes (I-IV), mean heart rate, blood pressure (systolic and diastolic), haemoglobin levels and HIV status (human immune deficiency virus infection).

Table 4. Demographic and clinical profile of patients with pulmonary hypertension

Variable	**All patients with PH (n=10)**
Gender	Female (8), Male (2)
Mean age (years)	37.70 ± 3.68
History of smoking (n)	4
BMI (kg/m^2)	23.24 ± 1.7
WHO functional class II/III-IV	II (2) III (4) IV (4)
Shortness of breath (n)	8
Fatigue (n)	6
Dizziness (n)	6
6 minute walking distance (m)	271.50 ± 56
Pulse at rest (bpm)	95.20 ± 6
Systolic blood pressure (mmHg)	121.30 ± 4.92
Diastolic blood pressure (mmHg)	77.80 ± 3.32
LV ejection fraction (%)	54.70 ± 4.33
RV systolic pressure (mmHg)	72.60 ± 6.58
Anaemia (Hemoglobin)	12.41 ± 0.63
HIV infection	6

PH: pulmonary hypertension, **BMI**: body mass index, **WHO:** World health organisation, **HIV:** Human immunodeficiency virus, **RV:** right ventricular. All the values are expressed as the mean ± standard error of the mean (SEM).

4.2. Evaluation of oxidative stress in controls and patients with pulmonary hypertension

Levels of blood plasma lipid peroxidation were measured by assessing the thiobarbituric acid reactive substances (TBARS) in patients with PH and healthy controls. PH patients had significantly higher levels of plasma lipid peroxidation compared to controls (1.23 ± 0.11 vs. 0.89 ± 0.02 µmol/mL, $p<0.008$) (Figure 25).

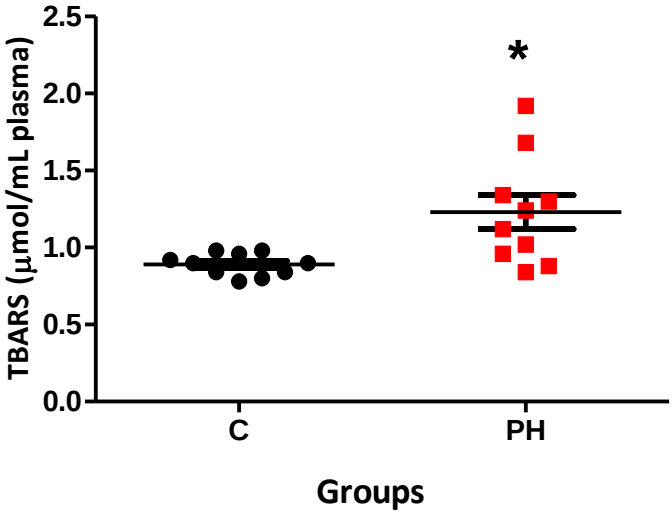


Figure 25. Plasma lipid peroxidation (TBARS) in control (C) and PH patients, *****$p<0.008$ (PH vs. C), n=10 per group.

Antioxidant capacity was measured using the ORAC assay. PH patients had a similar plasma antioxidant capacity compared to controls (10.86 ± 2.89 vs. 5.65 ± 0.94 nmol/L trolox equivalents) (Figure 26).

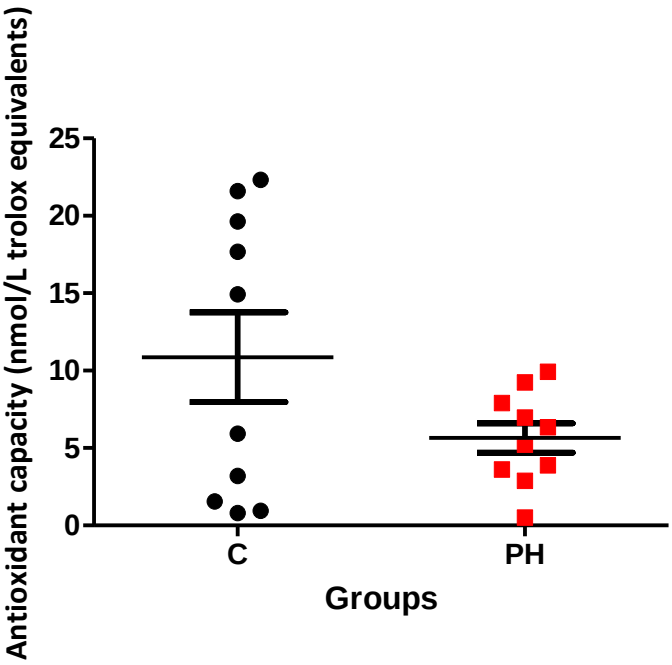


Figure 26. Plasma antioxidant capacity in control (C) and PH patients, n=10 per group.

Catalase activity of the PH patients was significantly higher compared to controls (9.03 ± 1.92 vs. 3.56 ± 0.33 IU/mg protein, $p<0.01$) (Figure 27).

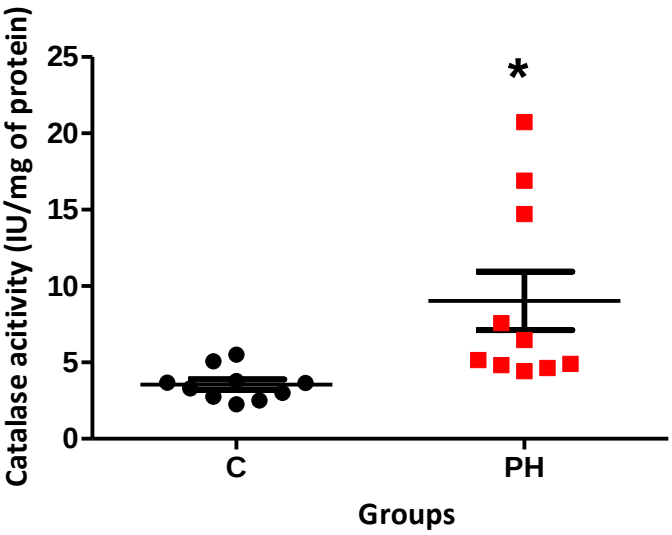


Figure 27. Plasma catalase activity in control (C) and PH patients, ***$p<0.01$** (PH vs. C), n=10 per group.

SOD activity of the PH patients was significantly higher compared to controls (14.41 ± 0.74 vs. 11.87 ± 0.25 IU/mg protein, $p<0.005$) (Figure 28).

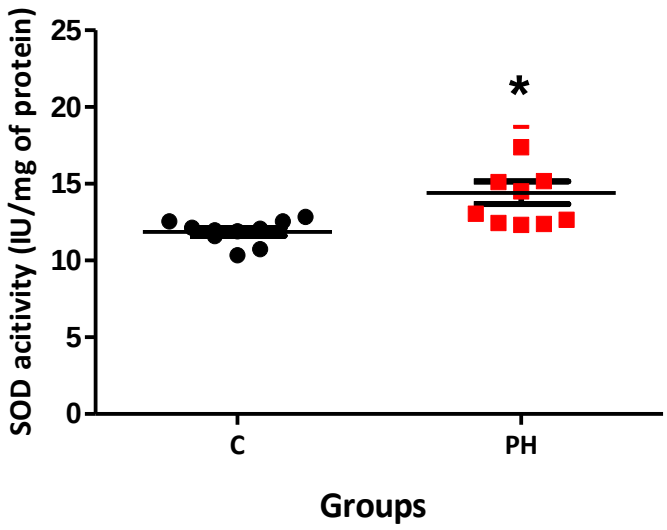


Figure 28. Plasma superoxide dismutase (SOD) activity in control (C) and PH patients, *****$p<0.005$ (PH vs. C), n=10 per group.

5. Discussion

PH and the resultant RV failure, enhances the production of ROS and may diminish antioxidant activity, resulting in oxidative damage to crucial cell components such as DNA, proteins and lipids [211]. Oxidative damage elevates the breakdown by-products of these oxidative processes which can be detected in plasma, serum or urine samples of patients and is referred to as oxidative stress [211]. In PH, oxidative stress is suspected to be involved in the initial "trigger" stages of the disease development where it may contribute to endothelial injury in the pulmonary vasculature [30,210,211]. The potential role for oxidative stress in PH is even more likely as all cells in the lung can generate ROS mediated by alveolar macrophages, via nicotinamide adenine dinucleotide phosphate-(NADPH)-oxidase [212].

In our study, we showed that plasma oxidative stress was increased in a South African patients with PH compared to healthy controls. These findings are corroborated by others who report similar findings [225,226,229]. Irodova et al, showed that plasma of patients with idiopathic PH had higher concentrations of malonic dialdehyde (lipid peroxidation) than healthy volunteers [213]. Also, urine levels of F2-isoprostane, another marker of oxidative stress were increased in PH patients in a mass spectrometry based study [227]. Furthermore, immunohistochemical studies by Bowers et al, showed that lung tissue from patients with idiopathic PH increased 8- hydroxyguanosine staining which was an indication of oxidative damage to DNA [210]. Preston et al, used high-performance liquid chromatography, and demonstrated that patients with idiopathic PH have lower levels of lipophilic plasma antioxidants such as alpha-tocopherol and beta-carotene [228]. These studies demonstrate that PH is associated with an increase in oxidative stress.

In our study, we also showed that the overall oxygen radical absorbance (antioxidant) capacity of the blood plasma of PH patients showed a trend towards decreased antioxidant capacity. However, this was not statistically different, which may have been due to considerable variation within the group and relatively small sample size for human samples. We also showed that plasma antioxidant enzyme (catalase and SOD) activity was increased in patients with PH compared to healthy controls. These findings are supported by others who reported similar findings [213,227,228]. In particular, Iridova et al, that assessed plasma erythrocyte antioxidant enzyme activity in patients with PH [213]. They showed that erythrocyte antioxidant enzyme (glutathione peroxidase, catalase and SOD) activity was increased in patients with PH. However, we are not aware of any study who investigated the antioxidant enzyme activities in blood plasma, as shown in our study.

6. Limitations of this section of the study

- No detailed clinical profile has been provided for the healthy patients, as a proper clinical investigation was not performed and these patients were not appropriately matched with their PH counterparts. This was mainly because the purpose of this section was just to show that oxidative stress is elevated in PH compared and not to perform an in-depth clinical trial.
- A large proportion of the PH patients were HIV positive in our patient sample. HIV is known to lead to oxidative stress and further studies will be required in order to delineate the exact role of HIV vs. PH in this increase of oxidative stress, observed in our patient sample.

7. Conclusion

In our experiments we were able to show that oxidative stress is increased in the blood plasma of our South African population with PH compared to controls. These patients' blood plasma samples also showed a trend towards a decrease in antioxidant capacity and a definite increase in catalase and SOD activities. This suggests that oxidative stress is increased with PH, despite simultaneous increase in antioxidant enzyme activities. However, while many of the patients were HIV positive, confounding factors may also result in changes in antioxidant status.

SECTION E

VALIDATION OF EXPERIMENTAL MODEL OF PULMONARY HYPERTENSION

1. Introduction

In order to better understand the pathophysiology of PH and discover novel therapies to treat the disease, several animal models for PH have been developed [333]. The rat model of PH using a single injection of MCT has been largely published in the literature and has been useful in better understanding the pulmonary vascular remodelling in PH [333]. This was mostly shown by studies exploring the role of a genetic mutation in genes that are responsible for the regulation of endothelial cell and pulmonary arterial smooth muscle cell apoptosis and proliferation. Researchers discovered a mutation in the gene that encodes for bone morphogenetic protein receptor-2 (BMPR-2) and forms part of the transforming growth factor superfamily of proteins [58]. Other studies done in the MCT model also highlighted the pivotal role of inflammatory cells (macrophages, dendritic cells, and mast cells) and cytokines (interleukin-6, interleukin-1) in the early stages of pulmonary vascular remodelling observed in PH. Macrophages and dendritic cells, both antigen-presenting cells, are believed to facilitate the inflammatory response in PH, thereby contributing to pulmonary vascular remodelling [333].

In this model, a single injection of MCT induces endothelial injury to the small pulmonary arteries and leads to PH and RV remodelling within 28 days [208,326]. The MCT model has been described in Sprague Dawley rats but unfortunately this particular rat strain is not available at our animal facility, which only breeds Long-Evans and Wistar rats. In the literature it has been shown that the strain can influence the pathological response to a stimulus [334]. Therefore, to know whether the injection with MCT will recapitulate the full spectrum of PH markers (RV hypertrophy, increased RV systolic pressure and increased oxidative stress) as observed in Sprague Dawley rats, our aim was to validate this model in Long Evans rats.

2. Materials and methods

We induced PH with a single subcutaneous injection of MCT which should lead to severe PH and RV remodelling after 28 days. Control (C) rats received a single subcutaneous injection of sterile saline on the same day the other rats were injected with MCT. After 28 days, echocardiography was performed, the hearts were isolated for the isolated heart perfusions, blood samples were collected, biometrical measurements were done and hearts were processed for histological staining. Blood plasma samples were used for the oxidative stress and antioxidant enzyme activity assays (TBARS, ORAC, catalase and SOD activity) (Figure 29).

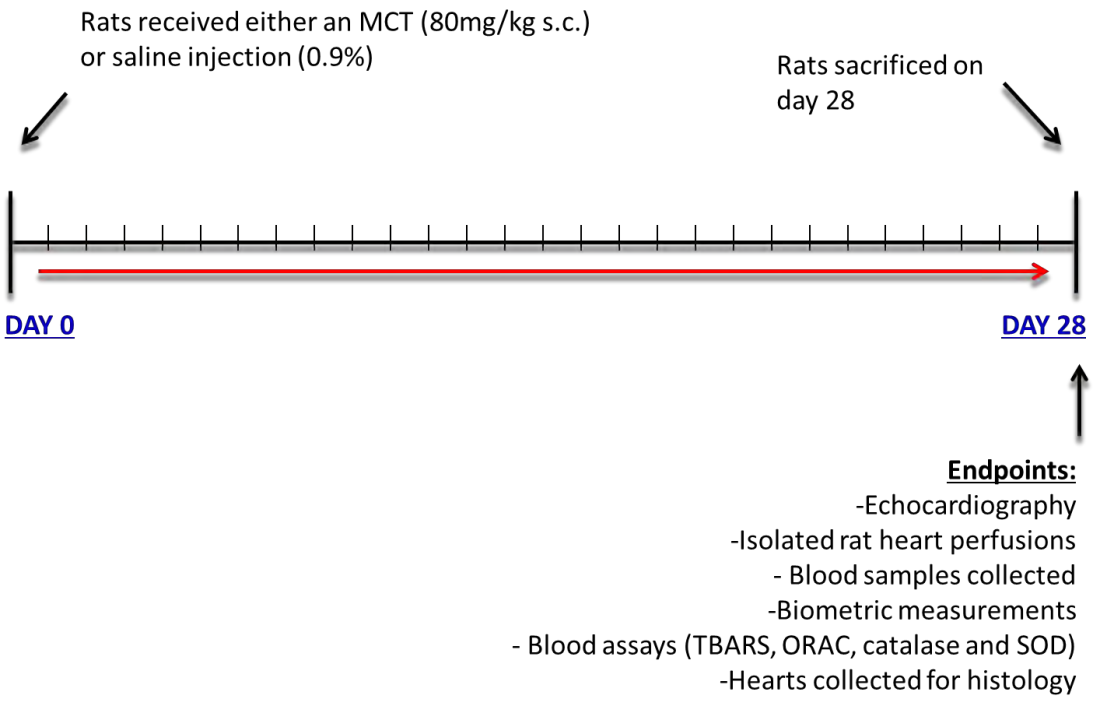


Figure 29. Experimental protocol for the validation of the model of PH. Male Long Evans rats were randomly assigned to various treatment groups: controls and monocrotaline. Control rats received a saline injection and monocroteline rats an injection of monocrotaline. Echocardiography measurements were done on the day of sacrifice (day 28). Then blood samples were collected and isolated rat heart perfusions performed. A separate group of rats were used for the collection and preparation of hearts for histological staining. Blood assays included TBARS, ORAC, catalase and SOD activity. Sample size was six rats group. **s.c**: subcutaneous, **mg/kg**: milligram per kilogram, **MCT**: monocrotaline, **TBARS:** thiobarbituric acids reactive substances, **ORAC:** oxygen radical absorbance capacity, **SOD:** superoxide dismutase.

3. Data analyses: Statistical tests

All data were expressed as mean ± standard error of the mean (SEM). For comparative studies, a Student's t-test (unpaired) was used performed. The experimental groups used for the fibrosis determination were statistically analysed by one-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. A $p<0.05$ was considered as significant.

4. Results

4.1. Biometric measurements

Table 5 summarizes the biometric measurements including body weights, weights of organs and weight of these organs expressed as a ratio of body weight or tibial length. MCT rats had significantly lower body weights (218.80 ± 18.41 vs. 308.70 ± 7.50 g, $p<0.0006$) and higher heart weights (1.62 ± 0.07 vs. 1.36 ± 0.07 g, $p<0.02$). MCT rats had higher heart weight over body weight (0.57±0.02 vs. 0.36±0.02 g/g, $p<0.0001$) and similar heart weight over tibial length ratios (0.37 ± 0.02 vs. 0.32 ± 0.02 g/cm, $p>0.05$). MCT rats had significantly higher RV weights (0.55 ± 0.02 vs. 0.20 ± 0.01 g/g, $p<0.0001$) and lower LV weights compared to control rats (0.65 ± 0.01 vs. 0.80 ± 0.03 g/g, $p<0.0001$). MCT rats had higher lung weights compared to controls (3.59 ± 0.13 vs. 1.85 ± 0.17 g, $p<0.0001$) and lung weight over body weight ratio (1.12±0.04 vs. 0.49±0.03 g/g, $p<0.0001$). MCT rats had also higher liver weights (19.60 ± 1.22 vs. 15.81 ± 0.59 g, $p<0.01$) and liver weight over body weight ratio (0.56 ± 0.03 vs. 0.42 ± 0.03 g/g, $p<0.05$).

Table 5. Biometric measurements

	C (at day 28)	MCT (at day 28)	*p*-value
Biometric measurements:			
Body weight (g)	308.70 ± 7.50	218.80 ± 18.41*	*<0.0006
Heart weight (g)	1.36 ± 0.07	1.62 ± 0.07*	*<0.02
Heart weight/body weight (g/g)	0.36±0.02	0.57±0.02*	*<0.0001
RV weight/LV+Septal weight (g/g)	0.20 ± 0.008	0.55 ± 0.02	<0.0001
LV+Septal weight/heart weight (g/g)	0.80 ± 0.03	0.65 ± 0.009	<0.0001
Heart weight/tibial length (g/cm)	0.32 ± 0.02	0.37 ± 0.02	>0.05
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13*	*<0.0001
Lung weight/body weight (g/g)	0.49±0.03	1.12±0.04*	*<0.0001
Liver weight (g)	15.81 ± 0.59	19.60 ± 1.22*	*<0.01
Liver weight/body weight (g/g)	0.42 ± 0.03	0.56 ± 0.03*	*<0.05

C: Control, **MCT:** Monocrotaline *p<0.0001 (MCT vs C, MCT+MEL vs. MCT), *p<0.05 (MCT vs C), *p<0.05 (MCT vs C), *p<0.0006 (MCT vs C, MCT+MEL vs. MCT), n≥ 6 per group. Values are mean ± standard error of the mean.

4.2. Echocardiography: left ventricular functional parameters

Echocardiography measurements showed that LV fractional shortening was significantly higher in MCT rats compared to controls (69.37 ± 2.17 vs. 36.13 ± 1.08 %, $p<0.0001$) (Figure 30).

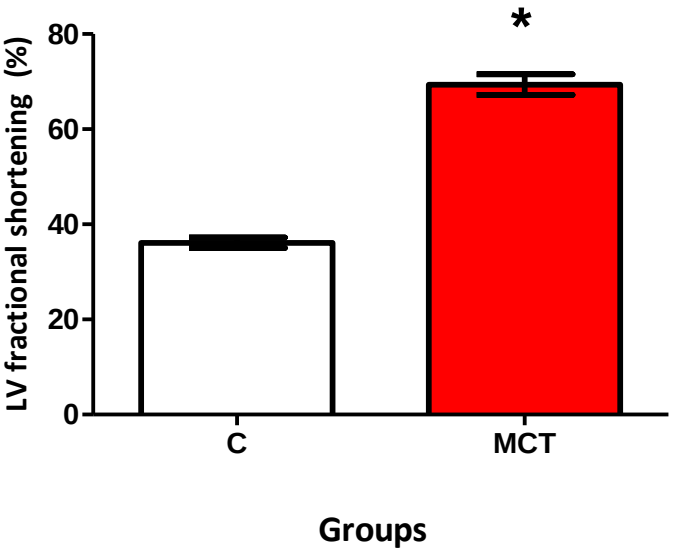


Figure 30. Effect of monocrotaline on left ventricular fractional shortening, *$p<0.0001$ (MCT vs. C), n≥6 per group.

Furthermore, MCT rats had significantly lower LV end diastolic volumes compared to controls (0.19 ± 0.01 vs. 0.68 ± 0.05 vs. mL, p<0.0001) (Figure 31 A). Representative images of LV echocardiography (M-Mode) are shown below (Figure 31 B).

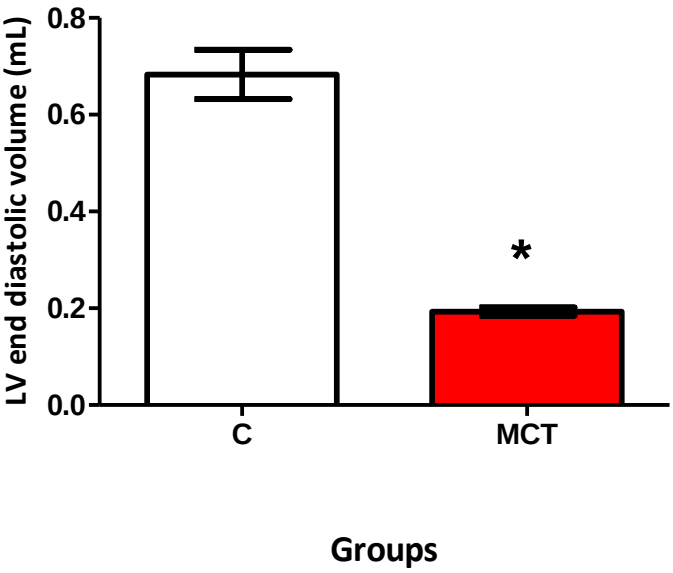


Figure 31 (A). Effect of monocrotaline on left ventricular end diastolic volume, ***p**<0.0001 (MCT vs. C), n≥6 per group.

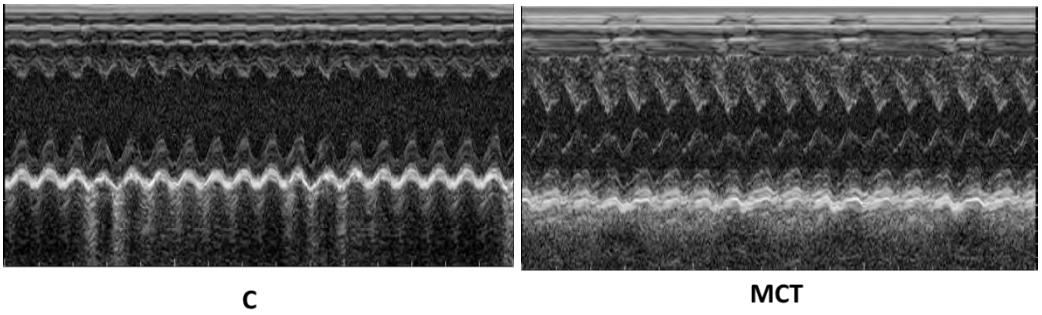


Figure 31 (B). Representative M-mode echocardiography images, for control (C) and monocrotaline (MCT) groups.

MCT rats had higher LV ejection fractions compared to controls (96.05 ± 0.63 vs. 69.09 ± 1.57 %, $p<0.0001$) (Figure 32).

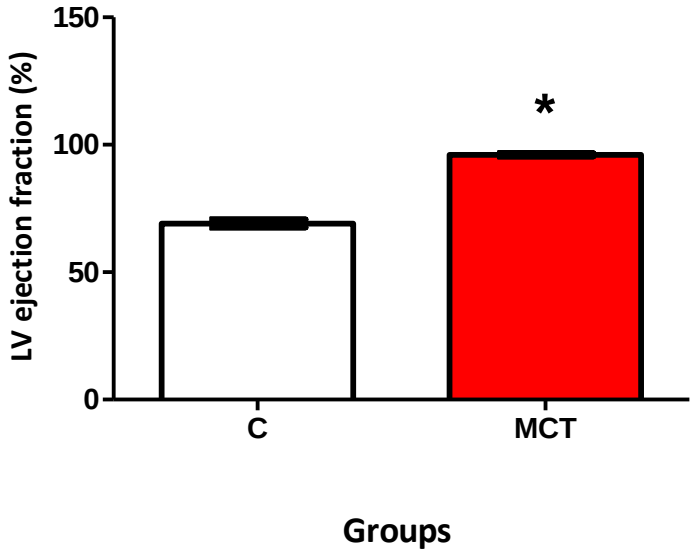


Figure 32. Effect of monocrotaline on left ventricular ejection fraction, ***$p<0.0001$** (MCT vs. C), n≥6 per group.

4.3. Isolated heart perfusions: Left ventricular function

Isolated heart perfusions showed that MCT rats had similar LV developed pressures compared to controls (96.18 ± 5.24 vs. 96.08 ± 6.66 mmHg) (Figure 33).

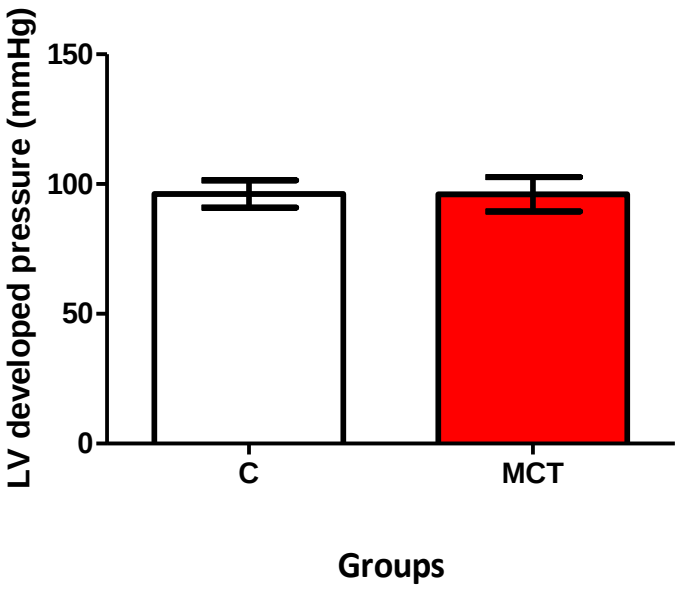


Figure 33. Effect of monocrotaline on left ventricular developed pressure, (MCT vs. C), n≥6 per group.

The LV systolic pressure was similar for MCT rats compared to controls (99.19 ± 6.63 vs. 103.80 ± 4.11 mmHg) (Figure 34).

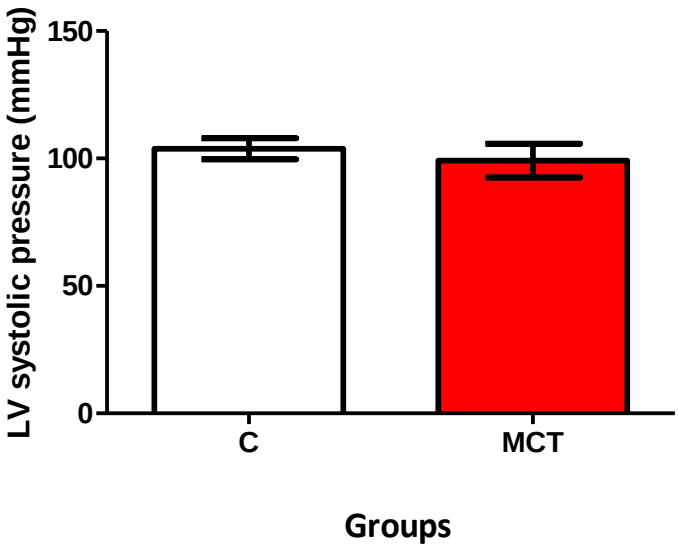


Figure 34. Effect of monocrotaline on left ventricular systolic pressure, (MCT vs. C), n≥6 per group.

The LV diastolic pressure was similar for MCT rats compared to controls (8.77 ± 0.25 vs. 8.42 ± 0.12 mmHg) (Figure 35).

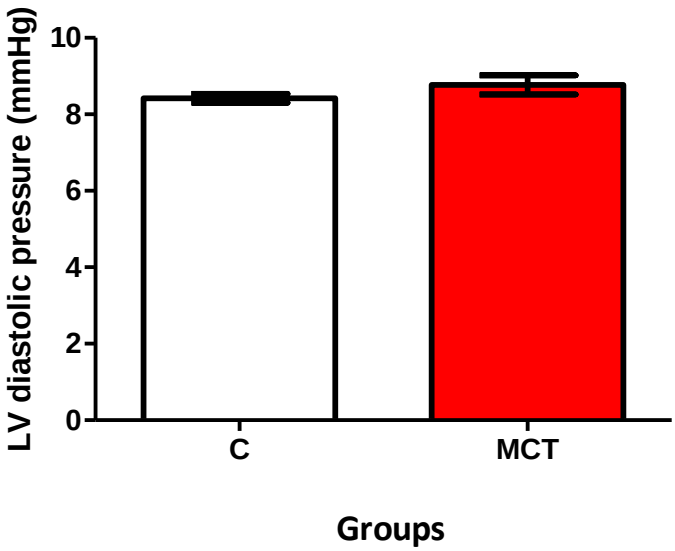


Figure 35. Effect of monocrotaline on left ventricular diastolic pressure, (MCT vs. C), n≥6 per group.

The heart rate was similar for MCT rats compared to controls (318.70 ± 9.29 vs. 303.30 ± 18.16 bpm) (Figure 36).

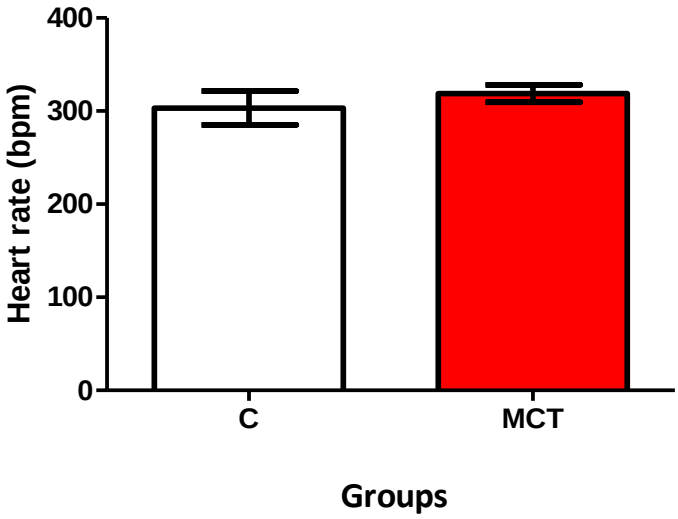


Figure 36. Effect of monocrotaline on heart rate, (MCT vs. C), n≥6 per group.

4.4. Isolated heart perfusions: Right ventricular function

The RV developed pressure was higher in the MCT rats compared to controls (81.22 ± 2.75 vs. 45.87 ± 2.11 mmHg, $p<0.0001$) (Figure 37).

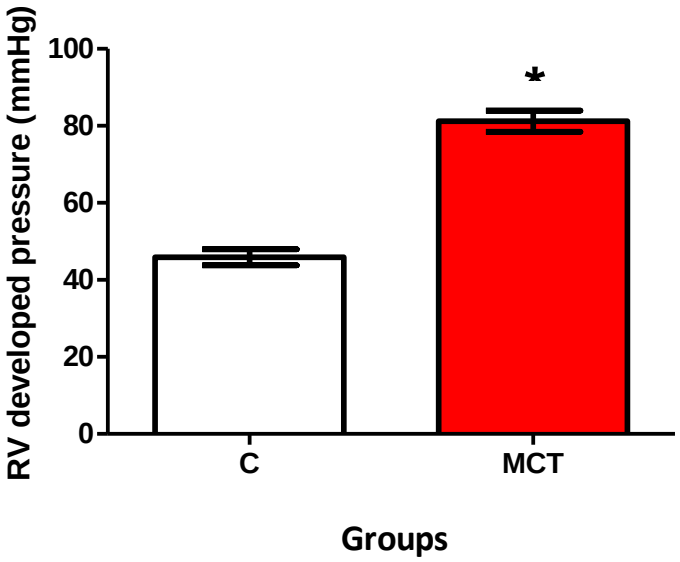


Figure 37. Effect of monocrotaline on right ventricular developed pressure, ***p*<0.0001 (MCT vs. C), n≥6 per group.

The RV systolic pressure was higher in the MCT rats compared to controls (87.91 ± 5.48 vs. 54.35 ± 2.28 mmHg, $p<0.0001$) (Figure 38).

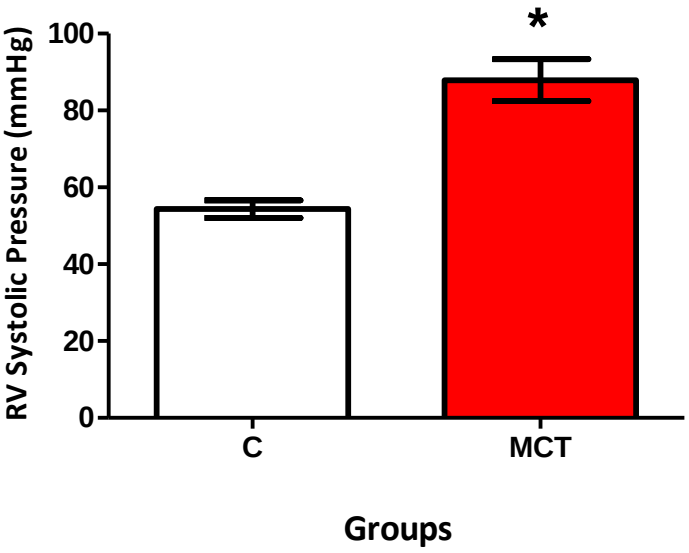


Figure 38. Effect of monocrotaline on right ventricular systolic pressure, ***p*<0.0001 (MCT vs. C), n≥6 per group.

RV diastolic pressure was similar in the MCT rats compared to the controls (9.06 ± 0.32 vs. 8.55 ± 0.09 mmHg) (Figure 39).

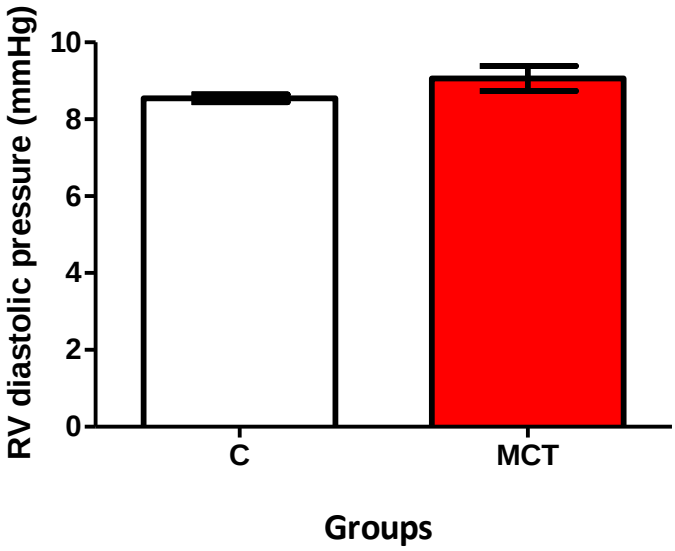


Figure 39. Effect of monocrotaline on right ventricular diastolic pressure, (MCT vs. C), n≥6 per group.

4.5. Oxidative stress in an experimental model of pulmonary hypertension

Levels of blood plasma lipid peroxidation was measured by assessing the thiobarbituric acid reactive substances (TBARS) in control rats and those with MCT induced PH. MCT rats had higher levels of plasma lipid peroxidation compared to controls (2.27 ± 0.22 vs. 0.84 ± 0.05 µmol/mL, $p<0.0001$) (Figure 40).

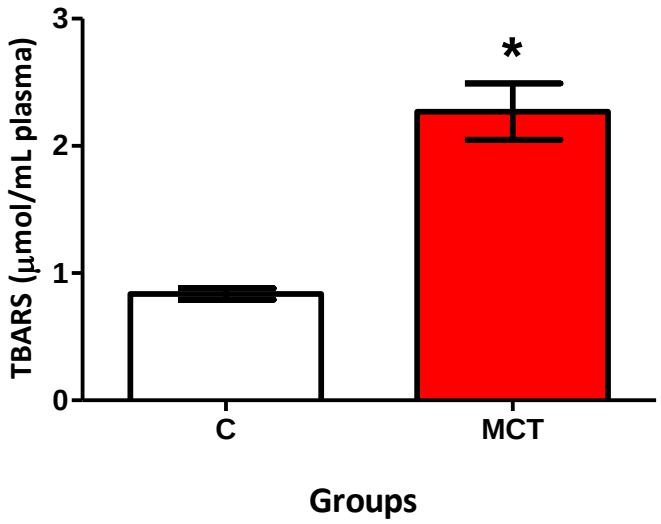


Figure 40. Effect of monocrotaline on plasma lipid peroxidation, *****$p<0.0001$ (MCT vs. C), n≥6 per group.

MCT rats was a significantly lower antioxidant capacity compared to controls (18.40 ± 2.29 vs. 30.67 ± 3.89 nmol/L trolox equivalents $p<0.02$) (Figure 41).

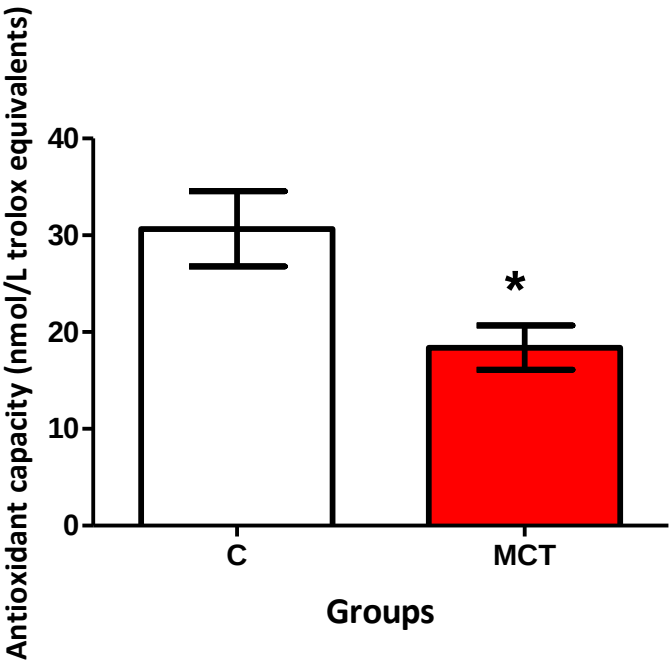


Figure 41. Effect of monocrotaline on plasma antioxidant capacity, *$p<0.02$ (MCT vs. C), n≥6 per group.

Catalase activity of the MCT rats was significantly higher than that of the controls (12.40 ± 1.80 vs. 3.72 ± 0.40 IU/mg protein, $p<0.0008$) (Figure 42).

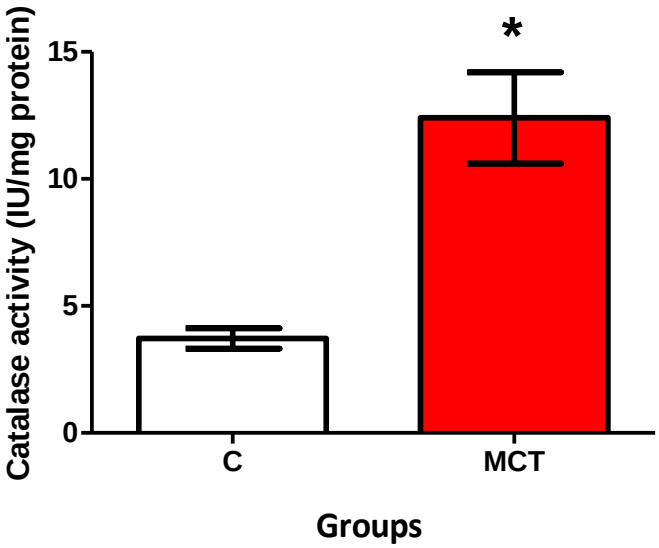


Figure 42. Effect of monocrotaline on plasma catalase activity, *****$p<0.0008$ (MCT vs. C), n≥6 per group.

SOD activity in the plasma of the MCT rats was significantly higher than that of the controls (9.97 ± 0.20 vs. 6.57 ± 0.83 IU/mg protein, $p<0.003$) (Figure 43).

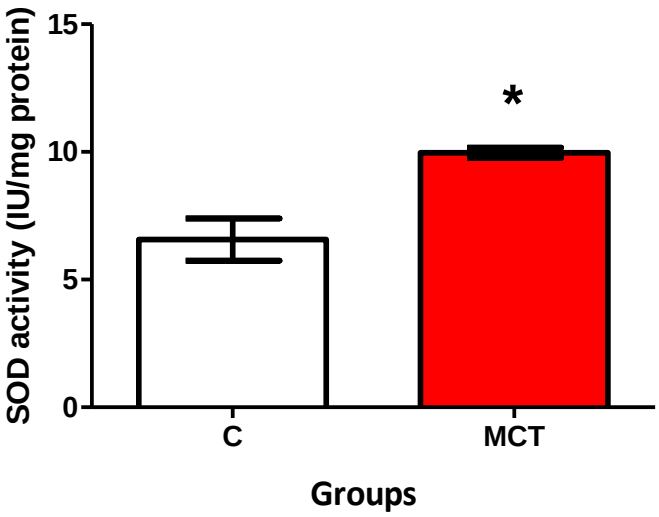


Figure 43. Effect of monocrotaline on plasma superoxide dismutase activity, ***$p<0.003$** (MCT vs. C), n≥6 per group.

4.6. Cardiac interstitial fibrosis in an experimental model of pulmonary hypertension

The percentage of collagen deposition in the LV sections of the MCT hearts were higher compared to the controls (2.18 ± 0.23 vs. 1.13 ± 0.15 % area, $p<0.009$) (Figure 44 A). (Figure 44 A). Representative images of all groups are shown below (Figure 44 B).

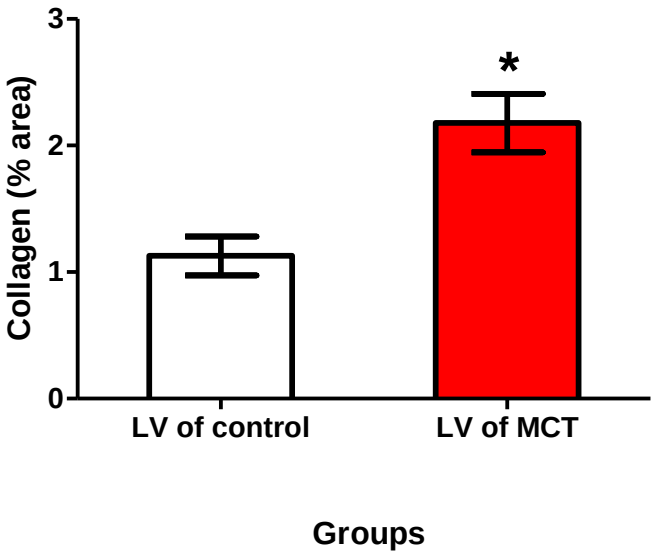


Figure 44 (A). Effect of monocrotaline on cardiac interstitial fibrosis, ***p*<0.009 (MCT vs. C for the LV sections), n=4 per group.

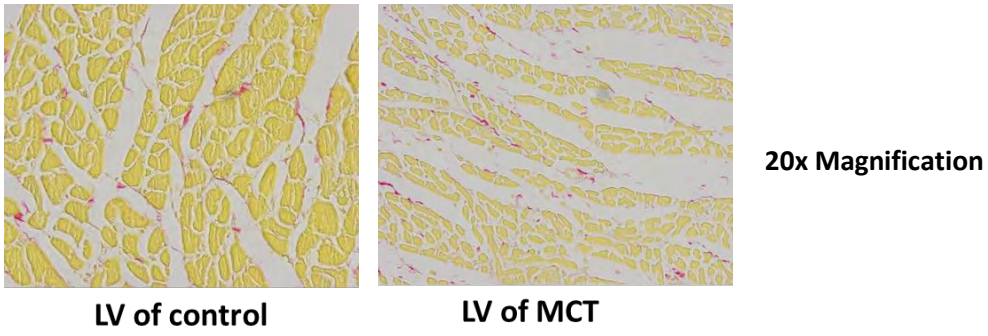


Figure 44 (B). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

The percentage of collagen deposition in the RV sections of the MCT hearts were higher compared to the controls (4.68 ± 0.75 vs. 2.54 ± 0.36 % area, $p<0.04$) (Figure 44 C). Representative images of all groups are shown below (Figure 44 D).

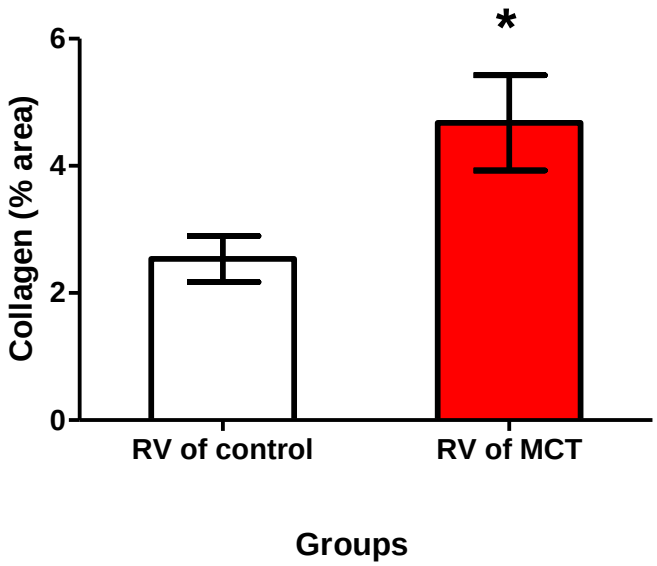


Figure 44 (C). Effect of monocrotaline on cardiac interstitial fibrosis, ***p*<0.04 (MCT vs. C for the RV sections), n=4 per group.

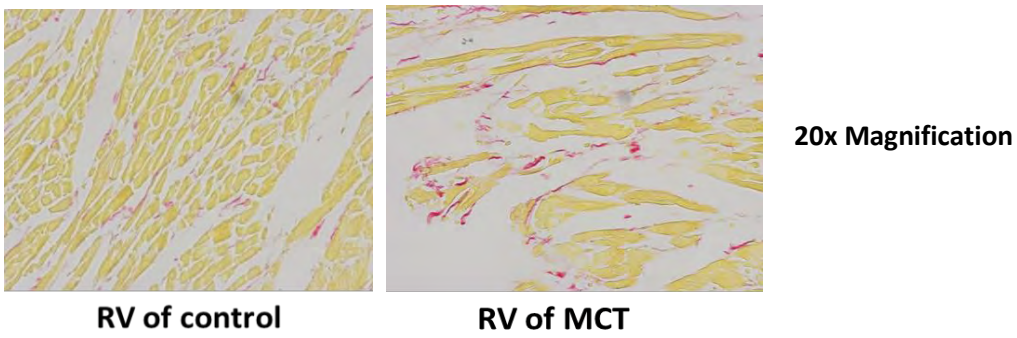


Figure 44 (D). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

5. Discussion

The experimental model of MCT-induced PH has only been performed in Sprague Dawley rats but unfortunately, we do not have these rats available in our animal facilities Therefore, our goal was to validate whether PH can be induced in the Long Evans rat strain, with all characteristics of experimental PH. In our study, we showed that a single subcutaneous injection of 80mg/kg MCT induced PH and RV hypertrophy after 28 days in the Long Evans rat strain as seen by the biometric measurements. Our rats also displayed RV and LV dysfunction as seen with echocardiography and isolated rat heart perfusions. Furthermore, the blood plasma analyses showed increased lipid peroxidation, decreased antioxidant capacity and increased antioxidant enzymes (catalase & SOD) activities.

Biometric measurements

With this experimental model of PH, MCT Sprague Dawley rats are characterized with a lower body weight, higher lung weight and higher liver weight compared to controls [208,219]. In our study, MCT had similar effects in Long Evans rats as seen in table 7. At the end of the 28 day period, MCT rats displayed severe weight loss or cachexia, while control rats were healthy. It appears that the increase in heart weight is mainly due to the increased RV weights or RV hypertrophy. During the 28 day period, we observed that MCT rats also had an obvious lack of physical activity, increased breathing rate (dyspnoea) and piloerection. It could be argued that the dose of 80mg/kg MCT was too high but this was chosen based on the study by Handoko et al, which was at the conception of our study, the only accessible paper where isolated rat heart perfusions to assess cardiac function were performed [208]. These observations were also made by Hessel et al, who induced PH with the same dose of MCT and examined these rats for the same time period [326]. Furthermore, the lung weights were significantly higher in MCT rats which could be a confirmation of extensive proliferative pulmonary response in oedema [326].

Our MCT rats had significantly higher liver weights which are also corroborated by Handoko et al, The reason for this increase is not clear but it has been reported that MCT induces damage of sinusoidal endothelial cells, central venular endothelial cells and hepatic parenchymal cells [324]. Although, these effects of MCT on the liver may not completely explain the increase in liver weights it does provide enough grounds for speculation that it may have caused hepatic damage associated with oedema.

RV hypertrophy

MCT induces pulmonary arterial remodelling and lumen narrowing which increases the, pulmonary artery pressure, RV afterload, RV hypertrophy and eventually leads to RV failure [324,325]. Therefore, this model has been characterized by RV hypertrophy which is underlined by microtubule proliferation in RV-cardiomyocytes [335]. An interesting finding in our study, was that MCT rats had higher RV weights (RV hypertrophy) but simultaneously, also lower LV weights than controls. This was also apparent in the study by Hessel et al, even though the changes weren't statistically significant [326]. However, this reduction in LV mass was completely visible in a study by Hardziyenka et al, who found that this reduction in LV mass was underlined by cardiomyocyte shrinkage due to atrophic remodelling [336].

Echocardiography: LV function

In our study, we assessed LV function with echocardiography and found that MCT rats had severely decreased LV end diastolic volumes. There is a paucity of publications that report the effects of MCT on both right and left ventricles. Nevertheless, Handoko et al, showed that MCT at a dose of 80mg/kg, caused both RV and LV dysfunction as assessed with cardiac magnetic resonance imaging [208]. This was supported by Hardziyenka et al, who showed that already at a dose of 60mg/kg, MCT caused RV and LV dysfunction and LV atrophy as assessed with both echocardiography and cardiac magnetic resonance imaging [336].

In our study, we also found that the LV fractional shortening and ejection fraction was increased in the MCT rats. There are not many available studies that report on these LV parameters for the MCT model but it does appear that these rats develop LV diastolic dysfunction and the changes in LV parameters may be due to severely altered LV filling. However, the increased LV fractional shortening does mean that the LV contractility is adversely affected by MCT-induced PH, as fractional shortening is a measure of the LV wall diameter during systole and diastole.

MCT seems to cause atrophy of the LV with concomitant contractile dysfunction in an *in vivo* setting. The LV ejection fraction is severely increased in the MCT rats compared to controls. A possible way to explain this is by taking a mathematical approach and to revisit the basic principles of LV ejection fraction, which is calculated by dividing the stroke volume with by the LV end diastolic volume. In our study, the LV end diastolic volumes in the MCT rats were decreased, so therefore, if one assumes the stroke volumes remains constant, and simply divide by the now, lower end diastolic volumes, this would equate to a higher ejection

fraction. Therefore, one can speculate that the LV ejection fraction in the MCT rats is higher due to decreased LV end diastolic volume. Usually, the MCT model is characterised by RV dysfunction as assessed with echocardiographic measurements [329]. This was seen in a study by Kosanovic et al, where MCT (60mg/kg) reduced RV function when it was assessed with echocardiography and right heart catheterisation [329]. Unfortunately, we were not able to measure RV function with echocardiography in our setting due to low probe sensitivity. For this reason, we assessed both LV and RV function with the isolated heart perfusion apparatus.

Isolated heart perfusions: LV and RV function

Data from the isolated heart perfusions generated very interesting results as we could not observe LV dysfunction as seen with echocardiography. These results are corroborated by the findings of Handoko et al, who also assessed LV function with the isolated heart perfusion apparatus [208]. Authors could not explain this phenomenon but this could be due to various reasons open to speculation. In our study, the hearts of MCT rats were bigger, the RV mass increased and the LV mass was smaller. It appears therefore, that the pressure overload with MCT-induced PH caused the RV to become hypertrophied and constitute a greater part of the actual heart. The LV mass was smaller in MCT rats and the LV function decreased when assessed with echocardiography. This could just mean that the isolated heart apparatus is not sensitive enough to pick up the LV functional changes as observed with the *in vivo* echocardiography. Furthermore, in our study we used the Langendorff, retrograde perfusion apparatus, which is not a true reflection of *in vivo* blood flow in a whole organism. Therefore, the better apparatus would have been the working heart perfusion apparatus and therefore the use of the Langendorff in our study could also account for the inability to pick up LV functional difference as with echocardiography.

We have also found that RV function was compromised as assessed with the isolated rat heart perfusion system. These findings are in line with that of Handoko et al, who showed that MCT-induced PH leads to increased RV developed and systolic pressure with the isolated heart perfusion apparatus [208]. This LV and RV dysfunction in the MCT model have been attributed to ventricular dyssynchrony and septal bulging [208]. In our study, the experimental model of PH was associated with concomitant LV and RV dysfunction as previously described in the literature [325,335,337].

Blood plasma oxidative and antioxidant capacity

Our study also showed that MCT increased plasma oxidative stress, decreased the total oxygen radical absorbance (antioxidant) capacity and increased antioxidant enzyme activity (catalase and SOD). Even though the antioxidant enzyme activities are increased in response to the oxidative stress, it is appears that this increase is not sufficient to limit the oxidative stress. This is supported by our findings that the total antioxidant capacity of the blood plasma was decreased in the MCT rats compared to control rats.

These findings are in line with work by Dorfmuller et al, who showed that MCT rats who also underwent left pneumonectomy, had increased NADPH-oxidase-4 and oxidative stress-induced lipid peroxidation [217]. These rats also had increased activity of plasma antioxidant enzymes [217]. On the other hand, Mohammadi et al, showed that rats only injected with MCT without an additional pathology such as pneumonectomy, had increased malonic dialdehyde levels and decreased plasma levels of catalase, superoxide dismutase and glutathione peroxidase [218]. In the light of both of these studies, it appears in a severe model of PH, the oxidative stress is elevated as well as the plasma antioxidant enzyme activity. In our present study, we also had a very severe model of PH as we injected rats with a relatively high dose of MCT. Therefore, this could be a possible explanation for the increased antioxidant enzyme activity in the blood plasma of MCT rats.

Furthermore, if one considers the generally accepted definition of oxidative stress, it holds that oxidants (such as ROS) are increased and the cell's inherent antioxidant systems (catalase, SOD activity) are decreased [76,210]. However, in either MCT-induced PH or that in humans, this is not always the case [213,225-229]. As with our results, it is clear that the plasma oxidative stress (lipid peroxidation) is increased while the antioxidant enzyme activity is also increased. This could mean that the oxidative stress provokes an increase in the antioxidant enzyme activity as an adaptive response, but despite the increased activity, this is insufficient to prevent or at least stunt the oxidative stress. This was then confirmed when the overall antioxidant capacity of the blood plasma was lower than that of healthy control rats. All these data mimic our findings of the plasma of the patients in the previous section.

Cardiac interstitial fibrosis

Our data show that MCT induced interstitial fibrosis exceeding control levels, in both the LV and RV. These data are corroborated by others who reported that a single injection of MCT induces interstitial fibrosis [12]. In this particular study, authors showed that the amount of RV fibrosis increased throughout the disease progression, reaching a peak when the RV starts to fail. They could not see an increase in LV fibrosis, as we did but this was most probably due to the differences in MCT dose as well as the degree of LV atrophy, which could have affected the degree of interstitial fibrosis [12]. The mechanism underlying the collagen deposition is believed to be an angiotensin-II-induced fibrosis mediated via the growth factor pathways and activation of the angiotensin receptor-1 [12]. The work of Campian et al, is also supported by others who showed similar levels of collagen deposition in RV's of MCT rats at the same dose [336].

Work by Redout et al, corroborates our data that 80mg/kg MCT induces extensive ventricular interstitial fibrosis [219]. In their study, the use of the antioxidant EUK-1, reduced fibrosis supporting the notion that matrix remodelling in the RV of MCT rats is at least partially due to ROS dependent activation of matrix metalloproteinases in PH-induced RV remodelling [219]. Therefore, it is possible that this increased interstitial fibrosis, also contributes to the cardiac dysfunction in the MCT model.

6. Conclusions

Our data demonstrate that a single subcutaneous injection of MCT (80mg/kg) induced experimental PH with RV hypertrophy, cardiac dysfunction, increased plasma oxidative stress, decreased plasma antioxidant capacity and abnormal antioxidant enzyme activity in Long Evans rats. Taken together, these data validated our experimental model of PH in the Long Evans rat strain which we aim to use in order to test the cardioprotective effect of melatonin in PH.

SECTION F

MELATONIN AS THERAPY IN PULMONARY HYPERTENSION

1. Introduction

In the previous sections of this thesis (sections D and E), we have been able to demonstrate that PH is associated with oxidative stress in a South African population with PH and in a Long Evans rats model of PH, induced by injection with MCT. Oxidative stress has been suggested to be a major player in the development of PH [30,210,211].

Melatonin is an endogenously produced hormone that is commonly available over the counter to regulate the sleeping pattern. It has powerful antioxidant properties by either directly scavenging ROS or indirectly stimulating the synthesis and activity of antioxidant enzymes. Experimental data have demonstrated that a chronic treatment of melatonin, at the concentration found in red wine (75ng/L) protects the heart against ischemia/reperfusion injury [250]. This cardioprotective effect was mediated mainly via the activation of the survivor activating factor enhancement (SAFE) pathway [338]. At much higher concentrations (4mg/kg corresponds approximately to 10^6 higher dose compared to the amount of melatonin absorbed via the drinking of red wine), melatonin presents antioxidant as well as antihypertensive properties, effects partly mediated via the nitric oxide pathway and scavenging superoxide ion [282].

Therefore, our aim was to investigate whether a daily oral, melatonin treatment at a concentration of 75ng/L or doses of 4mg/kg and 6mg/kg could confer cardioprotection in the MCT model of PH.

2. Materials and methods

To test our hypothesis, we used the Long Evans rat model of PH, induced by a single injection of MCT as described in Section E. Subsequently, rats were designated for treatment with three doses of melatonin based on previous literature. 75ng/L, corresponding to the concentration of melatonin obtained through the chronic and regular consumption of red wine. Furthermore, also 4mg/g and 6mg/kg of melatonin, which represents a much higher concentration and would mimic better the amount of melatonin received with melatonin capsules available over the counter.

Melatonin was given orally in the drinking water and the treatment started on day zero of the treatment protocol i.e. on the same day as that the MCT injection was given. Melatonin treatment continued until day 28. After the 28 day period, we assessed various endpoints including echocardiography, blood collection, isolated rat heart perfusions, biometric

measurements, blood plasma oxidative and antioxidant status assays (TBARS, ORAC, and catalase and SOD activity) and histology of hearts for fibrosis assessment (Figure 45).

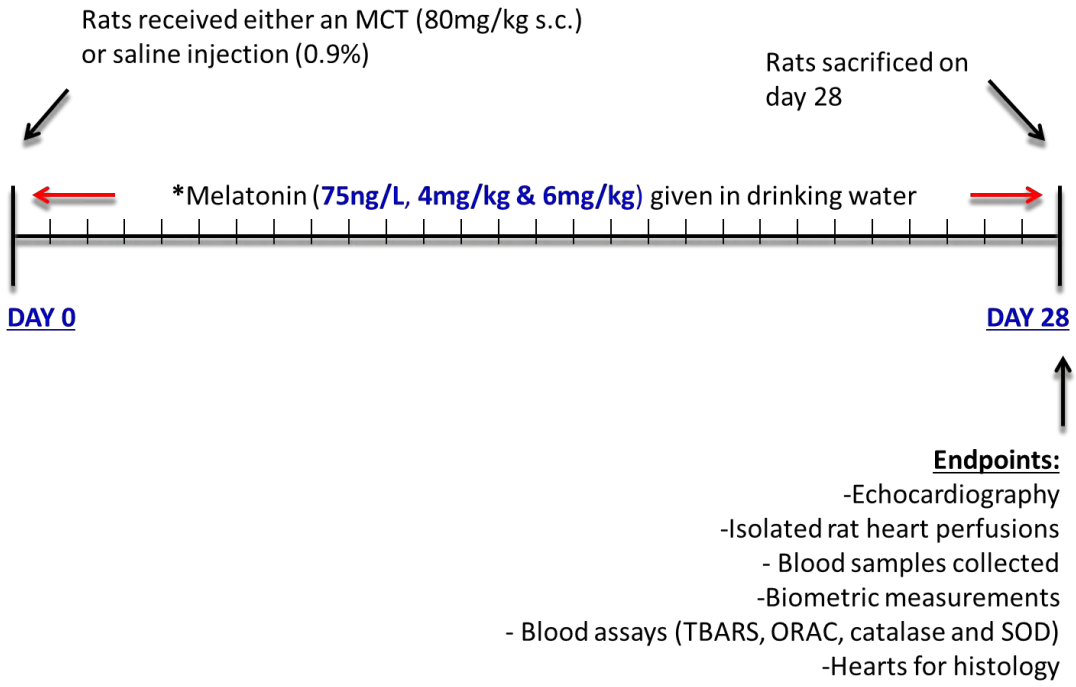


Figure 45. Melatonin as a therapy in PH: Experimental design. Male Long Evans rats were randomly assigned to various treatment groups: controls, monocrotaline, monocrotaline plus melatonin (doses of 75ng/L, 4mg/kg & 6mg/kg at different time points). Control rats received a saline injection and monocrotaline rats an injection of monocrotaline. Echocardiography measurements were done on the day of sacrifice (day 28). Then blood samples were collected and isolated rat heart perfusions performed. A separate group of rats were used for the collection and preparation of hearts for histological staining. Blood assays included TBARS, ORAC, catalase and SOD activity. Sample size was six rats group. **s.c**: subcutaneous, **mg/kg**: milligram per kilogram, **MCT**: monocrotaline, **TBARS:** thiobarbituric acids reactive substances, **ORAC:** oxygen radical absorbance capacity, **SOD:** superoxide dismutase.

3. Data analyses: Statistical tests

Unless stated otherwise, all data were expressed as mean ± standard error of the mean (SEM). For comparative studies a two-way ANOVA analysis was performed (with Bonferroni post-test, if $p< 0.05$). All statistical tests were performed using Graph Pad Prism-5. The experimental groups used for the fibrosis determination were statistically analysed by one-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. A $p<0.05$ was considered as significant.

4. Results: Effects of 75ng/L melatonin

4.1. Biometric measurements

Table 6 summarizes the biometric measurements including body weights, weights of organs and weight of these organs expressed as a ratio of body weight or tibial length. Melatonin treated MCT rats had significantly higher body weights (323.0 ± 5.46 vs. 218.80 ± 18.41 g, $p<0.0001$) while the heart weights were similar (1.64 ± 0.11 vs. 1.62 ± 0.07 g, $p>0.05$). Melatonin treated MCT rats had similar heart weights over body weights (0.58 ± 0.03 vs. 0.57 ± 0.02 g/g, $p>0.05$) and higher heart weight over tibial length ratios (0.47 ± 0.03 vs. 0.37 ± 0.02 g/cm, $p<0.09$). Melatonin treated MCT rats had significantly lower RV weights (0.33 ± 0.03 vs. 0.55 ± 0.02 g/g, $p<0.0001$) and higher LV weights compared to MCT rats (0.82 ± 0.07 vs. 0.65 ± 0.01 g/g, $p<0.09$). Melatonin treated MCT rats had lower lung weights compared to untreated MCT rats (2.87 ± 0.17 vs. 3.59 ± 0.13 g, $p<0.002$) and similar lung weight over body weight ratios (1.06 ± 0.08 vs. 1.12 ± 0.04 g/g, $p>0.05$). Melatonin treated MCT had also lower liver weights (11.30 ± 0.71 vs. 19.60 ± 1.22 g, $p<0.004$) and lower liver weight over body weight ratios (0.41 ± 0.02 vs. 0.56 ± 0.03 g/g, $p<0.05$).

Table 6. Biometric measurements

			Melatonin treatment (75ng/L MEL)		
	C (at day 28)	**(MCT at day 28)**	**C+MEL**	**MCT+MEL**	***p*-value**
Biometric measurements:					
Body weight (g)	323.0 ± 5.46	218.80 ± 18.41	320.20 ± 14.41	297.30 ± 11.31*	*<0.0001
Heart weight (g)	1.36 ± 0.07	1.62 ± 0.07	1.341 ± 0.09	1.63 ± 0.11	>0.05
Heart weight/body weight (g/g)	0.36 ± 0.02	0.57±0.02	0.38 ± 0.03	0.58 ± 0.03	>0.05
RV weight/LV+septal weight (g/g)	0.20 ± 0.01	0.55 ± 0.02	0.22 ± 0.01	0.33 ± 0.03*	*<0.0001
LV+septal weight/heart weight (g/g)	0.80 ± 0.03	0.65 ± 0.01	0.820 ± 0.008	0.82 ± 0.07*	*<0.09
Heart weight/tibial length (g/cm)	0.32 ± 0.02	0.37 ± 0.02	0.32 ± 0.03	0.47 ± 0.03	>0.05
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13	1.63 ± 0.14	2.87 ± 0.17*	*<0.002
Lung weight/body weight (g/g)	0.49 ± 0.03	1.12±0.04	0.50 ± 0.02	1.06 ± 0.08	>0.05
Liver weight (g)	15.81 ± 0.59	19.60 ± 1.22	15.51 ± 0.60	11.30 ± 0.71*	*<0.004
Liver weight/body weight (g/g)	0.42 ± 0.03	0.56 ± 0.03	0.50 ± 0.03	0.41 ± 0.02*	*<0.05

C: Control, **MCT:** Monocrotaline, **MEL:** Melatonin. *p<0.0001 (MCT vs C, MCT+MEL vs. MCT), *p<0.05 (MCT vs C), *p<0.05 (MCT vs C), *p<0.0006 (MCT vs C, MCT+MEL vs. MCT), n≥ 6 per group. Values are mean ± standard error of the mean.

4.2. Echocardiography: LV functional parameters

Echocardiography measurements showed that LV fractional shortening was significantly lower in the melatonin treated MCT rats compared to MCT rats (46.74 ± 4.44 vs. 69.37 ± 2.17 %, p<0.001) (Figure 46). Control rats treated with melatonin had similar LV fractional shortening compared to normal controls (39.10 ± 0.984 vs. 36.13 ± 1.08 %, p>0.05) (Figure 46).

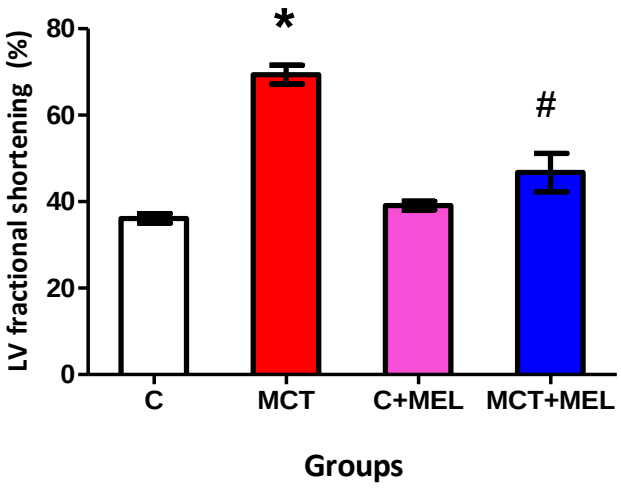


Figure 46. Effect of a chronic treatment with melatonin (75ng/L) on left ventricular fractional shortening in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), #p<0.001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly higher LV end diastolic volumes compared to MCT rats (0.56 ± 0.06 vs. 0.193 ± 0.01 mL, p<0.0001) (Figure 47). Control rats treated with melatonin had higher LV end diastolic volumes (0.82 ± 0.05 vs. 0.68 ± 0.05 mL, p<0.04) compared to controls (Figure 47).

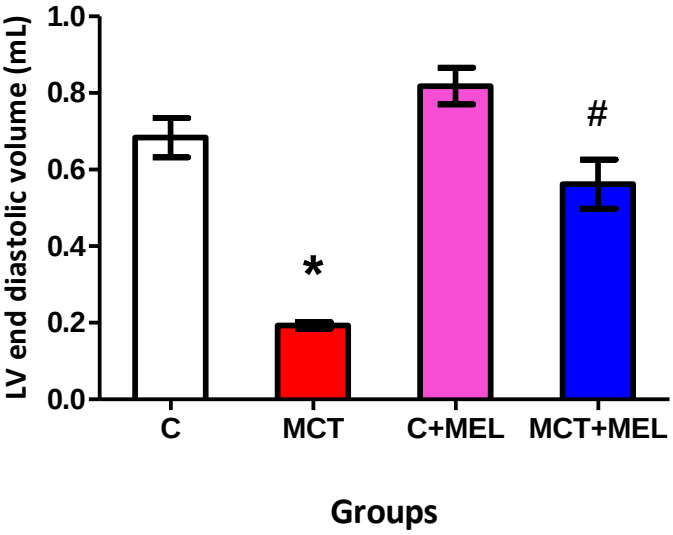


Figure 47. Effect of a chronic treatment with melatonin (75ng/L) on left ventricular end diastolic volume in a rat model of MCT-induced PH, *p<0.0001 (MCT vs. C), #p<0.0001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly lower LV ejection fractions compared to MCT rats (80.74 ± 3.96 vs. 96.05 ± 0.63%, p<0.02) (Figure 48). Control rats treated with melatonin had similar LV ejection fractions compared to controls (72.90 ± 1.49 vs. 69.09 ± 1.57 %) (Figure 48).

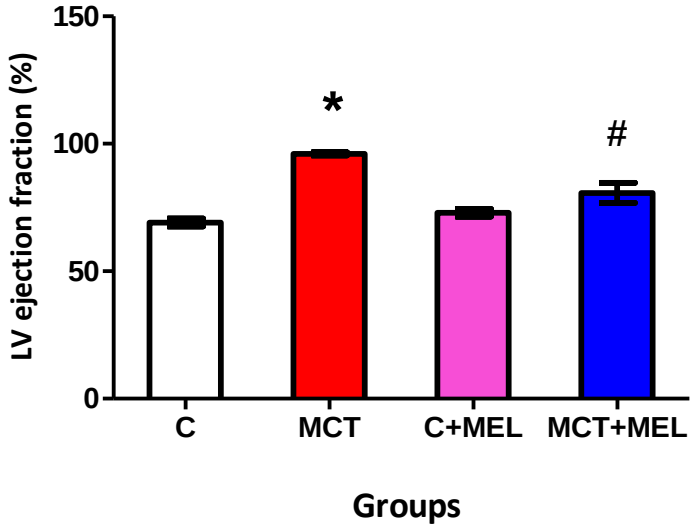


Figure 48. Effect of a chronic treatment with melatonin (75ng/L) on left ventricular ejection fraction in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), #p<0.02 (MCT+MEL vs. MCT), n≥6 per group.

4.3. Isolated heart perfusions: Left ventricular function

Isolated heart perfusions showed that melatonin treated MCT rats had similar LV developed pressures compared to MCT rats (96.18 ± 5.24 vs. 96.08 ± 6.66 mmHg) (Figure 49). Melatonin treated control rats had similar LV developed pressures compared to controls (106.30 ± 2.42 vs. 96.18 ± 5.24 mmHg) (Figure 49).

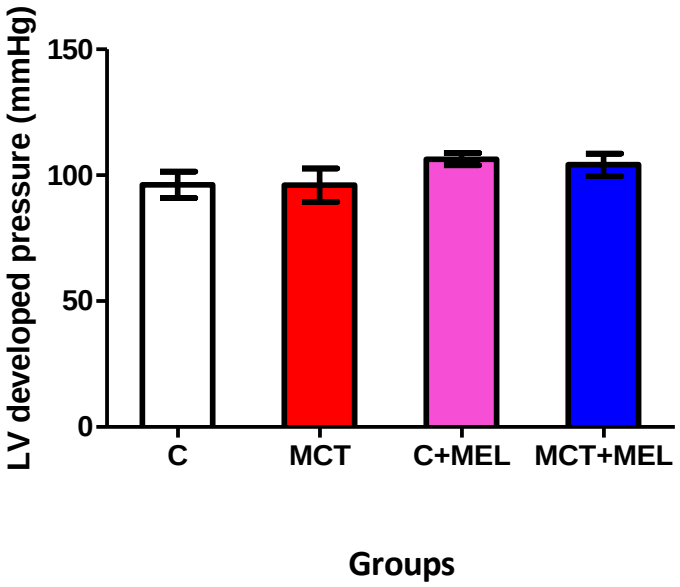


Figure 49. Effect of a chronic treatment with melatonin (75ng/L) on left ventricular developed pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV systolic pressure was similar for melatonin treated MCT rats compared to MCT rats (99.19 ± 6.63 vs. 103.80 ± 4.11 mmHg) (Figure 50). Melatonin treated control rats had similar LV systolic pressures compared to controls (115.50 ± 2.46 vs. 103.80 ± 4.11 mmHg) (Figure 50).

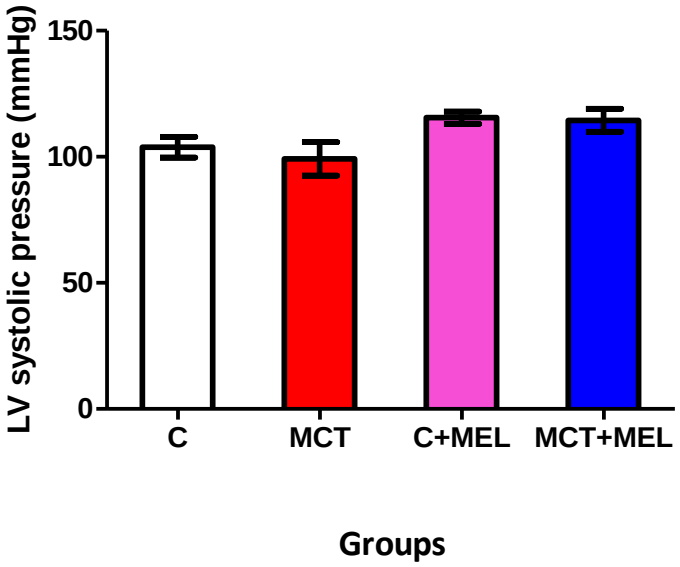


Figure 50. Effect of a chronic treatment with melatonin (75ng/L) on left ventricular systolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV diastolic pressure was similar for melatonin treated MCT rats compared to MCT rats (8.77 ± 0.25 vs. 8.42 ± 0.12 mmHg) (Figure 51). Melatonin treated control rats had similar LV systolic pressures compared to controls (8.53 ± 0.11 vs. 8.42 ± 0.12 mmHg) (Figure 51).

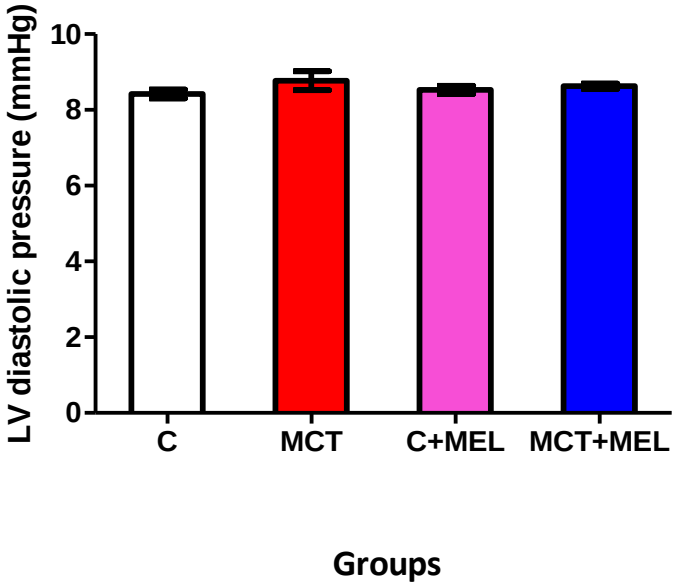


Figure 51. Effect of a chronic treatment with melatonin (75ng/L) on left ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had similar heart rates compared to the MCT rats (299.40 ± 12.62 vs. 283.90 ± 13.73 bpm) (Figure 52). Control rats treated with melatonin had similar heart rates compared to normal controls (274.90 ± 14.85 vs. 272.40 ± 14.04 bpm) (Figure 52).

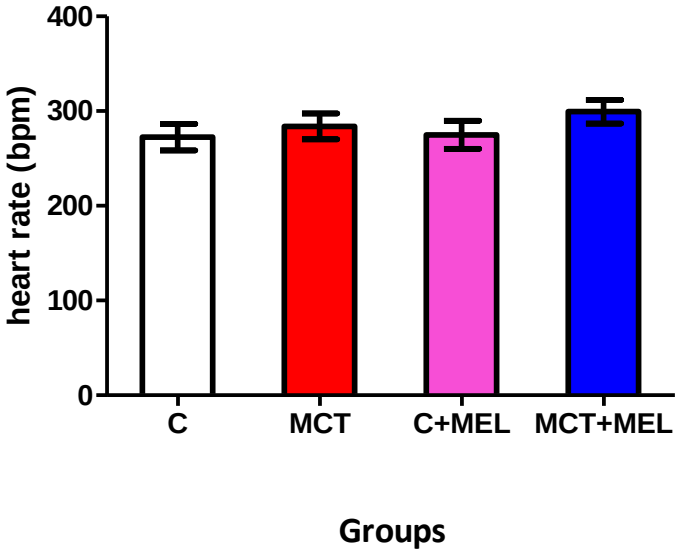


Figure 52. Effect of a chronic treatment with melatonin (75ng/L) on heart rate in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

4.4. Isolated heart perfusions: Right ventricular function

The RV developed pressure was lower in the melatonin treated MCT rats compared to MCT rats (71.95 ± 5.16 vs. 81.22 ± 2.75 mmHg, $p<0.04$) (Figure 53) Control rats treated with melatonin had similar RV developed pressures compared to normal controls (48.15 ± 2.21 vs. 45.87 ± 2.11 mmHg) (Figures 53).

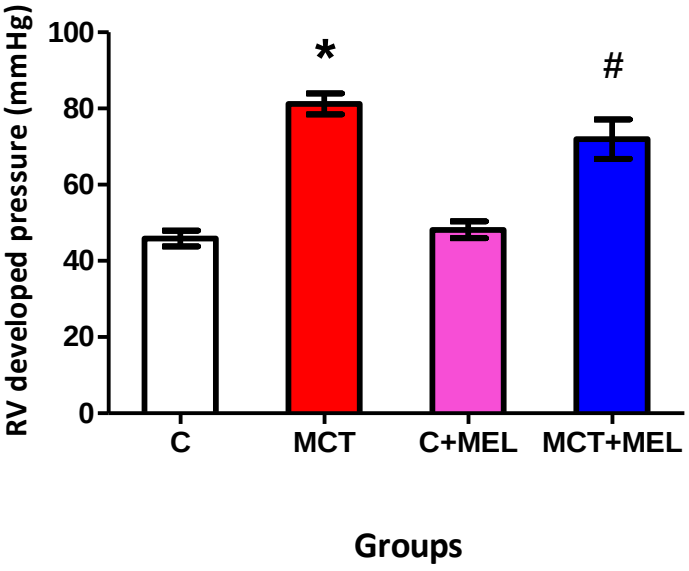


Figure 53. Effect of a chronic treatment with melatonin (75ng/L) on right ventricular developed pressure in a rat model of MCT-induced PH, **p*<0.0001 (MCT vs. C), #*p*<0.04 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had similar RV systolic pressures compared to the MCT rats (84.53 ± 4.83 vs. 87.91 ± 5.48 mmHg) (Figure 54). Control rats treated with melatonin had similar RV developed pressures compared to normal controls (50.44 ± 5.26 vs. 54.35 ± 2.28 mmHg, $p>0.05$) (Figure 54).

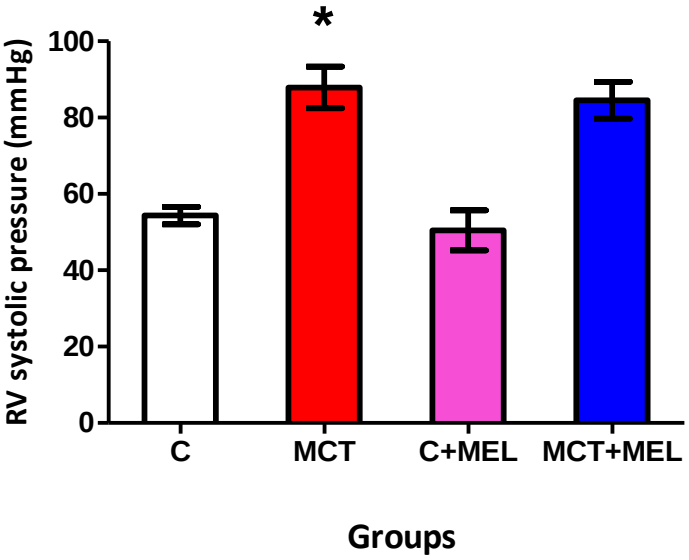


Figure 54. Effect of a chronic treatment with melatonin (75ng/L) on right ventricular systolic pressure in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had similar RV diastolic pressures compared to the MCT rats (8.49 ± 0.14 vs. 9.06 ± 0.32 mmHg) (Figure 55). Control rats treated with melatonin had similar RV diastolic pressures compared to normal controls (8.51 ± 0.11 vs. 8.55 ± 0.09 mmHg) (Figure 55).

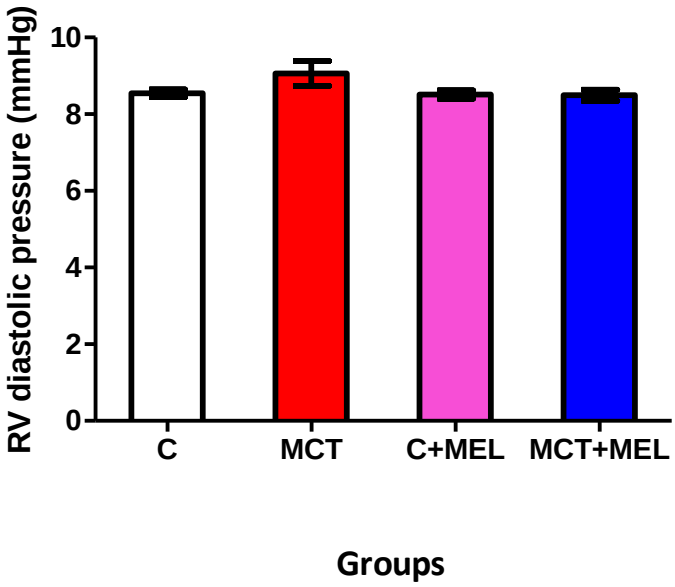


Figure 55. Effect of a chronic treatment with melatonin (75ng/L) on right ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

4.5. Plasma oxidative stress and antioxidant capacity

Levels of blood plasma lipid peroxidation was measured by assessing the thiobarbituric acid reactive substances (TBARS) in all treated groups. Melatonin treated MCT rats had significantly lower levels of plasma lipid peroxidation compared to MCT rats (1.30 ± 0.05 vs. 2.27 ± 0.22 µmol/mL, $p<0.002$) (Figure 56). Melatonin treated controls had similar levels of plasma lipid peroxidation compared to normal controls (0.87 ± 0.08 vs. 0.83 ± 0.05 µmol/mL) (Figure 56).

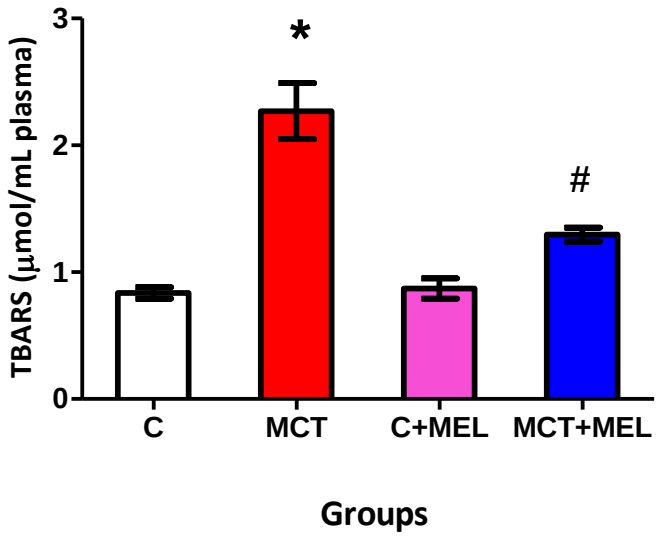


Figure 56. Effect of a chronic treatment with melatonin (75ng/L) on plasma lipid peroxidation in a rat model of MCT-induced PH, **p*<0.0001 (MCT vs. C), #*p*<0.002 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a similar antioxidant capacity compared to MCT rats (20.23 ± 2.61 vs. 18.40 ± 2.29 nmol/L trolox equivalents) (Figure 57). Melatonin treated controls had a similar antioxidant capacity compared to normal controls (29.50 ± 2.37 vs. 30.67± 3.88 nmol/L trolox equivalents) (Figure 57).

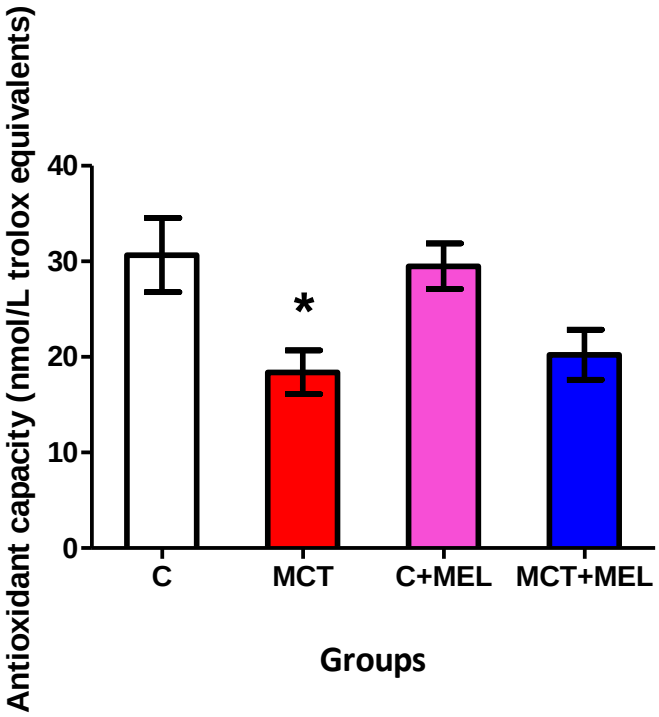


Figure 57. Effect of a chronic treatment with melatonin (75ng/L) on plasma antioxidant capacity in a rat model of MCT-induced PH, *****p<0.02 (MCT vs. C), (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower catalase activity compared to MCT (5.44 ± 0.75 vs. 12.40 ± 1.79 IU/mg protein, $p<0.0008$) (Figure 58). Melatonin treated controls had a similar catalase activity compared to controls (5.16 ± 0.87 vs. 3.72 ± 0.40 IU/mg protein (Figure 58).

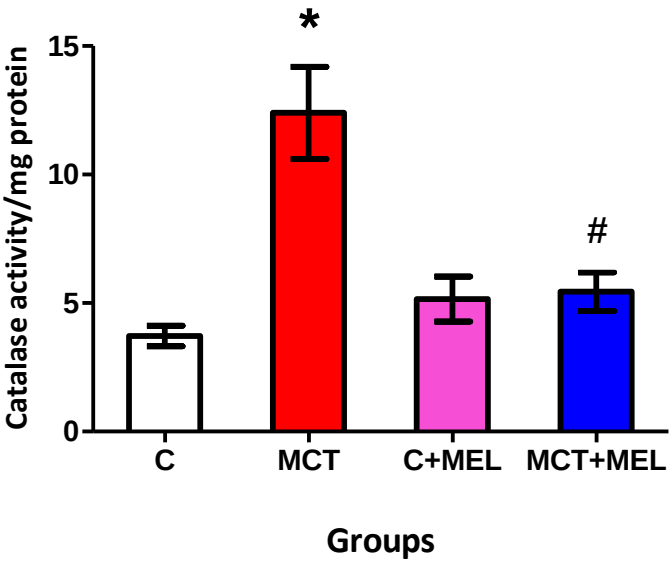


Figure 58. Effect of a chronic treatment with melatonin (75ng/L) on plasma catalase activity in a rat model of MCT-induced PH, *$p<0.0008$ (MCT vs. C), #$p<0.0008$ (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower SOD activity compared to MCT rats (8.75 ± 0.39 vs. 9.97 ± 0.20 IU/mg protein, $p<0.02$) (Figure 59). Melatonin treated control rats had a similar SOD activity compared to controls (7.69 ± 0.44 vs. 6.57 ± 0.83 IU/mg protein) (Figure 59).

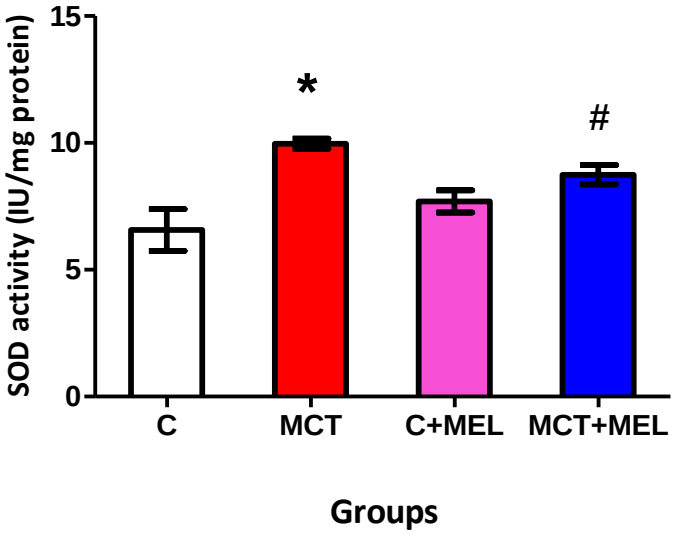


Figure 59. Effect of a chronic treatment with melatonin (75ng/L) on plasma superoxide dismutase (SOD) activity in a rat model of MCT-induced PH, *****$p<0.003$ (MCT vs. C), #$p<0.02$ (MCT+MEL vs. MCT), n≥6 per group.

4.6. Cardiac interstitial fibrosis: The effect of 75ng/L melatonin treatment

The percentage of collagen deposition in the RV sections of the MCT hearts were higher compared to the controls (4.68 ± 0.75 vs. 2.54 ± 0.36 % area, p<0.04) (Figure 60 A). The percentage of collagen deposition in the RV sections of the MCT+MEL hearts were similar to the MCT rats (4.20 ± 0.26 vs. 4.68 ± 0.75 %) (Figure 60A). Representative images of all groups are shown below (Figure 60 B).

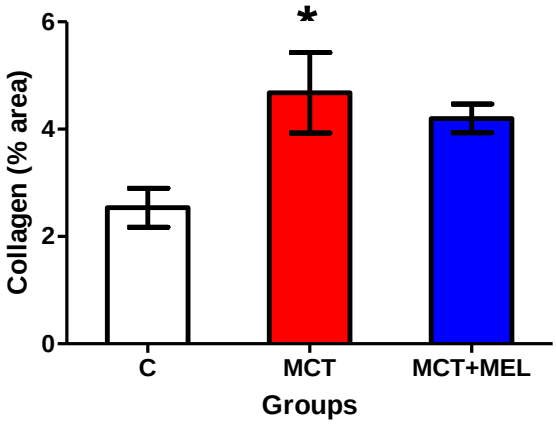


Figure 60 (A). Effect of a chronic treatment with melatonin (75ng/L) on cardiac interstitial fibrosis in a rat model of MCT-induced PH, ***p**<0.04 (MCT vs. C), (MCT+MEL vs. MCT), n=4 per group.

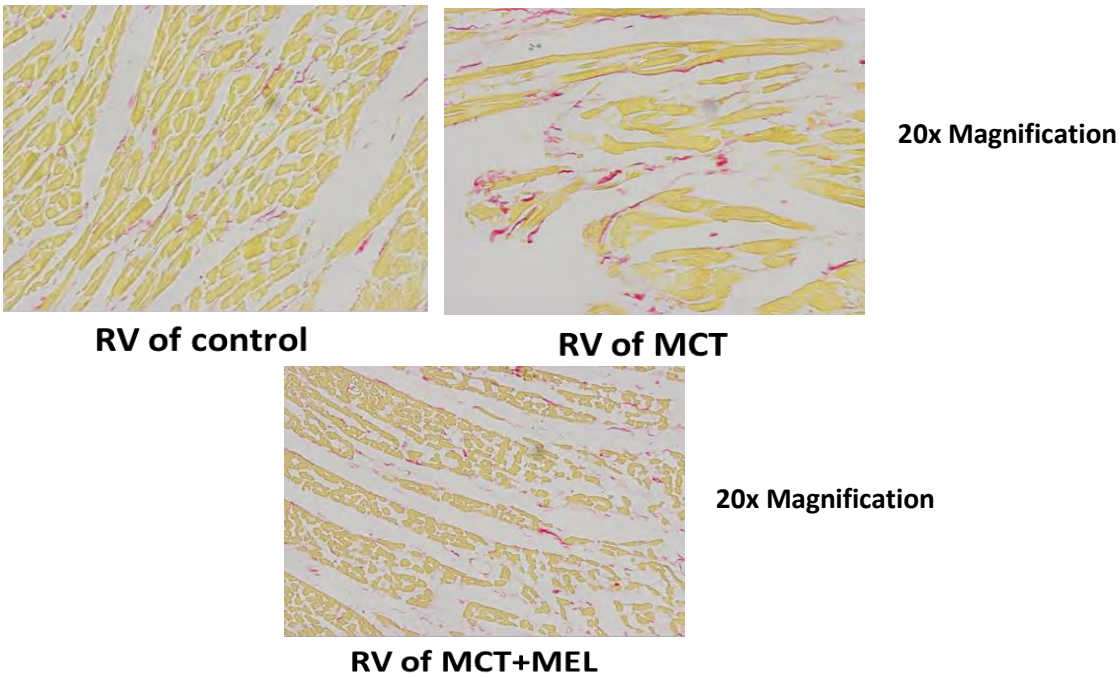


Figure 60 (B). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

5. Results: Effects of 4mg/kg melatonin

5.1. Biometric measurements

Table 7 summarizes the biometric measurements including body weights, weights of organs and weight of these organs expressed as a ratio of body weight or tibial length. Melatonin treated MCT rats had significantly higher body weights (323.0 ± 5.46 vs. 218.80 ± 18.41 g, $p<0.0002$) while the heart weights were significantly lower (1.24 ± 0.15 vs. 1.62 ± 0.07 g, $p>0.05$). Melatonin treated MCT rats had similar heart weights over body weights (0.46 ± 0.05 vs. 0.57 ± 0.02 g/g, $p>0.05$) and similar heart weight over tibial length ratios (0.31 ± 0.04 vs. 0.37 ± 0.02 g/cm, $p<0.09$). Melatonin treated MCT rats had significantly lower RV weights (0.28 ± 0.04 vs. 0.55 ± 0.02 g/g, $p<0.0001$) and higher LV weights compared to MCT rats (0.71 ± 0.04 vs. 0.65 ± 0.01 g/g, $p<0.04$). Melatonin treated MCT rats had lower lung weights compared to untreated MCT rats (1.80 ± 0.25 vs. 3.59 ± 0.13 g, $p<0.0001$) and lower lung weight over body weight ratios (0.70 ± 0.09 vs. 1.12 ± 0.04 g/g, $p<0.002$). Melatonin treated MCT had also lower liver weights (11.44 ± 1.15 vs. 19.60 ± 1.22 g, $p<0.0002$) and similar liver weight over body weight ratios (0.48 ± 0.08 vs. 0.56 ± 0.03 g/g, $p>0.05$).

Table 7. Biometric measurements

			Melatonin treatment (4mg/kg MEL)		
	C (at day 28)	**(MCT at day 28)**	**C+MEL**	**MCT+MEL**	***p*-value**
Biometric measurements:					
Body weight (g)	323.0 ± 5.46	218.80 ± 18.41	338.8 ± 11.68	280.40 ± 14.98*	*<0.0002
Heart weight (g)	1.36 ± 0.07	1.62 ± 0.07	1.48 ± 0.15	1.24 ± 0.15*	*<0.04
Heart weight/body weight (g/g)	0.36 ± 0.02	0.57 ± 0.02	0.43 ± 0.04	0.46 ± 0.05	>0.05
RV weight/LV+septal weight (g/g)	0.20 ± 0.01	0.55 ± 0.02	0.25 ± 0.02	0.28 ± 0.04*	*<0.0001
LV+septal weight/heart weight (g/g)	0.80 ± 0.03	0.65 ± 0.01	0.79 ± 0.01	0.71 ± 0.04*	*<0.04
Heart weight/tibial length (g/cm)	0.32 ± 0.02	0.37 ± 0.02	0.40 ± 0.05	0.31 ± 0.04	>0.05
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13	1.60 ± 0.10	1.80 ± 0.25*	*<0.0001
Lung weight/body weight (g/g)	0.49 ± 0.03	1.12 ± 0.04	0.60 ± 0.16	0.70 ± 0.09*	*<0.002
Liver weight (g)	15.81 ± 0.59	19.60 ± 1.22	17.27 ± 0.42	11.44 ± 1.15*	*<0.0002
Liver weight/body weight (g/g)	0.42 ± 0.03	0.56 ± 0.03	0.51 ± 0.01	0.48 ± 0.08	>0.05

C: Control, **MCT:** Monocrotaline, **MEL:** Melatonin. *p<0.0001 (MCT vs C, MCT+MEL vs. MCT), *p<0.0001 (MCT+MEL vs. MCT), *p<0.05 (MCT vs C), *p<0.0006 (MCT vs. C), n≥ 6 per group. Values are mean ± standard error of the mean.

5.2. Echocardiography: LV functional parameters

Echocardiography measurements showed that LV fractional shortening was significantly lower in the melatonin treated MCT rats compared to normal MCT rats (56.66 ± 4.65 vs. 69.37 ± 2.17 %, $p<0.01$) (Figure 61). Melatonin treated control rats had similar LV fractional shortening (39.69 ± 1.47 vs. 36.13 ± 1.08 %) (Figure 61).

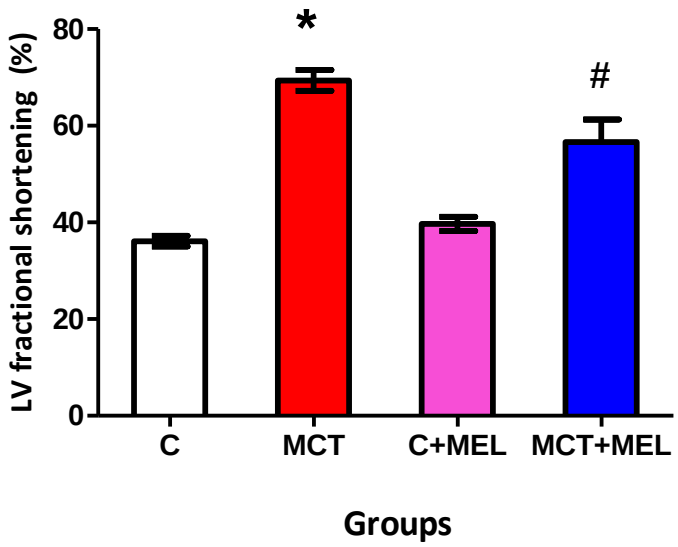


Figure 61. Effect of a chronic treatment with melatonin (4mg/kg) on left ventricular fractional shortening in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.01 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly higher LV end diastolic volumes compared to MCT rats (0.701 ± 0.05 vs. 0.193 ± 0.008 mL, $p<0.0001$) (Figure 62). Melatonin treated control rats had similar LV end diastolic volumes (0.76 ± 0.04 vs. 0.68 ± 0.05 mL) compared to normal controls (Figures 62).

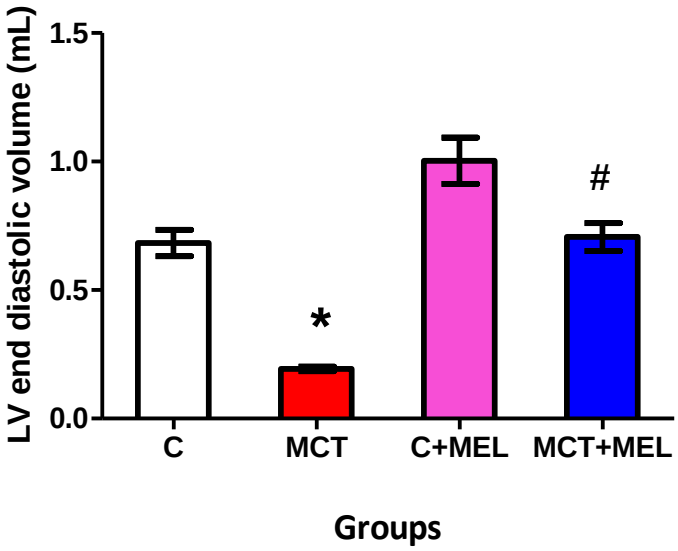


Figure 62. Effect of a chronic treatment with melatonin (4mg/kg) on left ventricular end diastole volume in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.0001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly lower LV ejection fractions (89.32 ± 2.55 vs. 96.05± 0.63 %, p<0.04) (Figure 63). Melatonin treated control rats had similar LV ejection fractions (72.90 ± 1.49 vs. 69.09 ± 1.57 %) (Figure 63).

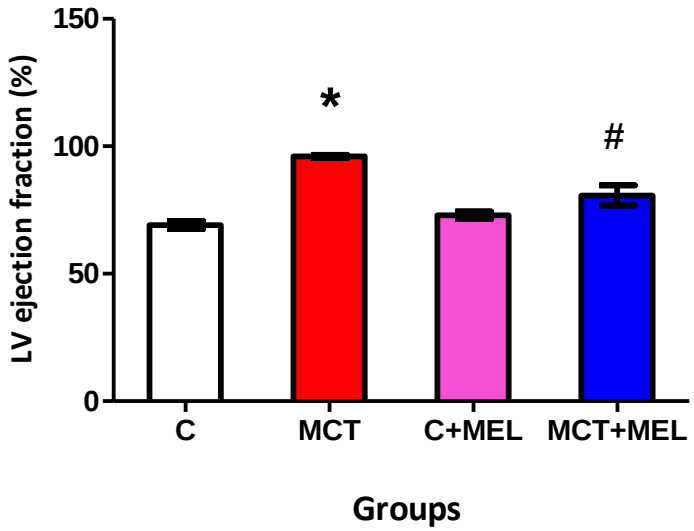


Figure 63. Effect of a chronic treatment with melatonin (4mg/kg) on left ventricular ejection fraction in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), #p<0.04 (MCT+MEL vs. MCT), n≥6 per group.

5.3. Isolated heart perfusions: Left ventricular function

Isolated heart perfusions showed that melatonin treated MCT rats had similar LV developed pressures compared to MCT rats (97.14 ± 3.47 vs. 96.08 ± 6.66 mmHg) (Figure 64). Melatonin treated control rats had similar LV developed pressures compared to controls (97.91 ± 1.86 vs. 100.30 ± 7.38 mmHg) (Figure 64).

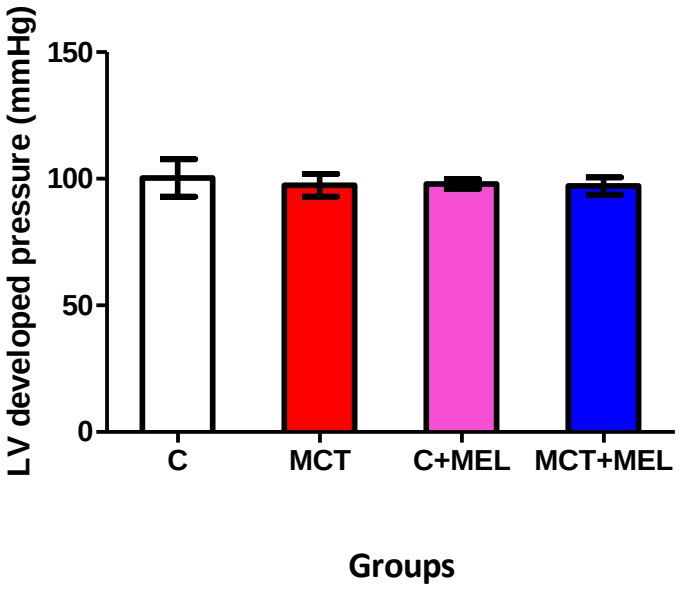


Figure 64. Effect of a chronic treatment with melatonin (4mg/kg) on left ventricular developed pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV systolic pressure was similar for melatonin treated MCT rats compared to MCT rats (103.50 ± 1.25 vs. 106.10 ± 4.44 mmHg) (Figure 65). Melatonin treated control rats had similar LV systolic pressures compared to controls (107.00 ± 1.86 vs. 103.80 ± 4.11 mmHg) (Figure 65).

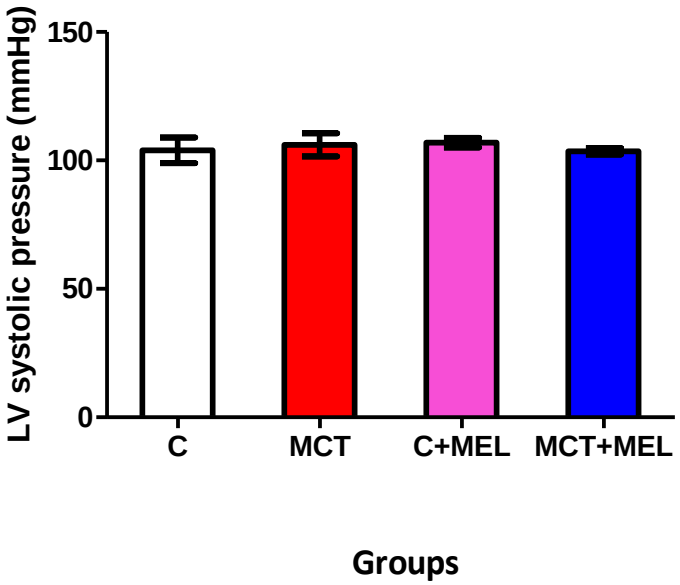


Figure 65. Effect of a chronic treatment with melatonin (4mg/kg) on left ventricular systolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV diastolic pressure was similar for melatonin treated MCT rats compared to MCT rats (8.27 ± 0.09 vs. 8.59 ± 0.08 mmHg) (Figure 66). Melatonin treated control rats had similar LV systolic pressures compared to controls (8.56 ± 0.12 vs. 8.56 ± 0.10 mmHg) (Figure 66).

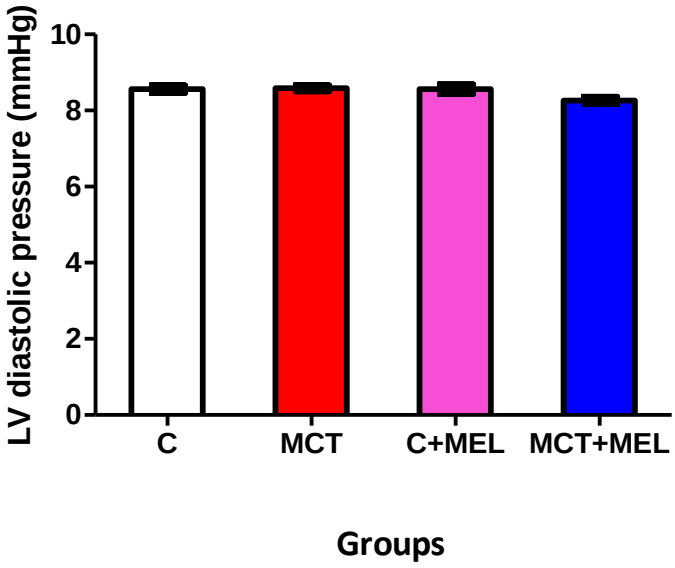


Figure 66. Effect of a chronic treatment with melatonin (4mg/kg) on left ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The heart rate was similar for melatonin treated MCT rats compared to MCT rats (304.60 ± 6.06 vs. 303.30 ± 18.16 bpm) (Figure 67). Melatonin treated control rats had similar heart rates compared to controls (307.1 ± 5.41 vs. 303.20 ± 14.77 bpm) (Figure 67).

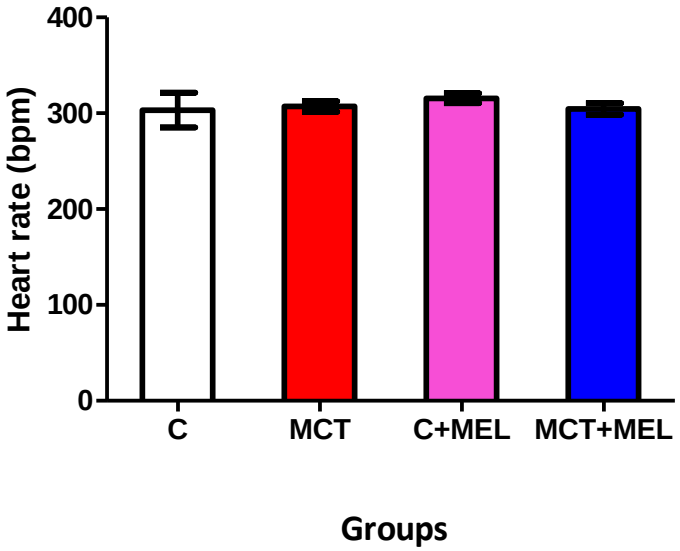


Figure 67. Effect of a chronic treatment with melatonin (4mg/kg) on heart rate in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

5.4. Isolated heart perfusions: RV function

The RV developed pressure was lower in the melatonin treated MCT rats compared to MCT rats (57.18 ± 3.04 vs. 81.22 ± 2.75 mmHg, $p<0.001$) (Figure 68) Control rats treated with melatonin had similar RV developed pressures compared to normal controls (45.89 ± 0.54 vs. 45.87 ± 2.11 mmHg) (Figures 68).

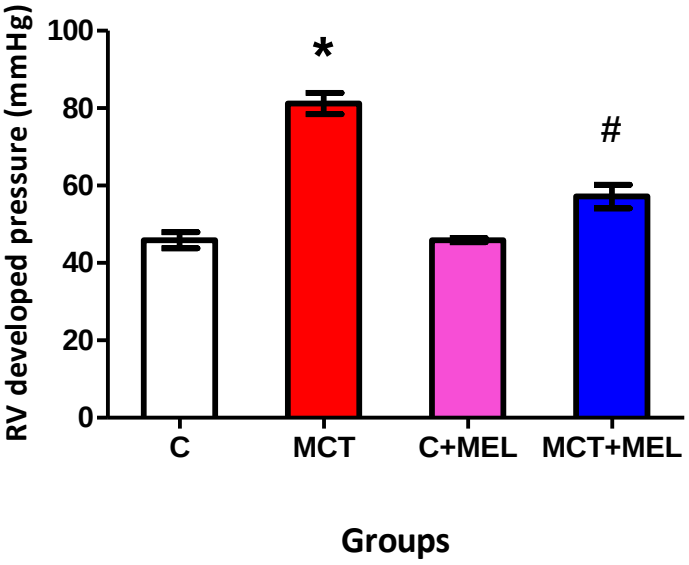


Figure 68. Effect of a chronic treatment with melatonin (4mg/kg) on right ventricular developed pressure in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had lower RV systolic pressures compared to the MCT rats (68.40 ± 3.59 vs. 87.91 ± 5.48 mmHg, $p<0.002$) (Figure 69). Control rats treated with melatonin had similar RV developed pressures compared to normal controls (54.37 ± 0.57 vs. 54.35 ± 2.28 mmHg) (Figure 69).

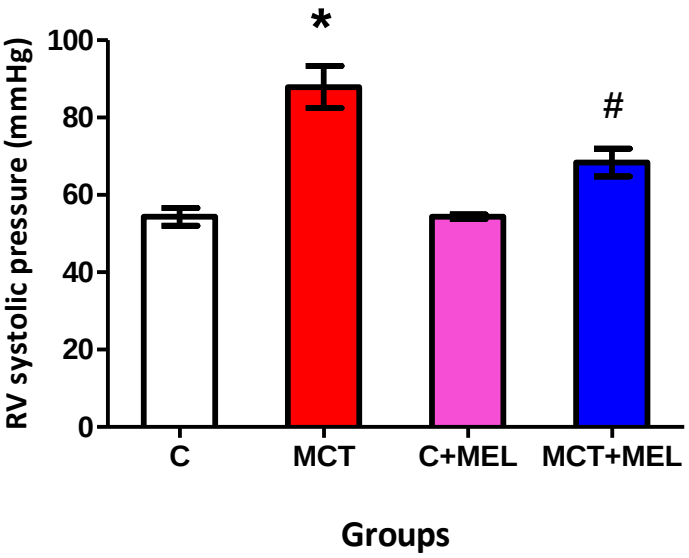


Figure 69. Effect of a chronic treatment with melatonin (4mg/kg) on right ventricular systolic pressure in a rat model of MCT-induced PH, *****$p<0.0001$ (MCT vs. C), #$p<0.002$ (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had similar RV diastolic pressures compared to the MCT rats (8.49 ± 0.08 vs. 8.74 ± 0.04 mmHg) (Figure 70). Control rats treated with melatonin had similar RV diastolic pressures compared to normal controls (8.48 ± 0.13 vs. 8.51 ± 0.12 mmHg) (Figure 70).

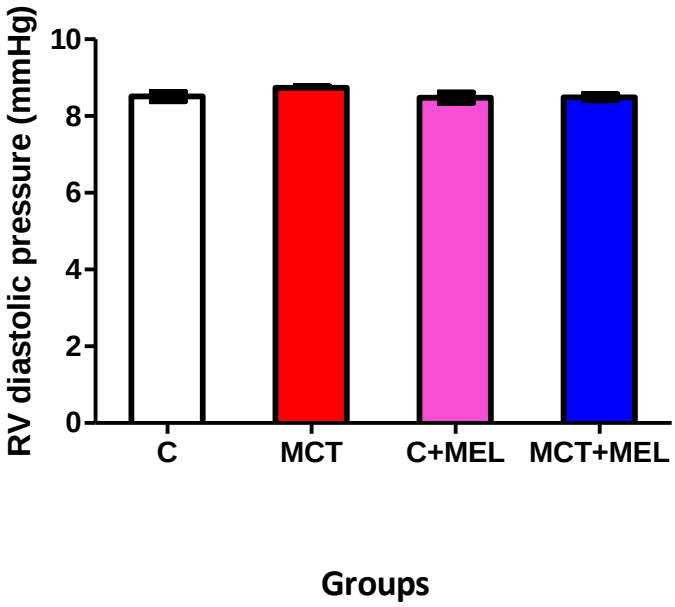


Figure 70. Effect of a chronic treatment with melatonin (4mg/kg) on right ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

5.5. Plasma oxidative stress and antioxidant capacity

Levels of blood plasma were measured by assessing the thiobarbituric acid reactive substances (TBARS) in all treated groups. Melatonin treated MCT rats had significantly lower levels of plasma lipid peroxidation compared to MCT rats (1.66 ± 0.07 vs. 2.27 ± 0.22 µmol/mL, $p<0.03$) (Figure 71). Melatonin treated controls had similar levels of plasma lipid peroxidation compared to normal controls (0.88 ± 0.05 vs. 0.83 ± 0.05 µmol/mL) (Figure 71).

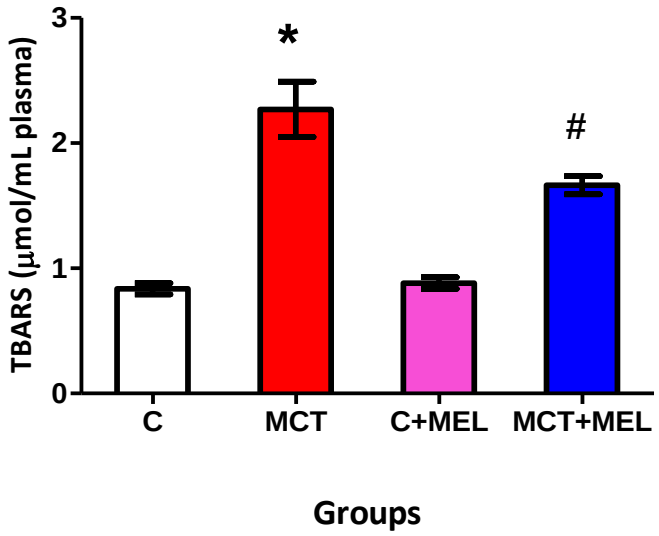


Figure 71. Effect of a chronic treatment with melatonin (4mg/kg) on plasma lipid peroxidation in a rat model of MCT-induced PH, ***$p<0.0001$** (MCT vs. C), #$p<0.03$ (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a higher antioxidant capacity compared to MCT rats (26.69 ± 1.84 vs. 18.40 ± 2.29 nmol/L trolox equivalents, $p<0.02$) (Figure 72). Melatonin treated controls had a similar antioxidant capacity compared to normal controls (23.23 ± 1.43 vs. 30.6 7± 3.88 nmol/L trolox equivalents) (Figure 72).

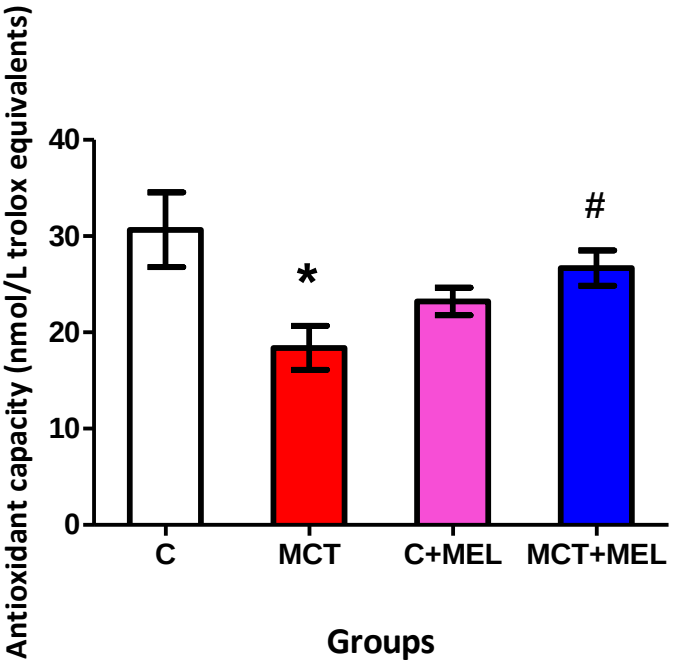


Figure 72. Effect of a chronic treatment with melatonin (4mg/kg) on plasma antioxidant capacity in a rat model of MCT-induced PH, ***p*<0.02 (MCT vs. C), #*p*<0.02 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower catalase activity compared to MCT (7.27 ± 0.88 vs. 12.40 ± 1.79 IU/mg protein, $p<0.006$) (Figure 73). Melatonin treated controls had a similar catalase activity compared to controls (4.71 ± 0.97 vs. 3.72 ± 0.40 IU/mg protein (Figure 73).

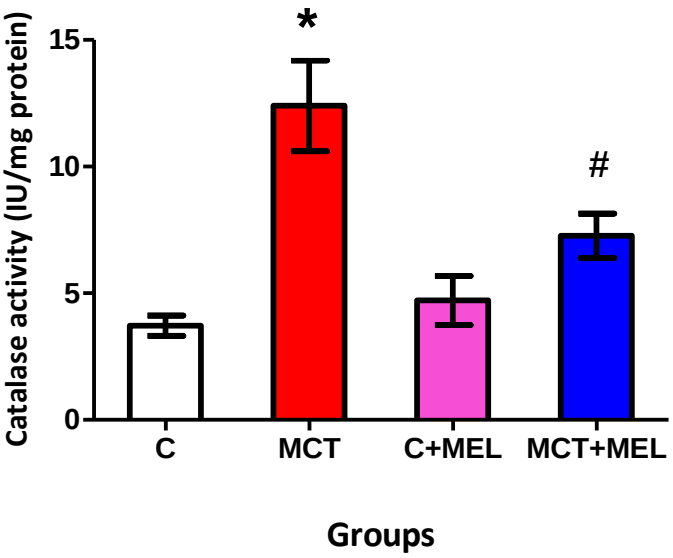


Figure 73. Effect of a chronic treatment with melatonin (4mg/kg) on plasma catalase activity in a rat model of MCT-induced PH, ***$p<0.0008$** (MCT vs. C), #$p<0.006$ (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower SOD activity compared to MCT rats (8.20 ± 0.71 vs. 9.97 ± 0.20 IU/mg protein, $p<0.04$) (Figure 74). Melatonin treated control rats had a similar SOD activity compared to controls (6.80 ± 0.65 vs. 6.57 ± 0.83 IU/mg protein) (Figure 74).

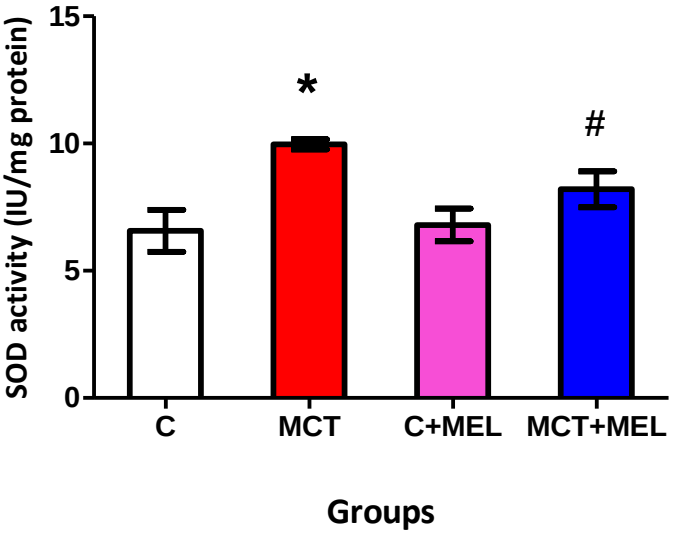


Figure 74. Effect of a chronic treatment with melatonin (4mg/kg) on plasma superoxide dismutase (SOD) activity in a rat model of MCT-induced PH, *****$p<0.003$ (MCT vs. C), #$p<0.04$ (MCT+MEL vs. MCT), n≥6 per group.

5.6. Cardiac interstitial fibrosis: The effect of 4mg/kg melatonin treatment

The percentage of collagen deposition in the RV sections of the MCT hearts were higher compared to the controls (4.68 ± 0.75 vs. 2.54 ± 0.36 % area, p<0.04) (Figure 75 A). The percentage of collagen deposition in the RV sections of the MCT+MEL hearts were similar to the MCT rats (3.82 ± 1.02 vs. 4.68 ± 0.75 %) (Figure 75 A). Representative images of all groups are shown below (Figure 75 B).

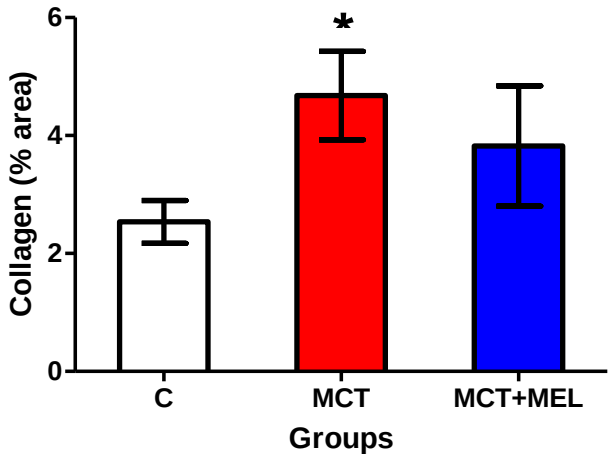


Figure 75 (A). Effect of a chronic treatment with melatonin (4mg/kg) on cardiac interstitial fibrosis in a rat model of MCT-induced PH, ***p**<0.04 (MCT vs. C), (MCT+MEL vs. MCT), n=4 per group.

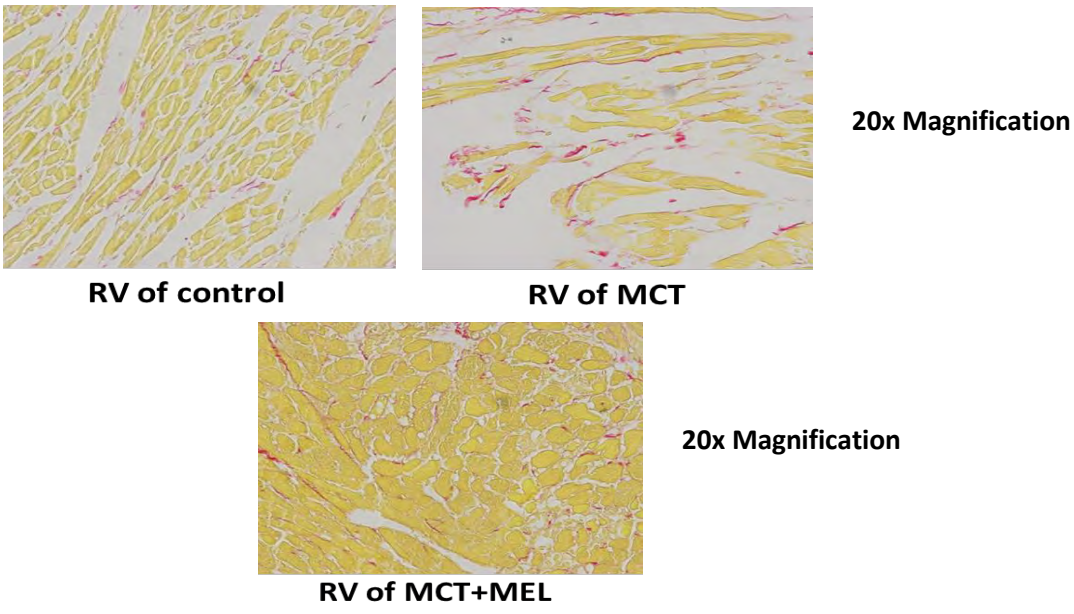


Figure 75 (B). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

6. Results: Effects of 6mg/kg melatonin

6.1. Biometric measurements

Table 8 summarizes the biometric measurements including body weights, weights of organs and weight of these organs expressed as a ratio of body weight or tibial length. Melatonin treated MCT rats had significantly higher body weights (323.00 ± 5.46 vs. 218.80 ± 18.41 g, $p<0.02$) while the heart weights were similar (1.28 ± 0.09 vs. 1.62 ± 0.07 g, $p<0.01$). Melatonin treated MCT rats had similar heart weights over body weights (0.41 ± 0.02 vs. 0.57 ± 0.02 g/g, $p>0.05$) and similar heart weight over tibial length ratios (0.28 ± 0.01 vs. 0.37 ± 0.02 g/cm, $p>0.05$). Melatonin treated MCT rats had significantly lower RV weights (0.30 ± 0.04 vs. 0.55 ± 0.02 g/g, $p<0.0001$) and higher LV weights compared to MCT rats (0.74 ± 0.04 vs. 0.65 ± 0.01 g/g, $p<0.04$). Melatonin treated MCT rats had lower lung weights compared to untreated MCT rats (2.65 ± 0.3 vs. 3.59 ± 0.13 g, $p<0.02$) and similar lung weight over body weight ratios (1.03 ± 0.14 vs. 1.12 ± 0.04 g/g, $p<0.03$). Melatonin treated MCT had also lower liver weights (12.77 ± 0.97 vs. 19.60 ± 1.22 g, $p<0.0001$) and lower liver weight over body weight ratios (0.39 ± 0.02 vs. 0.56 ± 0.03 g/g, $p<0.0002$).

Table 8. Biometric measurements

			Melatonin treatment (6mg/kg MEL)		
	C (at day 28)	**(MCT at day 28)**	**C+MEL**	**MCT+MEL**	***p*-value**
Biometric measurements:					
Body weight (g)	323.0 ± 5.46	218.80 ± 18.41	334.20 ± 6.53	265.60 ± 5.27*	*<0.02
Heart weight (g)	1.36 ± 0.07	1.62 ± 0.07	1.37 ± 0.08	1.28 ± 0.09*	*<0.01
Heart weight/body weight (g/g)	0.36 ± 0.02	0.57 ± 0.02	0.43 ± 0.02	0.41 ± 0.02*	*<0.001
RV weight/LV+septal weight (g/g)	0.20 ± 0.01	0.55 ± 0.02	0.19 ± 0.01	0.30 ± 0.04*	*<0.0001
LV+septal weight/heart weight (g/g)	0.80 ± 0.03	0.65 ± 0.01	0.83 ± 0.01	0.74 ± 0.04*	*<0.04
Heart weight/tibial length (g/cm)	0.32 ± 0.02	0.37 ± 0.02	0.33 ± 0.03	0.28 ± 0.01	>0.05
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13	1.50 ± 0.05	2.65 ± 0.3*	*<0.02
Lung weight/body weight (g/g)	0.49 ± 0.03	1.12 ± 0.04	0.45 ± 0.01	1.03 ± 0.14*	*<0.03
Liver weight (g)	15.81 ± 0.59	19.60 ± 1.22	15.28 ± 1.14	12.77 ± 0.97*	*<0.0001
Liver weight/body weight (g/g)	0.42 ± 0.03	0.56 ± 0.03	0.455 ± 0.03	0.39 ± 0.02*	*0.0002

C: Control, **MCT:** Monocrotaline, **MEL:** Melatonin. *p<0.0001 (MCT vs C), *p<0.0001 (MCT vs C, MCT+MEL vs. MCT), *p<0.05 (MCT vs. C), *p<0.0006 (MCT vs C, MCT+MEL vs. MCT), n≥ 6 per group. Values are mean ± standard error of the mean.

6.2. Echocardiography: LV functional parameters

Echocardiography measurements showed that LV fractional shortening was significantly lower in the melatonin treated MCT rats compared to MCT rats (39.08 ± 5.19 vs. 69.37 ± 2.17 %, p<0.0001) (Figure 76). Control rats treated with melatonin had similar LV fractional shortening compared to normal controls (36.52 ± 1.88 vs. 36.13 ± 1.08 %) (Figures 76).

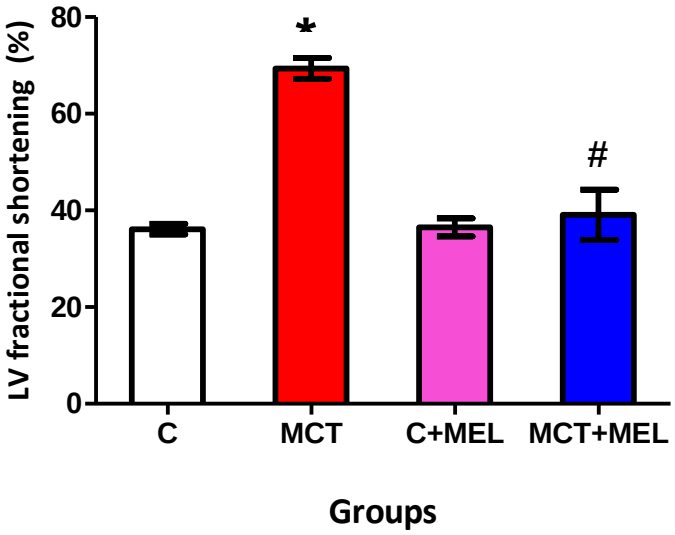


Figure 76. Effect of a chronic treatment with melatonin (6mg/kg) on left ventricular systolic fractional shortening in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.0001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly higher LV end diastolic volumes compared to MCT rats (0.64 ± 0.15 vs. 0.19 ± 0.008 mL, p<0.0001) (Figure 77). Control rats treated with melatonin had similar LV end diastolic volumes (0.78 ± 0.06 vs. 0.68 ± 0.05 mL) compared to controls (Figure 77).

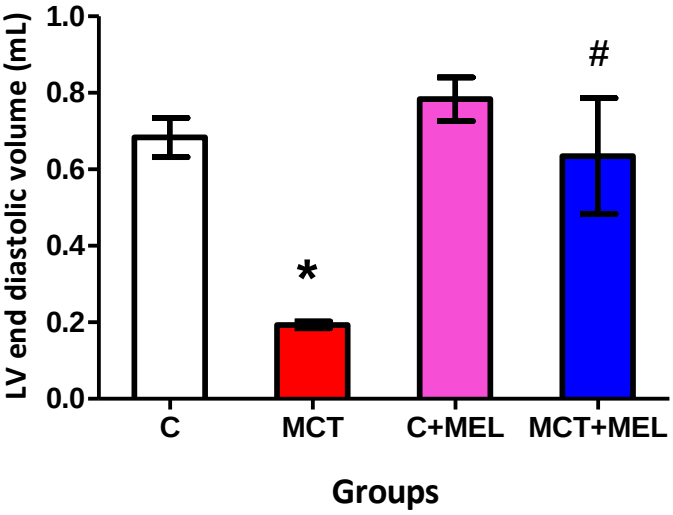


Figure 77. Effect of a chronic treatment with melatonin (6mg/kg) on left ventricular end diastole volume in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), #p<0.0001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly lower LV ejection fractions compared to MCT rats (80.74 ± 3.96 vs. 96.05 ± 0.63%, $p<0.02$) (Figure 78). Control rats treated with melatonin had similar LV ejection fractions compared to controls (72.83 ± 5.21 vs. 69.09 ± 1.57 %) (Figure 78).

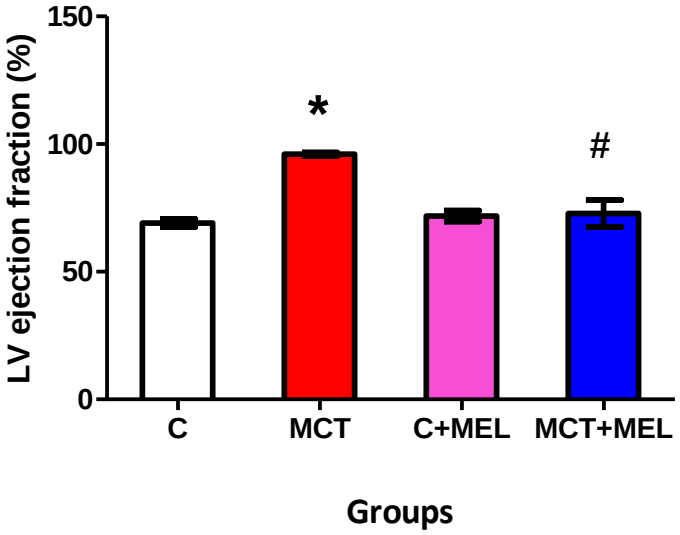


Figure 78. Effect of a chronic treatment with melatonin (6mg/kg) on left ventricular ejection fraction in a rat model of MCT-induced PH, ***$p<0.0001$** (MCT vs. C), #$p<0.02$ (MCT+MEL vs. MCT), n≥6 per group.

6.3. Isolated heart perfusions: Left ventricular function

Isolated heart perfusions showed that melatonin treated MCT rats had similar LV developed pressures compared to MCT rats (107.50 ± 1.53 vs. 98.41 ± 3.87 mmHg) (Figure 79). Melatonin treated control rats had similar LV developed pressures compared to controls (105.50 ± 2.75 vs. 104.20 ± 5.08 mmHg) (Figure 79).

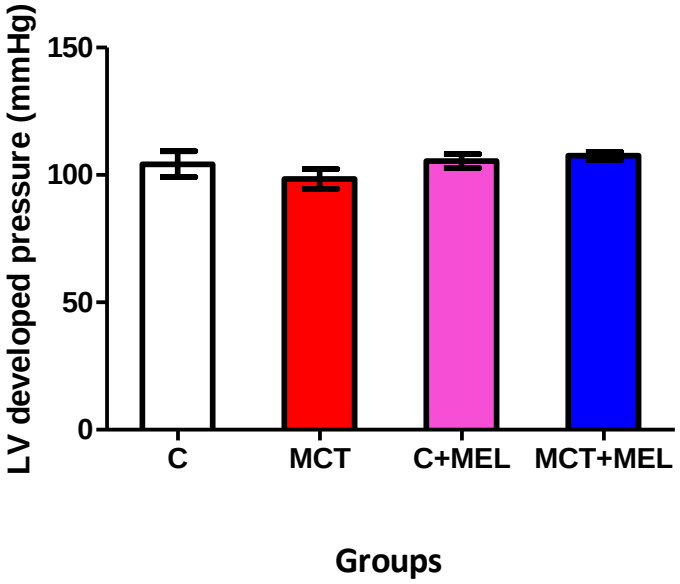


Figure 79. Effect of a chronic treatment with melatonin (6mg/kg) on left ventricular developed pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV systolic pressure was similar for melatonin treated MCT rats compared to MCT rats (109.70 ± 2.20 vs. 104.10 ± 5.82 mmHg) (Figure 80). Melatonin treated control rats had similar LV systolic pressures compared to controls (113.30 ± 2.70 vs. 110.00 ± 3.51 mmHg) (Figure 80).

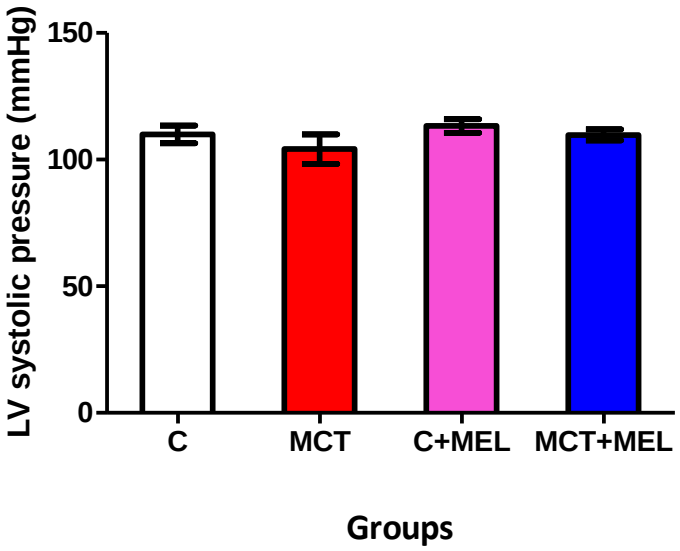


Figure 80. Effect of a chronic treatment with melatonin (6mg/kg) on left ventricular systolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV diastolic pressure was similar for melatonin treated MCT rats compared to MCT rats (8.59 ± 0.09 vs. 8.62 ± 0.11 ± 0.12 mmHg) (Figure 81). Melatonin treated control rats had similar LV systolic pressures compared to controls (8.39 ± 0.07 vs. 8.44 ± 0.15 mmHg) (Figure 81).

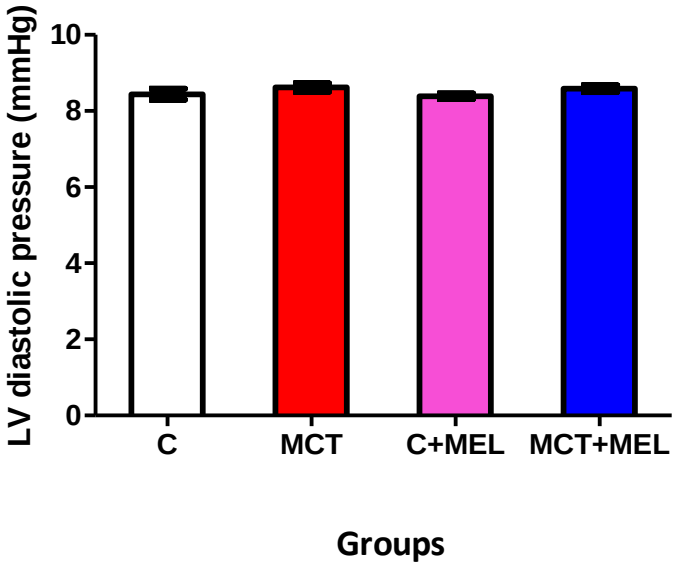


Figure 81. Effect of a chronic treatment with melatonin (6mg/kg) on left ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The heart rate was similar for melatonin treated MCT rats compared to MCT rats (318.40 ± 16.22 vs. 284.40 ± 13.23 bpm) (Figure 82). Melatonin treated control rats had similar heart rates compared to controls (312.90 ± 17.99 vs. 303.20 ± 14.77 bpm) (Figure 82).

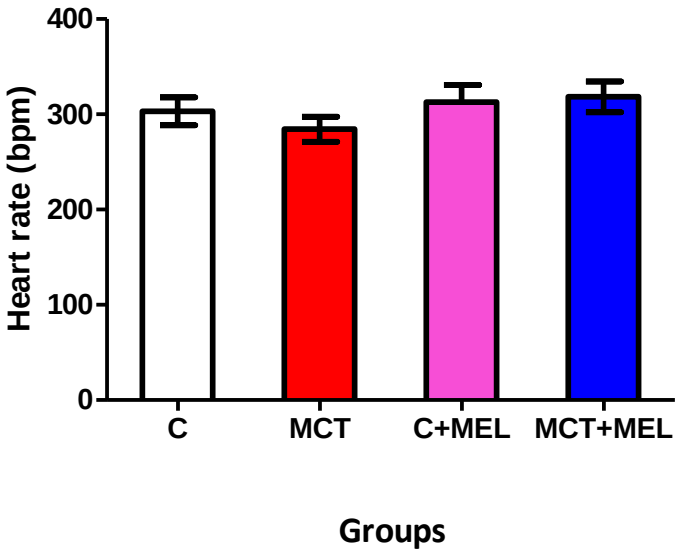


Figure 82. Effect of a chronic treatment with melatonin (6mg/kg) on heart rate in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

6.4. Isolated heart perfusions: Right ventricular function

The RV developed pressure was lower in the melatonin treated MCT rats compared to MCT rats (60.38 ± 3.10 vs. 81.22 ± 2.75 mmHg, $p<0.009$) (Figure 83) Control rats treated with melatonin had similar RV developed pressures compared to normal controls (48.72 ± 5.18 vs. 45.87 ± 2.11 mmHg) (Figures 83).

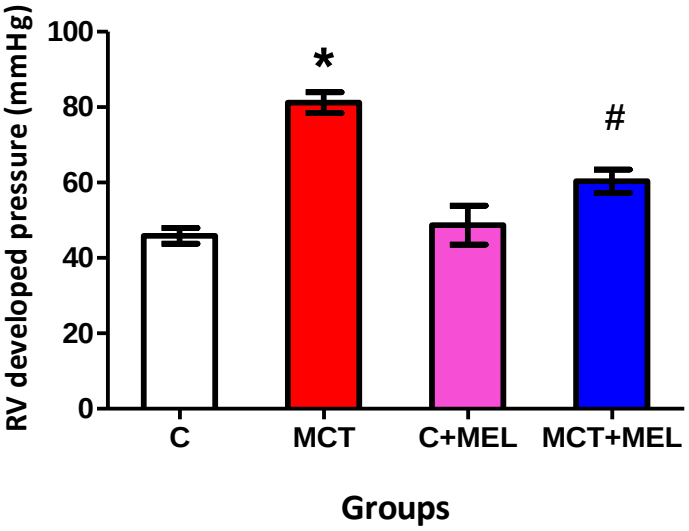


Figure 83. Effect of a chronic treatment with melatonin (6mg/kg) on right ventricular developed pressure in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.009 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had lower RV systolic pressures compared to the MCT rats (64.34 ± 2.55 vs. 87.91 ± 5.48 mmHg, p<0.0004) (Figure 84). Control rats treated with melatonin had similar RV systolic pressures compared to normal controls (54.50 ± 3.73 vs. 54.35 ± 2.28 mmHg) (Figure 84).

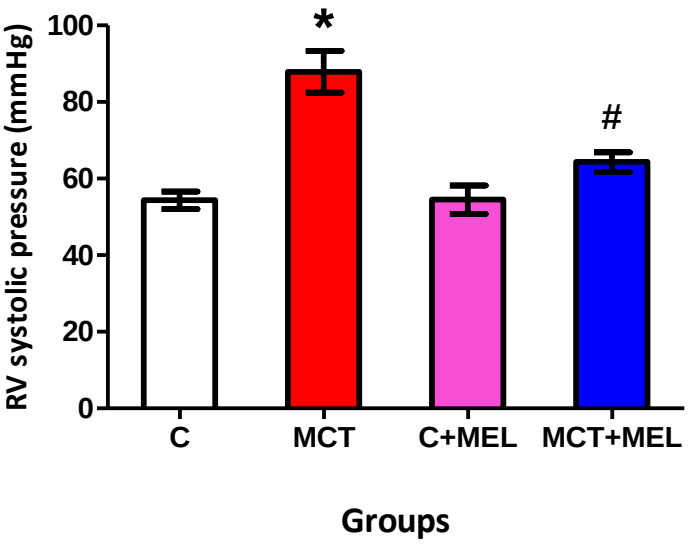


Figure 84. Effect of a chronic treatment with melatonin (6mg/kg) on right ventricular systolic pressure in a rat model of MCT-induced PH, *****p<0.0001 (MCT vs. C), #p<0.0004 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had similar RV diastolic pressures compared to the MCT rats (8.44 ± 0.05 vs. 8.73 ± 0.08 mmHg) (Figure 85). Control rats treated with melatonin had similar RV diastolic pressures compared to normal controls (8.51 ± 0.10 vs. 8.55 ± 0.10 mmHg) (Figure 85).

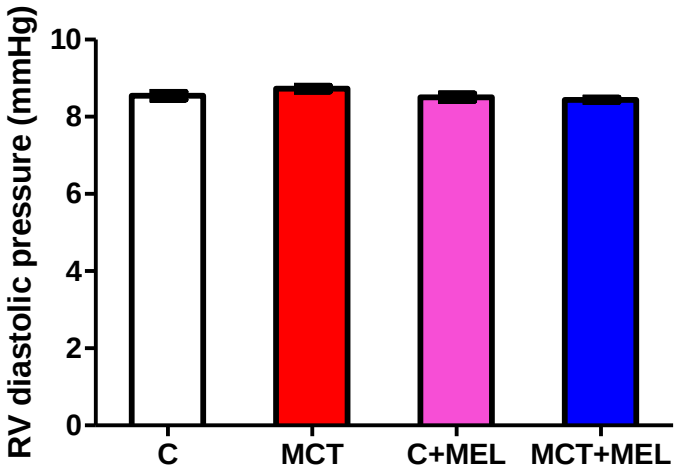


Figure 85. Effect of a chronic treatment with melatonin (6mg/kg) on right ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

6.5. Plasma oxidative stress and antioxidant capacity

Levels of blood plasma were measured by assessing the thiobarbituric acid reactive substances (TBARS) in all treated groups. Melatonin treated MCT rats had significantly lower levels of plasma lipid peroxidation compared to MCT rats (1.65 ± 0.12 vs. 2.27 ± 0.22 µmol/mL, $p<0.005$) Figure 86). Melatonin treated controls had similar levels of plasma lipid peroxidation compared to normal controls (0.93 ± 0.05 vs. 0.83 ± 0.05 µmol/mL) (Figure 86).

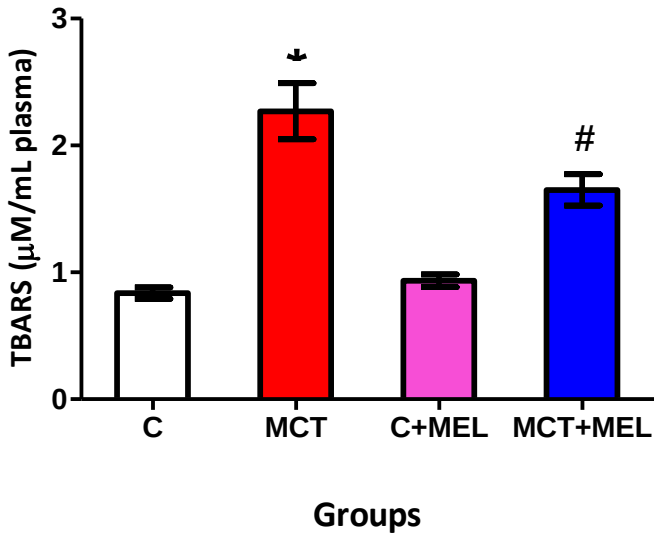


Figure 86. Effect of a chronic treatment with melatonin (6mg/kg) on plasma lipid peroxidation in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.005 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a higher antioxidant capacity compared to MCT rats (29.32 ± 1.35 vs. 18.40 ± 2.29 nmol/L trolox equivalents, p<0.004) (Figure 87). Melatonin treated controls had a similar antioxidant capacity compared to normal controls (32.20 ± 3.31 vs. 30.6 7 ± 3.88 nmol/L trolox equivalents) (Figure 87).

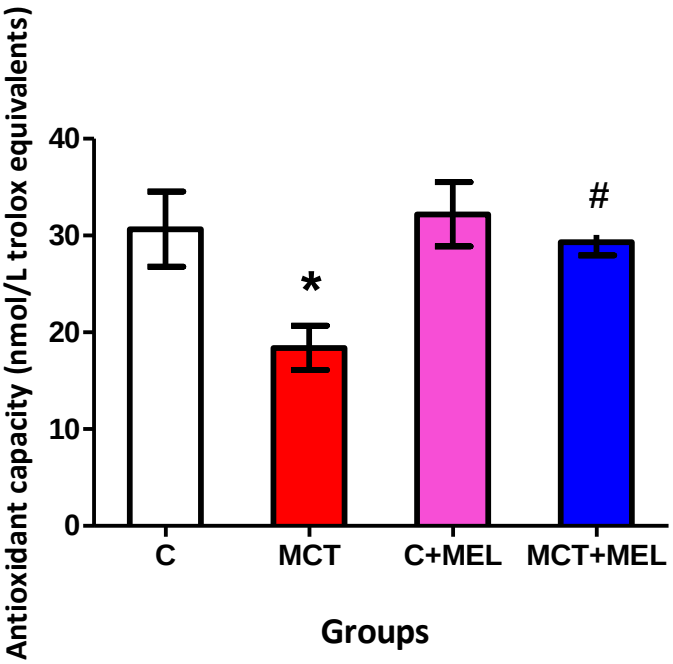


Figure 87. Effect of a chronic treatment with melatonin (6mg/kg) on plasma antioxidant capacity in a rat model of MCT-induced PH, *p<0.02 (MCT vs. C), #p<0.004 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower catalase activity compared to MCT (4.98 ± 0.68 vs. 12.40 ± 1.79 IU/mg protein, $p<0.0009$) (Figure 88). Melatonin treated controls had a similar catalase activity compared to controls (4.90 ± 1.04 vs. 3.72 ± 0.40 IU/mg protein, $p>0.05$) (Figure 88).

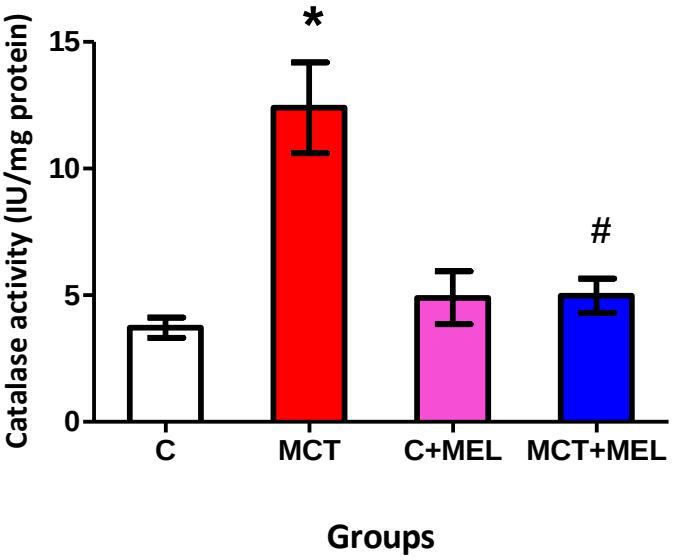


Figure 88. Effect of a chronic treatment with melatonin (6mg/kg) on plasma catalase activity in a rat model of MCT-induced PH, *$p<0.0008$ (MCT vs. C), #$p<0.0009$ (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower SOD activity compared to MCT rats (6.15 ± 0.73 vs. 9.97 ± 0.20 IU/mg protein, $p<0.01$) (Figure 89). Melatonin treated control rats had a similar SOD activity compared to controls (7.44 ± 0.52 ± 0.44 vs. 6.57 ± 0.83 IU/mg protein) (Figure 89).

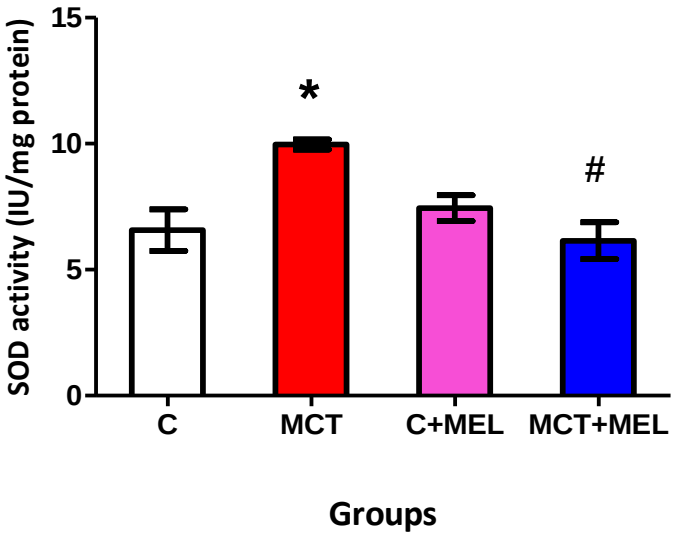


Figure 89. Effect of a chronic treatment with melatonin (6mg/kg) on plasma superoxide dismutase (SOD) activity in a rat model of MCT-induced PH, ***$p<0.003$** (MCT vs. C), #$p<0.01$ (MCT+MEL vs. MCT), n≥6 per group.

6.6. Cardiac interstitial fibrosis: The effect of 6mg/kg melatonin treatment

The percentage of collagen deposition in the RV sections of the MCT hearts were higher compared to the controls (4.68 ± 0.75 vs. 2.54 ± 0.36 % area, p<0.04) (Figure 90 A). The percentage of collagen deposition in the RV sections of the MCT+MEL hearts were similar to the MCT rats (3.89 ± 0.99 vs. 4.68 ± 0.75 %) (Figure 90 A). Representative images of all groups are shown below (Figure 90 B).

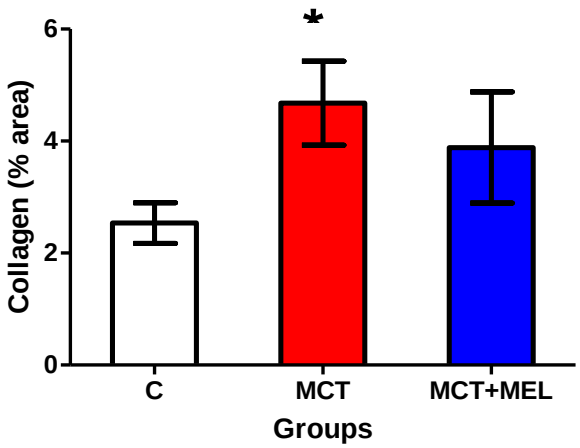


Figure 90 (A). Effect of a chronic treatment with melatonin (6mg/kg) on cardiac interstitial fibrosis in a rat model of MCT-induced PH, ***p**<0.04 (MCT vs. C), (MCT+MEL vs. MCT), n=4 per group.

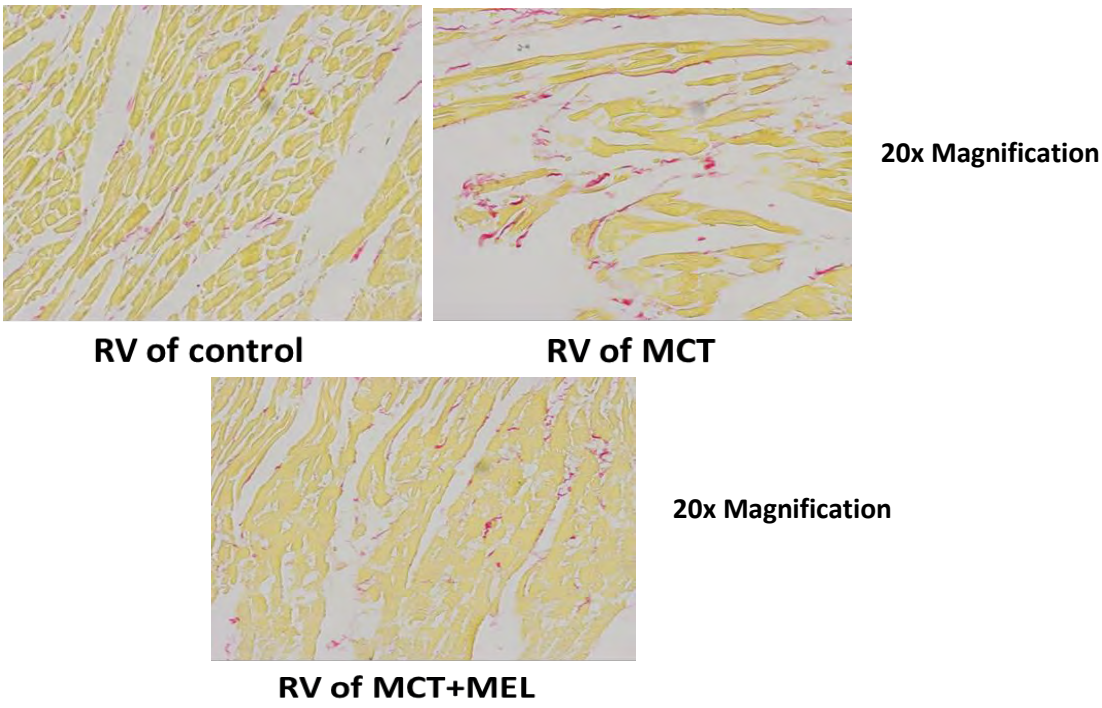


Figure 90 (B). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

7. Discussion for the effects of the different doses of melatonin

In the previous sections, we were able to show that oxidative stress is increased in the plasma of patients with PH and controls, as well as in the experimental model of MCT-induced PH. Our next goal was to test whether melatonin, a powerful antioxidant, could confer cardioprotection in experimental PH. In order to do this, we administered three different doses of melatonin (75ng/L, 4m/kg and 6mg/kg) in the drinking water of the experimental rats. Melatonin treatment commenced on the same day as the induction of experimental PH (i.e. day zero) and continued until the last day of the experimental protocol (i.e. day 28). Our results showed that melatonin treatment reduced RV hypertrophy, oxidative stress and improved cardiac function in the MCT model of PH.

Biometric measurements

Melatonin treatment for 28 days resulted in increased body weights compared to the MCT rats. There are currently no reports in the literature reporting similar findings, as melatonin has not been tested in this experimental model of PH. From the literature, it is clear that MCT causes weight loss due to a combination of factors including, growth retardation, and lack of physical activity, reduced food consumption, skeletal muscle wasting and heart failure associated cachexia [208,326,339]. Melatonin treatment may have modulated these factors to result in higher body weights. However, it's imperative to keep in mind that we started melatonin treatment on the same day as the induction of MCT-PH, which may have interfered with the disease induction and thus modulated pathological processes that normally cause body weight loss.

In the light of the above argument, it's possible that melatonin inhibited the disease induction, development and progression, at least partially, and by doing so, prevented further weight loss as would occur in the absence of melatonin. Thus, when melatonin treated MCT rats were weighed on day 28, they had higher body weights than untreated MCT rats. This effect of melatonin was achieved by all three doses of melatonin in our experiments.

The 75ng/L dose of melatonin had no effect on the heart weights while the higher doses (4mg/kg and 6mg/kg) decrease these measurements back to normal values. This was also reflected in the ratios of heart weight over body weight and heart weight over tibial length. As mentioned previously, it seems that the higher heart weights are mainly due to the marked RV hypertrophy and therefore, a reduction in the heart weight measurements reflects reduced RV hypertrophy caused by melatonin treatment. It is clear, that the 75ng/L dose of melatonin corresponding to nutritional values is not sufficient to modulate heart weights compared to the higher doses.

All of the doses of melatonin reduced lung weights and lung weight over body weight ratios. This could mean that melatonin treatment attenuated pulmonary oedema or damage, underlined by endothelial injury, oxidative stress and inflammation; however this was not assessed in our study. The protective effects of melatonin against lung injury and damage are widely reported for animal models of cigarette smoke exposure-induced lung damage, ventilator induced lung injury, hyperbaric oxygen exposure-induced lung injury and allergic lung inflammation [302-304]. Therefore, the reduction of lung weight by melatonin may have occurred because melatonin had direct pulmonary protective effects. Lastly, all doses of melatonin normalised liver weight and liver weight over body weight ratios. This suggests that melatonin also had a protective effect on the livers of MCT rats.

As melatonin has been shown to have antihypertensive properties, it could be argued that we should have measured the blood pressures of the melatonin treated rats. The blood pressure lowering effects of melatonin has been shown in healthy women and men in three classic studies in the literature [340-342]. These studies looked at the acute effects of melatonin, within 90 minutes post-administration and they used carotid atery pulsatility as an index of vasodilation. An interesting observation in these studies was that individuals who had higher baseline values, had greater reduction in pulsatility and lower blood pressures. Therefore, these data does not necessarily mean that melatonin reduces blood pressure of healthy individuals, but at the time, rather suggested that melatonin could reduce blood pressure in individuals perhaps already predisposed to hypertension. If these findings are extrapolated to our animal study, it becomes highly unlikely that laboratory bred rats would have varying baseline blood pressures or carotid artery pulsatility. Another interesting observation, is that in these three classic studies, these individuals were placed a “low light” room. These low light conditions could have contributed to the reduced blood pressure, as different types of light have been shown to affect plasma/saliva melatonin levels [343]. Furthermore, these investigators administered 1mg of melatonin to the individuals, which is three times lower

than the dose of melatonin available at pharmacies for jetlag or 5 times lower than the doses tested in healthy volunteers, yet without any reported effect on blood pressure [255-257,295]. Xia et al, have shown that melatonin causes a slight decrease in blood pressure in normotensive rats but this effect lasted for only 15 minutes, after which blood pressure returned to normal levels [344]. It is therefore clear that one should cautiously interpret these data of melatonin on blood pressure in healthy individuals or healthy animals. Nevertheless, it would have been relevant to measure the blood pressures in our animals in order to explore the possible effects of melatonin on blood pressure.

RV hypertrophy

In our experiments, RV hypertrophy was prevalent as displayed by the increased RV weight over LV weight plus septal weight ratios in the MCT rats. With this experimental model of PH, the RV hypertrophy is solely due to PH or increased RV afterload. Together with these findings, all three doses of melatonin reduced the RV hypertrophy. Therefore suggesting that melatonin reduced RV hypertrophy by attenuating PH, however, we can only assume its effects of PH as we did not measure pulmonary haemodynamic parameters. In line with this notion, melatonin's antihypertensive properties may also have come into play in our study. This is highly likely as melatonin can cause vasodilation by increasing plasma L-arginine levels (a substrate used by nitric oxide synthase to produce nitric oxide) and decreasing asymmetric dimethyl arginine (a natural inhibitor of nitric oxide) [251,252]. Furthermore, the antihypertensive effects of melatonin are also achieved by activation of its receptors, reduction of intracellular calcium, elevation of cyclic-GMP levels and consequent vasodilation [290]. However, these mechanisms would require further investigation in our study.

With this experimental model of PH, there are also direct oxidative stress-induced adverse effects on the RV which could also have been stunted with melatonin treatment. There are three interesting studies, where melatonin was administered at 10mg/kg or 30mg/kg for either four or 6 weeks to spontaneously hypertensive rats [294,345,346]. In all three these studies, spontaneous hypertensive rats developed LV hypertrophy and had marked systemic hypertension. Melatonin reduced systemic blood pressure in the rats with heart disease, but did not affect LV hypertrophy and these studies, concluded that melatonin did not have a direct effect on the hypertrophic process despite a reduction of systemic blood pressure. However, this phenomenon could have been due to the fact that in these studies, melatonin only achieved minimal effect on systemic blood pressure. In a rat model of hyperthyroid-

induced cardiac hypertrophy, melatonin treatment ameliorated cardiac hypertrophy [292]. Yeung et al, gave rats a daily intraperitoneal injection of 10mg/kg melatonin and subsequently exposed these rats to chronic hypoxia (10% oxygen) for 28 days [347]. Melatonin reduced RV hypertrophy compared to the vehicle treated rats. It appears therefore that the anti-hypertrophic effects of melatonin are achieved by attenuating the underlying disease and depends on the aetiology of the cardiac hypertrophy.

Echocardiography and Isolated heart perfusions: LV and RV function

Our echocardiography data showed that all three doses of melatonin increased LV fractional shortening and ejection fraction and also increased LV end diastolic volumes towards control levels. LV dysfunction in the MCT model, as measured with echocardiography, has been attributed to LV atrophy caused by the MCT toxin. [336] Melatonin attenuates the LV atrophy as seen by the normalised LV weight ratios, which could be the reason for the improved LV function.

We did not find any cardiac functional abnormalities when we assessed LV function with isolated heart perfusions. Therefore, also no effects were seen with melatonin treatment. We ascribe this to the insensitivity of the Langendorff isolated heart perfusion apparatus to detect up functional changes which echocardiography can detect. Nevertheless, the ability of melatonin to improve cardiac function has been shown in other models of cardiac dysfunction [250,273]. The mechanisms underlining these cardioprotective effects of melatonin have been ascribed to its direct antioxidant properties, antiadrenergic and receptor mediated effects [273]. Furthermore, melatonin has also been shown to confer cardioprotection by activating the RISK pathway, which is an inherent, cellular pro-survival signalling pathway [273]. Melatonin also improves cardiac function, by improving calcium handling at the level of the sarcoplasmic reticulum in the setting of ischemia/reperfusion [347]. Lastly, Lamont et al, demonstrated that melatonin confers cardioprotection via the activation of the survivor activating factor enhancement (SAFE) pro-survival signalling pathway that involves the activation of tumour necrosis factor alpha and the signal transducer and activator of transcription 3 (STAT3) [250]. It is possible that all these functions of melatonin could have contributed to improvement of cardiac function seen in our study.

Blood plasma oxidative stress and antioxidant capacity

All three doses of melatonin reduced plasma lipid peroxidation, increased plasma antioxidant capacity and normalised plasma catalase and SOD activity. Melatonin is well known for its powerful antioxidant effect in various animal models of cardiovascular disease [288]. This antioxidant effect of melatonin is either achieved by increasing the activity of antioxidant enzymes via promoting a preferable reduced state or by increasing antioxidant enzyme expression [282]. In our study it became apparent that despite an increase in catalase and SOD activity, oxidative stress (lipid peroxidation) was still increased in the MCT rats. Therefore, the fact that oxidative stress was reduced in the plasma of melatonin treated MCT rats means that melatonin might have had a direct effect on the oxidative stress by scavenging free radicals either independent or in concert with antioxidant enzymes. Melatonin can directly bind hydroxyl radical to the C-2 position of its indole ring and interact with hydrogen peroxide and form water as a by-product and N-acetyl-n-formyl-5-methoxy-kenuramine (AFMK) [284,285]. These antioxidant abilities of melatonin are even further amplified by its by-products which have also been shown to scavenge oxygen free radicals more effectively than melatonin itself [284,285]. It has been demonstrated by Venegas et al, that high doses of melatonin (10mg/kg-200mg/kg) seem necessary to reach subcellular concentrations sufficient and exert pharmacological therapeutic effects [348]. However, our data show clearly that even the low concentration of melatonin (75ng/L) can modulate antioxidant capacity and enzyme activity.

It is possible that the antioxidant properties of melatonin, its metabolites and by-products could be responsible for the antioxidant and cardioprotective effects seen in our study. Other antioxidant effects could also be mediated via activation of the melatonin receptors 1, 2 and 3 [282]. With these receptor mediated effects, melatonin can bind to its membrane bound, G-protein coupled receptors, activate cytoplasmic molecules, which translocate to the nucleus and increase the expression of antioxidant enzymes [288]. However, we did not assess antioxidant enzyme expression and furthermore, the increase in enzyme expression does not necessarily mean increased activity. Nevertheless, the receptor mediated effects of melatonin could also be involved in the cardioprotective effects in our study.

Cardiac interstitial fibrosis

The involvement of melatonin in the process of cardiac remodelling, is supported by findings of Simko et al, who showed that pinealectomized rats and constant exposure to light reduced circulating melatonin levels and led to the development of LV interstitial fibrosis [349]. It is

suggested that the anti-fibrotic effect of melatonin is mediated via its antioxidant effects and modulation of the sympathetic nervous and renin-angiotensin systems [349]. However, in our study, none of the three doses of melatonin affected cardiac interstitial fibrosis. One would assume that improved cardiac function after melatonin treatment would also be due to improved interstitial fibrosis, but the fact the melatonin treatment did not affect fibrosis could mean otherwise. It is possible that cardiac dysfunction in the MCT model of PH is mainly due to the pressure overload, RV hypertrophy, oxidative stress and the adverse consequences of all these parameters.

The fact that these three doses of melatonin did not modulate interstitial fibrosis may mean that starting melatonin treatment on the same day as the induction of PH, does either not have an effect on fibrosis or the effects is not seen due to a small sample size. The latter argument is very likely as all three doses showed a trend towards a decrease. The fact that we only measured fibrosis in only 5 fields of each heart section could also be a reason we did not see statistically significant differences in the effect of melatonin on fibrosis. More fields could have yielded more significant results. Furthermore, Simko et al, demonstrated the anti-fibrotic effect of melatonin at a dose of 10mg/kg per day for 6 weeks, while we used much lower doses for shorter time periods [349].

8. Conclusions

In this section, our data suggest that melatonin given at a concentration similar to nutritional values (75ng/L) or at doses similar to the dose received with pills (4mg/kg or 6mg/kg) confers cardioprotection. This cardioprotective effect was validated by reduction of RV hypertrophy, improved cardiac function, antioxidant capacity and antioxidant enzyme activity. Our findings are important as even the low concentration of melatonin was found to cause substantial improvement of cardiac parameters. The data suggest that cardioprotection could be offered via a diet consisting of food enriched with melatonin. Our data also demonstrate that the higher doses of melatonin, conferred greater cardioprotective benefit as seen by their greater effect on cardiac parameters and plasma antioxidant parameters. Our data therefore suggest that melatonin could be used as a simple therapy for cardioprotection in PH.

SECTION G

MELATONIN AS A THERAPEUTIC OF PREVENTIVE TREATMENT IN PH

1. Introduction

In section F, we were able to demonstrate that all three doses of melatonin conferred cardioprotection against PH, as seen with reduced RV hypertrophy, improved cardiac function, decreased oxidative stress, normalised antioxidant capacity and antioxidant enzyme activity. Melatonin treatment was started on the same day as the induction of experimental PH, which poorly mimics the clinical situation. Furthermore, one could argue that administration of melatonin on the same day as the induction of experimental PH may interfere with the disease development and this may display the true efficacy of melatonin in PH. Therefore, in order to truly test the efficacy of melatonin as a cardioprotective agent in PH, we proposed to take two different approaches including therapeutic (melatonin treatment started fourteen days after the induction of experimental PH, when the disease already manifests) and preventive (melatonin treatment started five days before the induction of experimental PH). For these protocols, we used 6mg/kg of melatonin as our previous data suggested that this dose conferred the greatest degree of cardioprotection

2. Materials and methods

In order to assess the efficacy of melatonin treatment in a protocol that better mimics the clinical setting, we started melatonin treatment fourteen days after the induction of PH or five days before. For these experiments, we used 6mg/kg of melatonin which corresponds to the range of melatonin absorbed via pills. Echocardiography was done on the first day of experiments, day 14 and day 28.

After the melatonin treatment protocols, rats were sacrificed on day 14 and 28 for the therapeutic approach or day 28 for the preventive approach. Hereafter, we assessed cardiac function with echocardiography, isolated heart perfusions, we collected blood samples, performed biometric measurements and assessed oxidative/antioxidant status with blood assays for lipid peroxidation (TBARS), antioxidant capacity (ORAC), and catalase and superoxide dismutase (SOD) activity. Lastly, hearts were collected for histology (Figure 91).

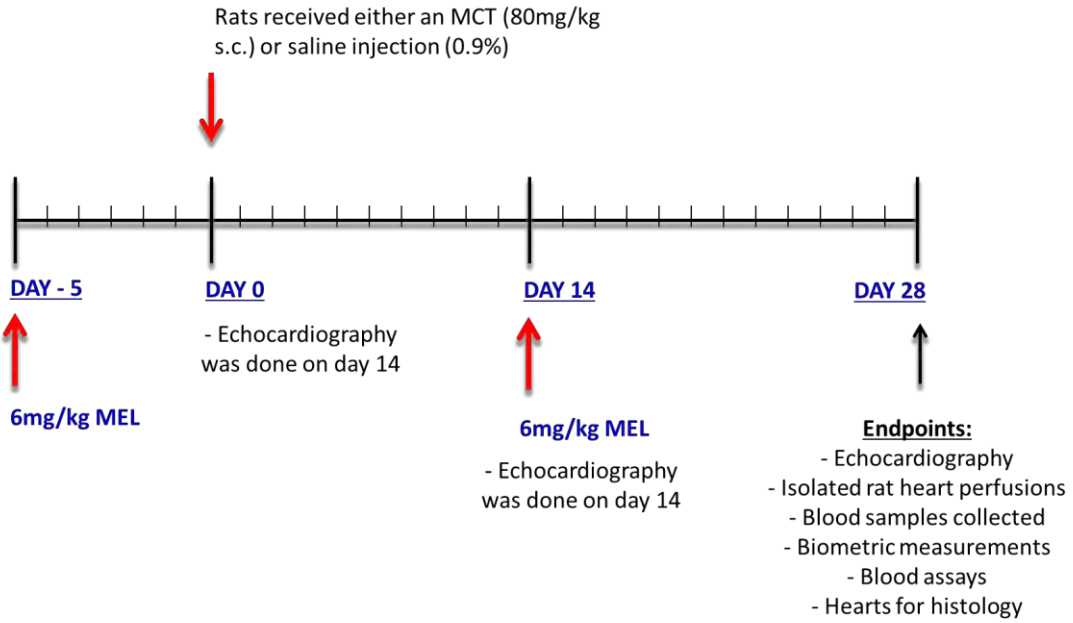


Figure 91. Melatonin as therapeutic or preventative treatment in PH: Experimental design. Male Long Evans rats were randomly divided into three groups: C, MCT and monocrotaline plus MEL. Two melatonin treatment protocols were followed: **(A)** Melatonin (6mg/kg) treatment started on DAY 14 (14 days after the MCT injection) until DAY 28. **(B)** Melatonin (6mg/kg) pretreatment started five days before MCT injection until Day 28. Sample size (n) was six rats per group. **C**: control, **MCT**: monocrotaline, **MEL**: melatonin, **PH:** pulmonary hypertension.

3. Data analyses: Statistical tests

Unless stated otherwise, all data were expressed as mean ± standard error of the mean (SEM). For comparative studies two-way ANOVA analyses (with Bonferroni post-test, if $p<0.05$) were used respectively. All statistical tests were performed using Graph Pad Prism-5. The experimental groups used for the fibrosis determination were statistically analysed by one-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. A $p<0.05$ was considered as significant.

4. Results: Effects of therapeutic treatment with 6mg/kg melatonin

To test the cardioprotective effect of melatonin as therapeutic treatment, daily oral treatment of melatonin (6mg/kg) was given fourteen days after the induction of experimental PH and treatment continued until the end of the 28 days protocol.

4.1. Biometric measurements

Table 9 summarizes the biometric measurements including body weights, weights of organs and weight of these organs expressed as a ratio of body weight or tibial length. MCT rats at day 14 had lower body weights and LV+septal weight over heart weight ratios compared to the control rats at day 14. MCT rats at day 14 also had higher heart weight over body weight, RV weight over LV+septal weight over heart weight, heart weight over tibial length ratios. Furthermore, MCT rats at day 14 had higher lung weights, liver weight over body weights and live weights over body weight ratios. Melatonin treated MCT rats had significantly higher body weights (297.30 ± 11.31 vs. 218.80 ± 18.41 g, $p<0.0001$) while the heart weights were similar (1.64 ± 0.11 vs. 1.62 ± 0.07 g, $p>0.05$). Melatonin treated MCT rats had similar heart weights over body weights (0.58 ± 0.03 vs. 0.57 ± 0.02 g/g, $p>0.05$) and higher heart weight over tibial length ratios (0.47 ± 0.03 vs. 0.37 ± 0.02 g/cm, $p<0.09$). Melatonin treated MCT rats had significantly lower RV weights (0.33 ± 0.03 vs. 0.55 ± 0.02 g/g, $p<0.0001$) and higher LV weights compared to MCT rats (0.75 ± 0.07 vs. 0.65 ± 0.01 g/g, $p<0.09$). Melatonin treated MCT rats had lower lung weights compared to untreated MCT rats (2.87 ± 0.17 vs. 3.59 ± 0.13 g, $p<0.002$) and similar lung weight over body weight ratios (1.06 ± 0.08 vs. 1.12 ± 0.04 g/g, $p>0.05$). Melatonin treated MCT had also lower liver weights (11.30 ± 0.71 vs. 19.60 ± 1.22 g, $p<0.004$) and lower liver weight over body weight ratios (0.41 ± 0.02 vs. 0.56 ± 0.03 g/g, $p<0.05$).

Table 9. Biometric measurements

					Melatonin treatment (6mg/kg MEL)		
	C (at day 14)	**MCT (at day 14)**	**C (at day 28)**	**MCT (at day 28)**	**C+MEL**	**MCT+MEL**	***p*-value**
Biometric measurements:							
Body weight (g)	290.40 ± 2.23	284.20 ± 14.59*	323.0 ± 5.46	230.80 ± 10.11	276.20 ± 25.02	304.00 ± 21.02*	*<0.0001
Heart weight (g)	1.21 ± 0.08	1.42 ± 0.04*	1.36 ± 0.07	1.63 ± 0.07	1.32 ± 0.10	1.28 ± 0.11*	*<0.01
Heart weight/body weight (g/g)	0.47 ± 0.04	0.50 ± 0.01	0.36 ± 0.02	0.57 ± 0.02	0.42± 0.05	0.62±0.08	*<0.0001
RV weight/LV+septal weight (g/g)	0.19 ± 0.01	0.35 ± 0.06*	0.20 ± 0.008	0.55 ± 0.02	0.21 ± 0.008	0.34 ± 0.03*	*<0.004
LV+septal weight/heart weight (g/g)	0.83 ± 0.008	0.75 ± 0.03*	0.84 ± 0.006	0.65 ± 0.04	0.83 ± 0.01	0.75 ± 0.02	*<0.03
Heart weight/tibial length (g/cm)	0.33 ± 0.02	0.46 ± 0.02*	0.32 ± 0.02	0.37 ± 0.02	0.28 ± 0.04	0.32 ± 0.03	*0.03
Lung weight (g)	1.28 ± 0.07	1.59 ± 0.01*	2.21 ± 0.03	4.13 ± 0.21	1.33 ± 0.11	2.39 ± 0.22*	*<0.002
Lung weight/body weight (g/g)	0.54 ± 0.06	0.53 ± 0.01	0.49±0.03	1.12±0.04*	0.43 ± 0.09	0.98 ± 0.14	*<0.0001
Liver weight (g)	11.77 ± 0.39	15.08 ± 0.41*	11.67 ± 0.26	16.81 ± 0.54*	12.36 ± 1.39	12.28 ± 0.84*	*<0.0002
Liver weight/body weight (g/g)	0.49 ± 0.03	0.51 ± 0.02*	0.42 ± 0.03	0.56 ± 0.03*	0.41 ± 0.11	0.49 ± 0.04	*<0.05

C: Control, **MCT:** Monocrotaline, **MEL:** Melatonin, n≥ 6 per group. Values are mean ± standard error of the mean.

4.2. Echocardiography: LV functional parameters

Echocardiography measurements showed that LV fractional shortening was higher in the MCT rats at day 14 compared to the controls rats at day 14 (60.29 ± 5.41 vs. 48.02 ± 1.28 %, p<0.001) (Figure 92 A). LV fractional shortening was also higher in the MCT+MEL group (who was designated to receive melatonin treatment on day 14) compared to the controls on day 14 (62.65 ± 5.10 vs. 48.02 ± 1.28, p<0.001). The LV fractional shortening was similar between melatonin treated control rats (50.98 ± 3.40 %) and the untreated controls (48.14 ± 1.28 %) (Figure 92 A).

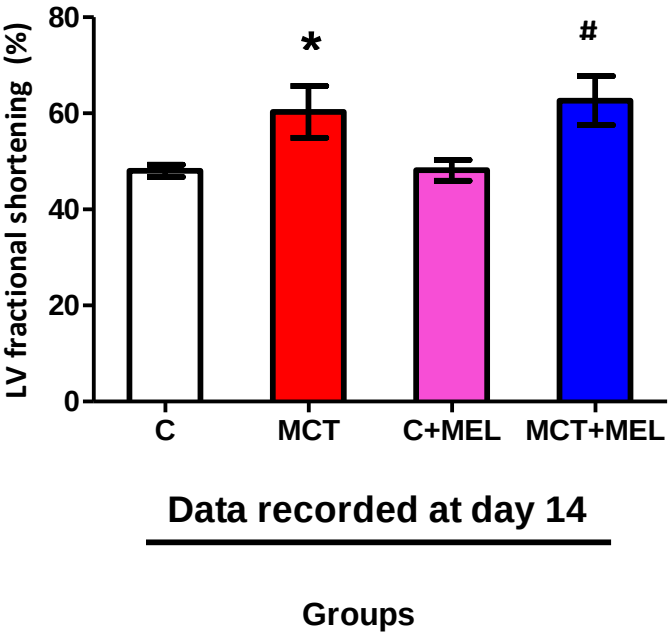


Figure 92 (A). Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular systolic fractional shortening measured at day 14 in a rat model of MCT-induced PH, *p<0.001 (MCT at day 14 vs. C at day 14), #p<0.001 (MCT+MEL vs. MCT), n≥6 per group.

LV fractional shortening was significantly higher in the melatonin treated MCT rats compared to MCT rats at day 28 (36.61 ± 1.31 vs. 69.37 ± 2.17 %, p<0.0001) (Figure 92 B). Control rats treated with melatonin had similar LV fractional shortening compared to normal controls at day 28 (39.78 ± 1.14 ± 0.98 vs. 32.95 ± 1.08 %) (Figures 92 B).

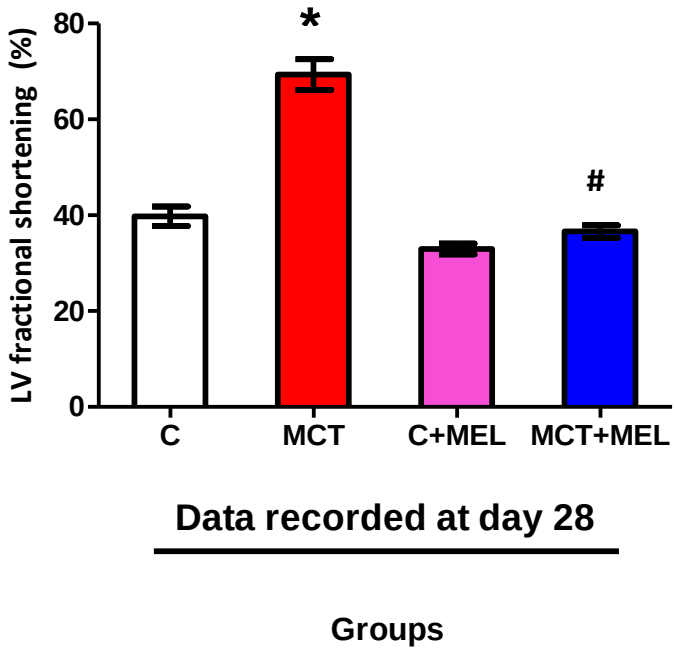


Figure 92 (B). Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular systolic fractional shortening measured at day 28 in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C at day 28), #*p*<0.0001 (MCT+MEL vs. MCT at day 28), n≥6 per group.

LV end diastolic volumes were similar in the MCT rats at day 14 compared to the controls rats at day 14 (0.57 ± 0.03 vs. 0.64 ± 0.07 mL) (Figure 92 C). The melatonin treated MCT rats had similar LV end diastolic volumes at day 14 (0.55 ± 0.04 vs. 0.57 ± 0.03 mL). (Figure 92 C). Furthermore, the melatonin treated controls had similar LV end diastolic volumes compared to the untreated controls at day 14 (0.64 ± 0.04 vs. 0.64 ± 0.07 mL) (Figure 92 C).

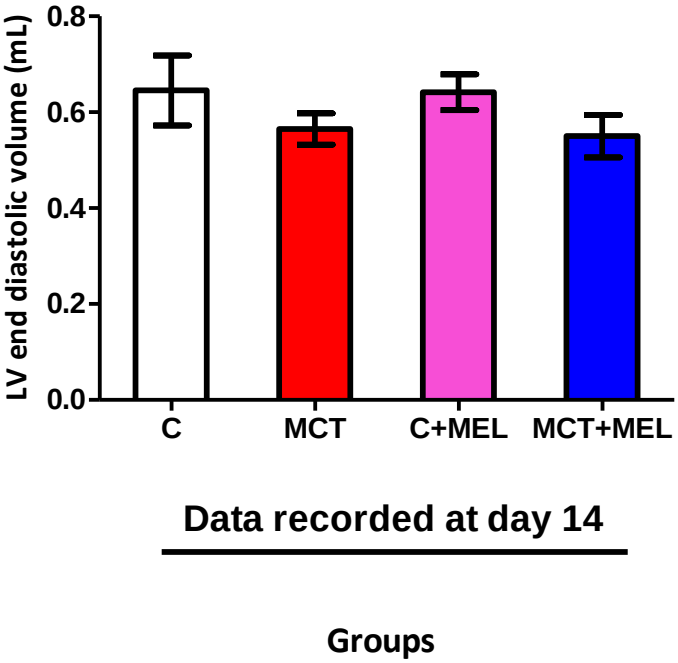


Figure 92 (C). Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular end diastolic volume measured at day 14 in a rat model of MCT-induced, (MCT at day 14 vs. C at day 14), (MCT+MEL vs. MCT at day 14), n≥6 per group.

Melatonin treated MCT rats had significantly higher LV end diastolic volumes compared to MCT rats at day 28 (0.65 ± 0.08 vs. 0.193 ± 0.01 mL, p<0.0001) (Figure 92 D). Control rats treated with melatonin had higher LV end diastolic volumes (0.73 ± 0.05 vs. 0.68 ± 0.05 mL, p<0.04) compared to controls at day 28 (Figure 92 D).

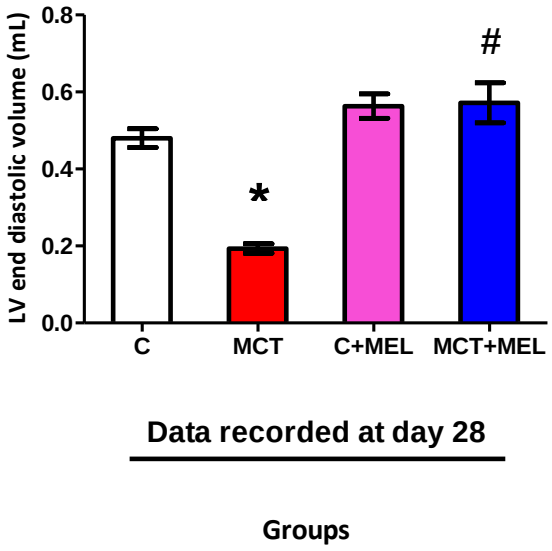


Figure 92 (D). Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular end diastolic volume measured at day 28 in a rat model of MCT-induced PH, *p>0.0001 (MCT at day 28 vs. C at day 28), *p>0.0001 (MCT+MEL vs. MCT at day 28), n≥6 per group.

LV ejection fractions were similar in the MCT rats at day 14 compared to the controls rats at day 14 (91.01 ± 2.73 vs. 84.24 ± 1.15 %) (Figure 93 A). LV ejection fractions were similar between the melatonin treated MCT rats and controls at day 14 (86.00 ± 2.61 vs. 91.01 ± 2.731 %) (Figure 93 A).

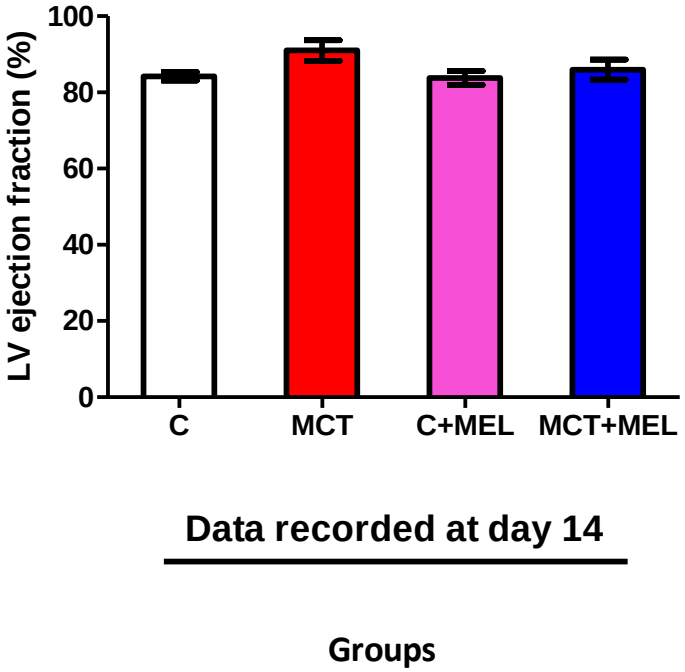


Figure 93 (A). Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular ejection fraction measured at day 14 in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14), (MCT+MEL vs. MCT at day 14), n≥6 per group.

Melatonin treated MCT rats had significantly lower LV ejection fractions compared to MCT rats at day 28 (72.14 ± 1.68 vs. 96.05 ± 0.63%, p<0.002) (Figure 93 B). Control rats treated with melatonin had similar LV ejection fractions compared to controls at day 28 (67.25 ± 1.67 vs. 69.09 ± 1.57 %) (Figure 93 B).

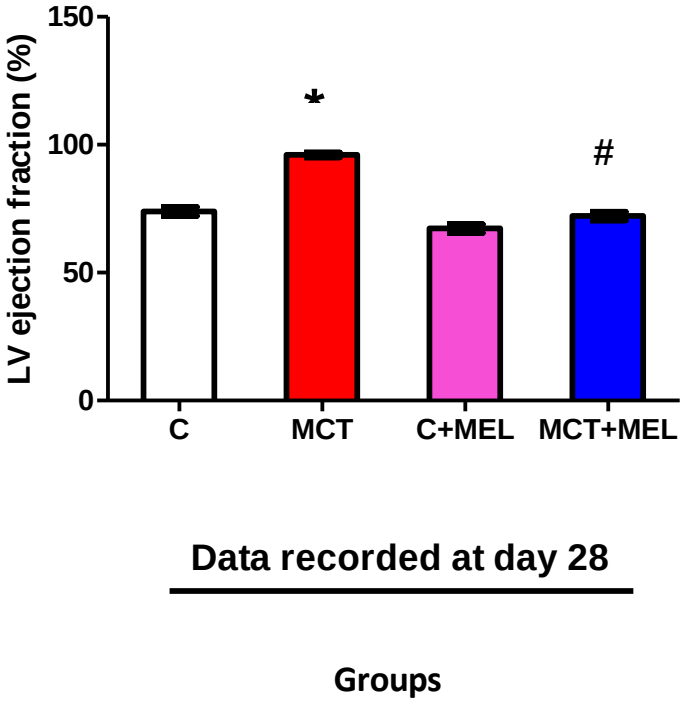


Figure 93 (B). Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular ejection fraction measured at day 28 in a rat model of MCT-induced PH, *p<0.0001 (MCT at day 28 vs. C at day 28), **#**p<0.002 (MCT+MEL vs. MCT at day 28), n≥6 per group.

4.3. Isolated heart perfusions: Left ventricular function

LV developed pressure was similar in the MCT rats at day 14 compared to control rats at day 14 (92.62 ± 2.33 vs. 87.39 ± 1.67 mmHg) (Figure 94). Isolated heart perfusions showed that melatonin treated MCT rats had similar LV developed pressures compared to MCT rats at day 28 (92.62 ± 5.04 vs. 85.93 ± 0.99 mmHg) (Figure 94). Melatonin treated control rats had similar LV developed pressures compared to controls at day 28 (94.70 ± 3.78 ± 2.42 vs. 92.73 ± 3.25 mmHg) (Figure 94).

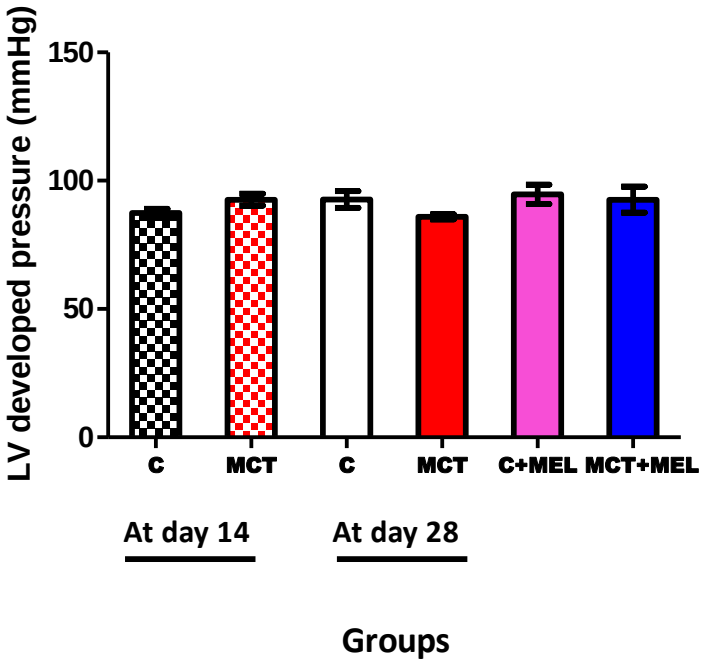


Figure 94. Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular developed pressure in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14), (MCT+MEL vs. MCT), n≥6 per group.

LV systolic pressure was similar in the MCT rats at day 14 compared to control rats at day 14 (99.15 ± 3.51 vs. 93.15 ± 2.61 mmHg) (Figure 95). The LV systolic pressure was similar for melatonin treated MCT rats compared to MCT rats at day 28 (107.80 ± 2.39 vs. 98.24 ± 6.75 mmHg) (Figure 95). Melatonin treated control rats had similar LV systolic pressures compared to controls at day 28 (109.20 ± 1.97 vs. 103.60 ± 4.37 mmHg) (Figure 95).

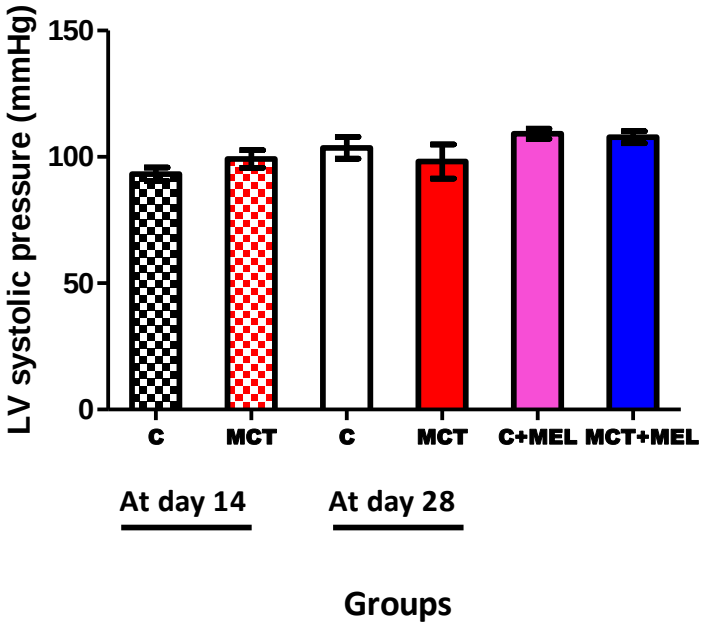


Figure 95. Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular systolic pressure in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14), (MCT+MEL vs. MCT), n≥6 per group.

LV diastolic pressure was similar in the MCT rats at day 14 compared to control rats at day 14 (8.53 ± 0.17 vs. 8.713 ± 0.04 mmHg) (Figure 96). The LV diastolic pressure was similar for melatonin treated MCT rats compared to MCT rats at day 28 (8.48 ± 0.12 vs. 8.62 ± 0.13 mmHg) (Figure 96). Melatonin treated control rats had similar LV systolic pressures compared to controls at day 28 (8.30 ± 0.09 vs. 8.38 ± 0.08 mmHg) (Figure 96).

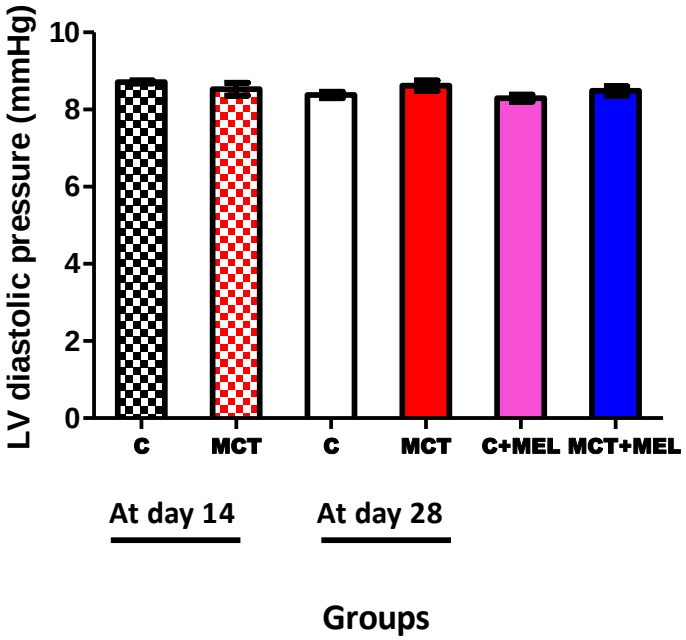


Figure 96. Effect of a therapeutic treatment with melatonin (6mg/kg) on left ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14), (MCT+MEL vs. MCT), n≥6 per group.

Heart rate was similar in the MCT rats at day 14 compared to control rats at day 14 (279.20 ± 18.24 vs. 337.90 ± 19.80 bpm) (Figure 97). The heart rate was similar for melatonin treated MCT rats compared to MCT rats at day 28 (293.60 ± 5.80 vs. 296.30 ± 14.25 bpm) (Figure 97). Melatonin treated control rats had similar heart rates compared to controls at day 28 (284.80 ± 11.41 vs. 271.20 ± 8.79 bpm) (Figure 97).

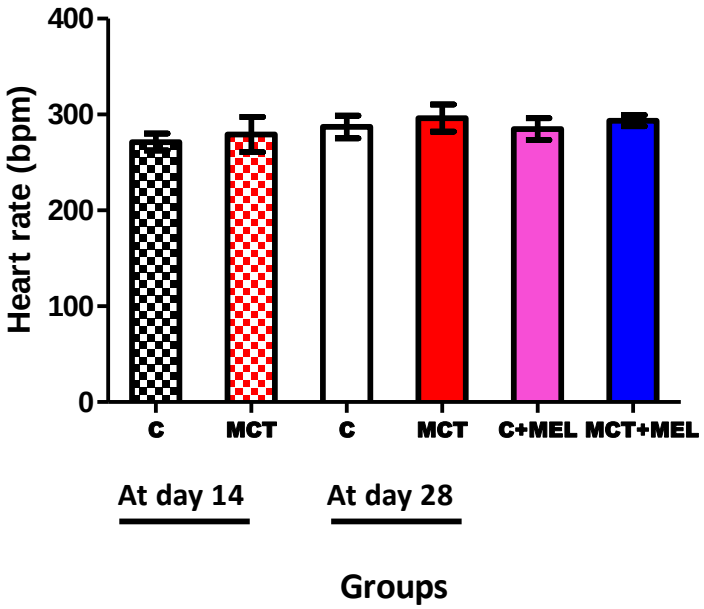


Figure 97. Effect of a therapeutic treatment with melatonin (6mg/kg) on heart rate in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14), (MCT+MEL vs. MCT), n≥6 per group.

4.4. Isolated heart perfusions: Right ventricular function

RV developed pressure was significantly higher in the MCT rats at day 14 compared to control rats at day 14 (56.57 ± 2.47 vs. 37.95 ± 2.34 mmHg, $p<0.0009$) (Figure 98). The RV developed pressure was lower in the melatonin treated MCT rats compared to MCT rats at day 28 (71.39 ± 0.57 vs. 86.28 ± 2.61 mmHg, $p<0.0002$) (Figure 98) Control rats treated with melatonin had similar RV developed pressures compared to normal controls at day 28 (42.62 ± 4.13 vs. 45.87 ± 2.11 mmHg) (Figures 98).

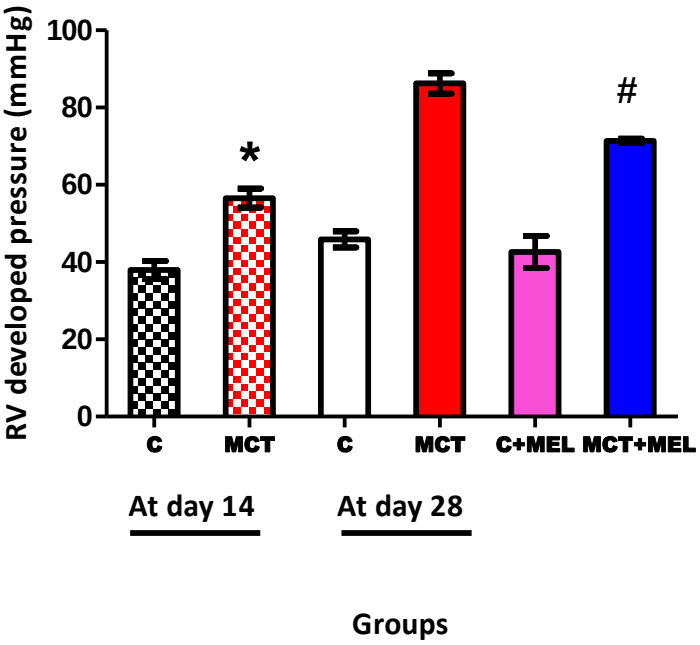


Figure 98. Effect of a therapeutic treatment with melatonin (6mg/kg) on right ventricular developed pressure in a rat model of MCT-induced PH, *$p<0.0009$ (MCT at day 14 vs. C at day 14), #$p<0.0002$ (MCT+MEL vs. MCT), n≥6 per group.

RV systolic pressure was significantly higher in the MCT rats at day 14 compared to control rats at day 14 (96.18 ± 5.24 vs. 96.08 ± 6.66 mmHg, p<0.005) (Figure 99). Melatonin treated MCT rats had lower RV systolic pressures compared to the MCT rats at day 28 (79.82 ± 0.51 vs. 95.00 ± 7.07 mmHg, p<0.005) (Figure 99). Control rats treated with melatonin had similar RV developed pressures compared to controls at day 28 (50.95 ± 4.16 vs. 54.35 ± 2.28 mmHg) (Figure 99).

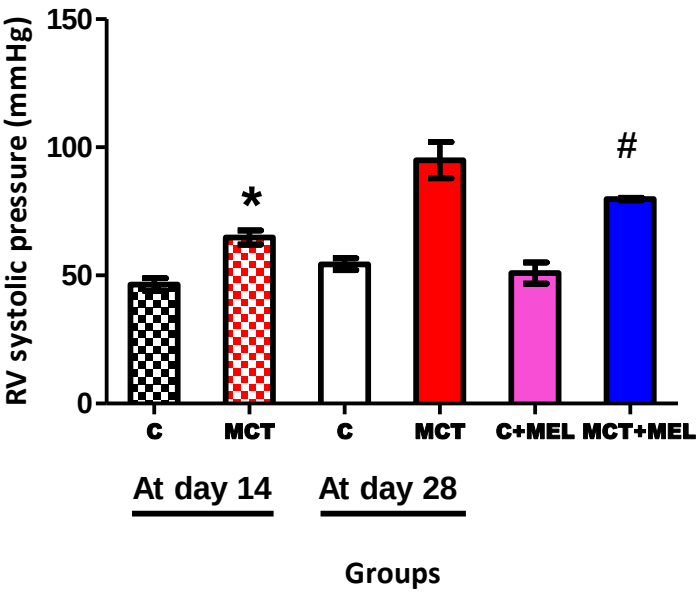


Figure 99. Effect of a therapeutic treatment with melatonin (6mg/kg) on right ventricular systolic pressure in a rat model of MCT-induced PH, *p<0.005 (MCT at day 14 vs. C at day 14), #p<0.005 (MCT+MEL vs. MCT), n≥6 per group.

RV diastolic pressure was similar in the MCT rats at day 14 compared to control rats at day 14 (8.53 ± 0.19 vs. 8.59 ± 0.30 mmHg) (Figure 100).Melatonin treated MCT rats had similar RV diastolic pressures compared to the MCT rats at day 28 (8.48 ± 0.10 vs. 8.47 ± 0.15 mmHg) (Figure 100). Control rats treated with melatonin had similar RV diastolic pressures compared to normal controls at day 28 (8.33 ± 0.07 vs. 8.58 ± 0.11 mmHg) (Figure 100).

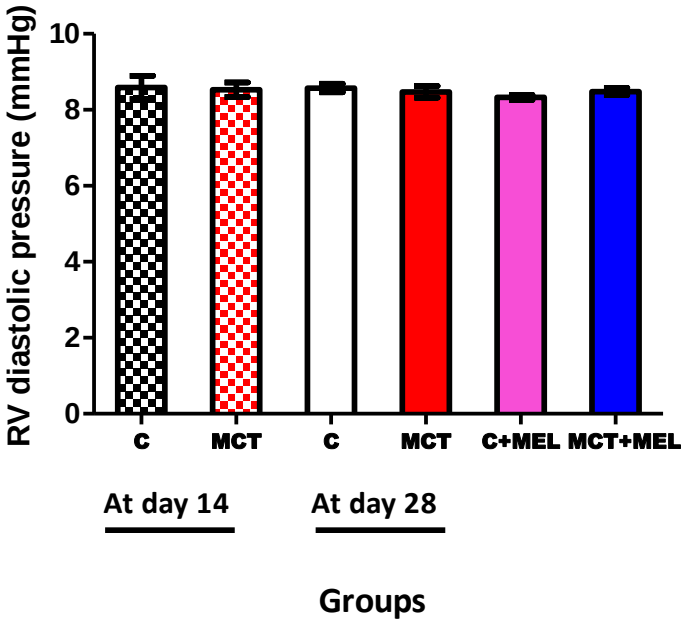


Figure 100. Effect of a therapeutic treatment with melatonin (6mg/kg) on right ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14 and MCT+MEL vs. MCT), n≥6 per group.

4.5. Plasma oxidative stress and antioxidant capacity

Levels of blood plasma were measured by assessing the thiobarbituric acid reactive substances (TBARS) in all treated groups. Plasma lipid peroxidation was significantly higher in the MCT rats at day 14 compared to control rats at day 14 (1.60 ± 0.15 vs. 0.74 ± 0.12 mmHg, $p<0.0009$) (Figure 101). Melatonin treated MCT rats had significantly lower Levels of plasma lipid peroxidation compared to MCT rats at day 28 (1.48 ± 0.07 vs. 2.19 ± 0.26 µmol/mL, $p<0.006$) (Figure 101). Melatonin treated controls had similar levels of plasma lipid peroxidation compared to normal controls at day 28 (0.97 ± 0.04 vs. 0.84 ± 0.05 µmol/mL) (Figure 101).

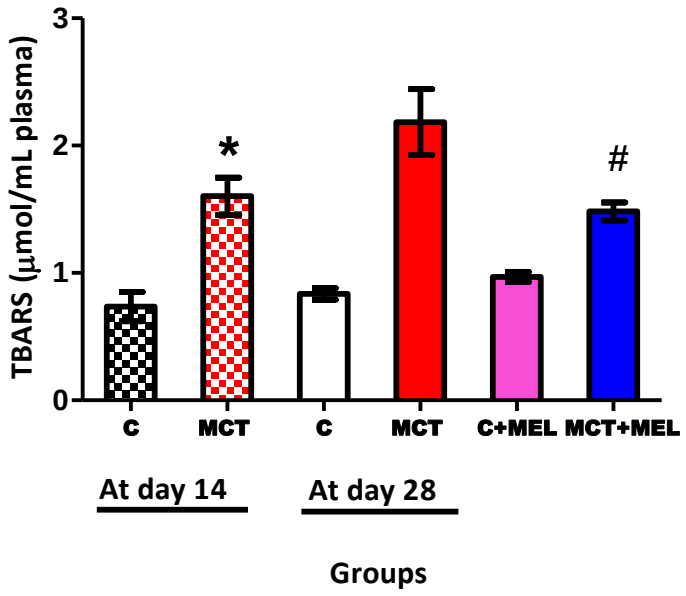


Figure 101. Effect of a therapeutic treatment with melatonin (6mg/kg) on plasma lipid peroxidation in a rat model of MCT-induced PH, *$p<0.0009$ (MCT at day 14 vs. C at day 14), #$p<0.006$ (MCT+MEL vs. MCT), n≥6 per group.

Plasma antioxidant capacity was significantly lower in the MCT rats at day 14 compared to control rats at day 14 (18.01 ± 2.64 vs. 25.77 ± 1.94 mmHg, p<0.04) (Figure 102). Melatonin treated MCT rats had a higher antioxidant capacity compared to MCT rats at day 28 (42.10 ± 1.28 vs. 18.40 ± 2.29 nmol/L trolox equivalents, p<0.0001) (Figure 102). Melatonin treated controls had a similar antioxidant capacity compared to normal controls at day 28 (25.77 ± 1.94 vs. 30.67 ± 3.88 nmol/L trolox equivalents) (Figure 102).

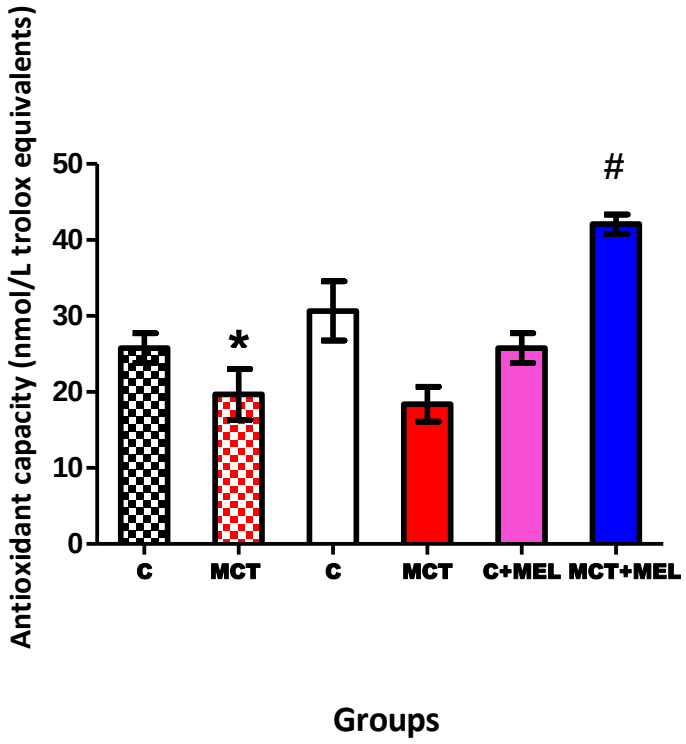


Figure 102. Effect of a therapeutic treatment with melatonin (6mg/kg) on plasma antioxidant capacity in a rat model of MCT-induced PH, *p<0.04 (MCT at day 14 vs. C at day 14), #p<0.0001 (MCT+MEL vs. MCT), n≥6 per group.

Plasma catalase activity was significantly higher in the MCT rats at day 14 compared to control rats at day 14 (8.19 ± 0.90 vs. 5.11 ± 0.61 mmHg, p<0.02) (Figure 103). Melatonin treated MCT rats had a significantly lower catalase activity compared to MCT at day 28 (6.81 ± 0.68 vs. 12.40 ± 1.80 IU/mg protein, p<0.004) (Figure 103). Melatonin treated controls had a similar catalase activity compared to controls at day 28 (2.62 ± 0.60 vs. 3.72 ± 0.40 IU/mg protein) (Figure 103).

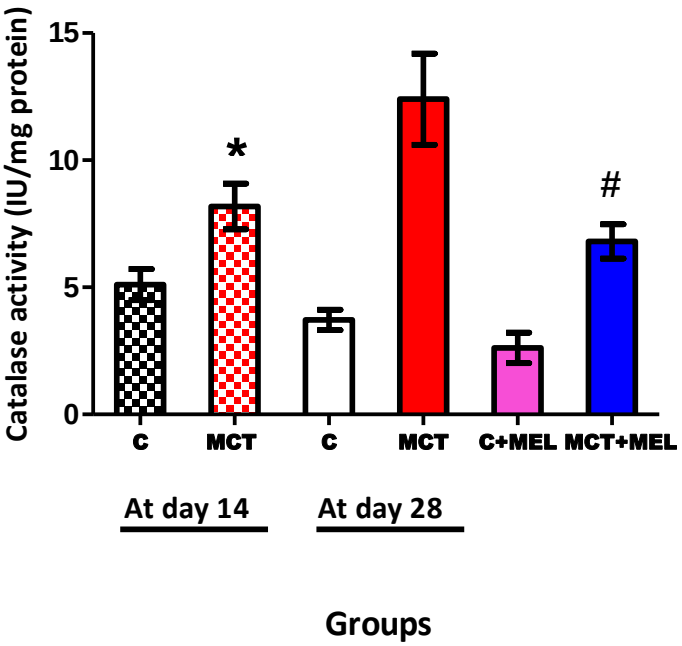


Figure 103. Effect of a therapeutic treatment with melatonin (6mg/kg) on plasma catalase activity in a rat model of MCT-induced PH, *p<0.02 (MCT at day 14 vs. C at day 14), #p<0.004 (MCT+MEL vs. MCT), n≥6 per group.

Plasma SOD activity was similar in the MCT rats at day 14 compared to control rats at day 14 (8.68 ± 0.31 vs. 9.20 ± 0.40 mmHg) (Figure 104). Melatonin treated MCT rats had a significantly lower SOD activity compared to MCT rats at day 28 (8.19 ± 0.52 vs. 9.97 ± 0.20 IU/mg protein, p<0.02) (Figure 104). Melatonin treated control rats had a similar SOD activity compared to controls at day 28 (6.94 ± 0.30 vs. 6.57 ± 0.83 IU/mg protein) (Figure 104).

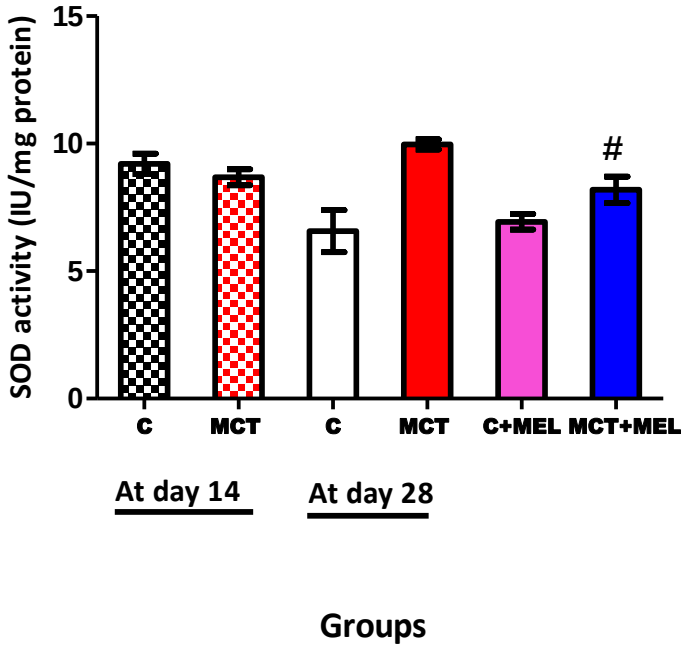


Figure 104. Effect of a therapeutic treatment with melatonin (6mg/kg) on plasma superoxide dismutase (SOD) activity in a rat model of MCT-induced PH, (MCT at day 14 vs. C at day 14), #p<0.02 (MCT+MEL vs. MCT), n≥6 per group.

4.6. Cardiac interstitial fibrosis: The effect of 6mg/kg therapeutic melatonin treatment

The percentage of collagen deposition in the RV sections of the MCT hearts were higher compared to the controls (4.68 ± 0.75 vs. 2.54 ± 0.36 % area, $p<0.04$) (Figure 105 A). The percentage of collagen deposition in the RV sections of the MCT+MEL hearts was significantly lower than in the MCT rats (2.23 ± 0.43vs. 4.68 ± 0.75 %, $p<0.03$) (Figure 105 A). Representative images of all groups are shown below (Figure 105 B).

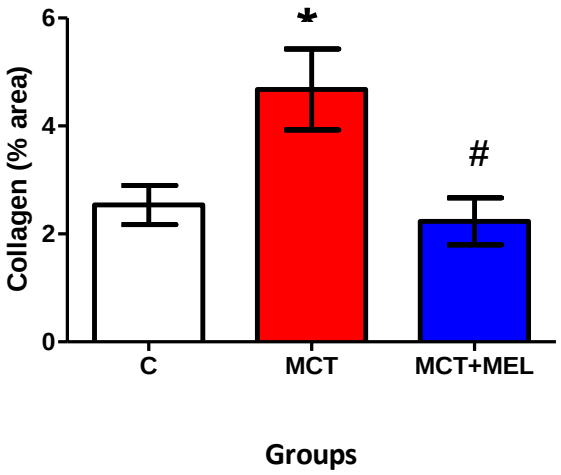


Figure 105 (A). Effect of a therapeutic treatment with melatonin (6mg/kg) on cardiac interstitial fibrosis in a rat model of MCT-induced PH, ***p**<0.04 (MCT vs C), #*p*<0.03 (MCT+MEL vs. MCT), n=3-4 per group.

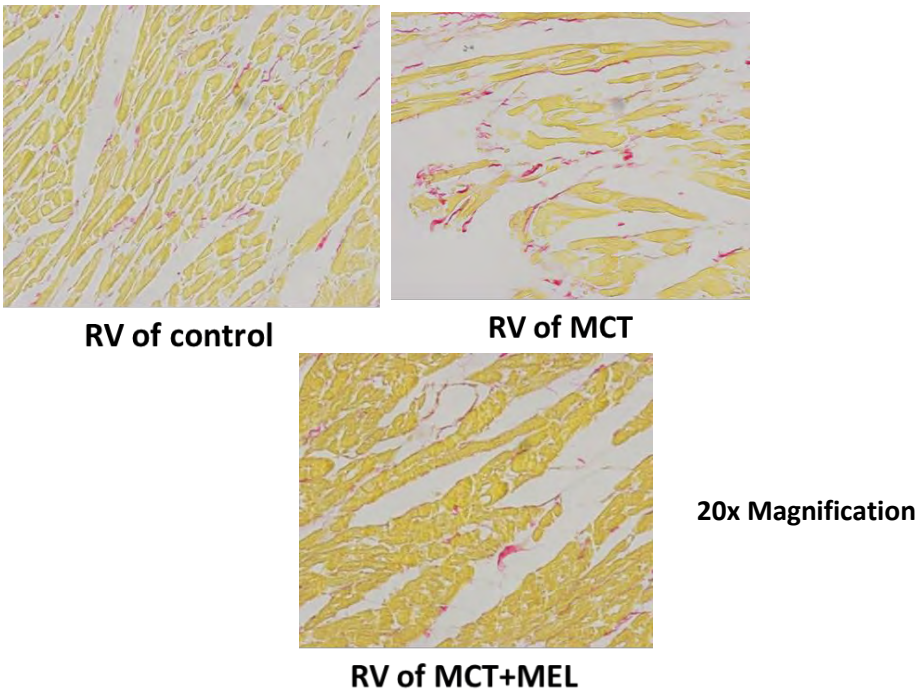


Figure 105 (B). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

5. Results: Effects of preventive treatment with 6mg/kg melatonin

In these experiments, the daily, oral melatonin treatment was started five days prior to the induction of experimental PH with a single subcutaneous injection of MCT and was continued until day 28.

5.1. Biometric measurements

Table 10 summarizes the biometric measurements including ratios of the heart weight over body weight, lung weight over body weight, liver weight over body weight and body weight respectively. Compared to the MCT group, preventive melatonin treatment (6mg/kg) did not affect the heart weight over body weight (0.52 ± 0.04 vs. 0.57 ± 0.02 g/g, $p<0.0001$) but decreased the lung weight over body weight (0.84 ± 0.06 vs. 1.12±0.04 g/g, $p<0.0001$). Preventive melatonin treatment decreased liver weight over body weight compared to the MCT group (0.47 ± 0.03 vs. 0.56 ± 0.03 g/g, $p<0.05$). Furthermore, preventive melatonin treatment increased the body weight compared to the MCT group (301.2 ± 28.24 vs. 218.8 ± 18.41 gram, $p<0.0006$).

Table 10. Biometric measurements

			Melatonin treatment (6mg/kg MEL)		
	C (at day 28)	**(MCT at day 28)**	**C+MEL**	**MCT+MEL**	***p*-value**
Biometric measurements:					
Body weight (g)	323.0 ± 5.46	218.80 ± 18.41	317.80 ± 16.76	317.80 ± 16.76*	*<0.003
Heart weight (g)	1.36 ± 0.07	1.62 ± 0.07	1.44 ± 0.08	1.52 ± 0.08	>0.05
Heart weight/body weight (g/g)	0.36 ± 0.02	0.57 ± 0.02	0.41± 0.01	0.49 ± 0.03*	*<0.04
RV weight/LV+septal weight (g/g)	0.20 ± 0.01	0.55 ± 0.02	0.18± 0.01	0.30 ± 0.03*	*<0.0001
LV+septal weight/heart weight (g/g)	0.80 ± 0.03	0.65 ± 0.01	0.85 ± 0.01	0.77 ± 0.02*	*<0.0001
Heart weight/tibial length (g/cm)	0.32 ± 0.02	0.37 ± 0.02	0.30 ± 0.05	0.28 ± 0.01	>0.05
Lung weight (g)	1.85 ± 0.17	3.59 ± 0.13	1.40 ± 0.07	2.44 ± 0.11*	*<0.0001
Lung weight/body weight (g/g)	0.49 ± 0.03	1.12 ± 0.04	0.39 ± 0.02	0.84 ± 0.10*	*<0.03
Liver weight (g)	15.81 ± 0.59	19.60 ± 1.22	16.56 ± 0.42	14.08 ± 1.30*	*<0.009
Liver weight/body weight (g/g)	0.42 ± 0.03	0.56 ± 0.03	0.45 ± 0.01	0.47 ± 0.03	>0.05

C: Control, **MCT:** Monocrotaline, **MEL:** Melatonin. *p<0.0001 (MCT vs C), *p<0.0001 (MCT+MEL vs. MCT), *p<0.05 (MCT vs C), *p<0.0006 (MCT vs C, MCT+MEL vs. MCT), n≥ 6 per group. Values are mean ± standard error of the mean.

5.2. Echocardiography: LV functional parameters

Echocardiography measurements showed that LV fractional shortening was significantly lower in the melatonin treated MCT rats compared to MCT rats (51.18 ± 2.34 vs. 69.37 ± 2.17 %, p<0.0001) (Figure 106). Control rats treated with melatonin had similar LV fractional shortening compared to normal controls (36.45 ± 1.74 vs. 36.13±1.08 %) (Figures 106).

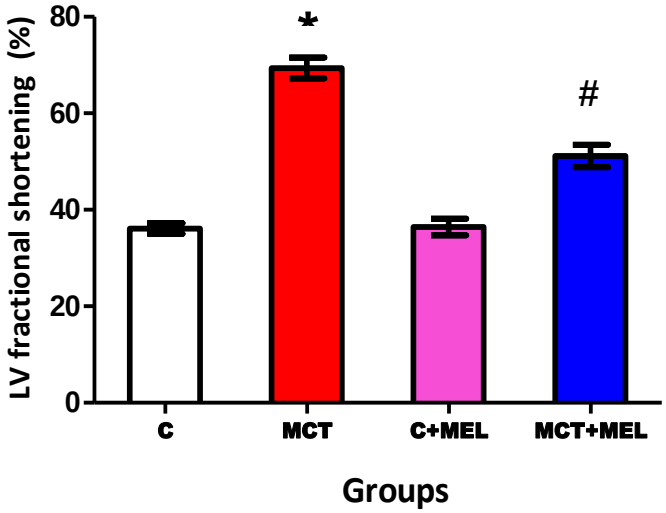


Figure 106. Effect of a preventive treatment with melatonin (6mg/kg) on left ventricular systolic fractional shortening in a rat model of MCT-induced PH, *p<0.0001 (MCT vs. C), #p<0.0001 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly higher LV end diastolic volumes compared to MCT rats (0.39 ± 0.03 ± 0.06 vs. 0.19 ± 0.01 mL, p<0.0001) (Figure 107). Control rats treated with melatonin had similar LV end diastolic volumes (0.55 ± 0.06 vs. 0.68± 0.05 mL, p<0.04) compared to controls (Figure 107).

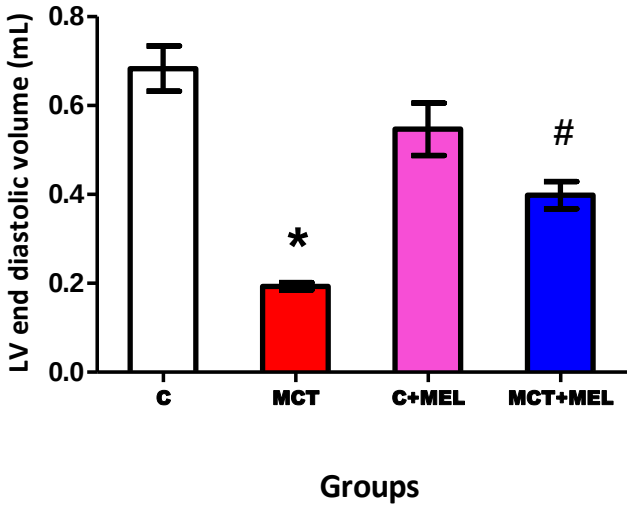


Figure 107. Effect of a preventive treatment with melatonin (6mg/kg) on left ventricular end diastolic volume in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), #p<0.004 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had significantly lower LV ejection fractions compared to MCT rats (86.50 ± 1.80 vs. 96.05± 0.63 %, p<0.003) (Figure 108). Control rats treated with melatonin had similar LV ejection fractions compared to controls (71.78 ± 2.14 vs. 69.09± 1.57 %) (Figure 108).

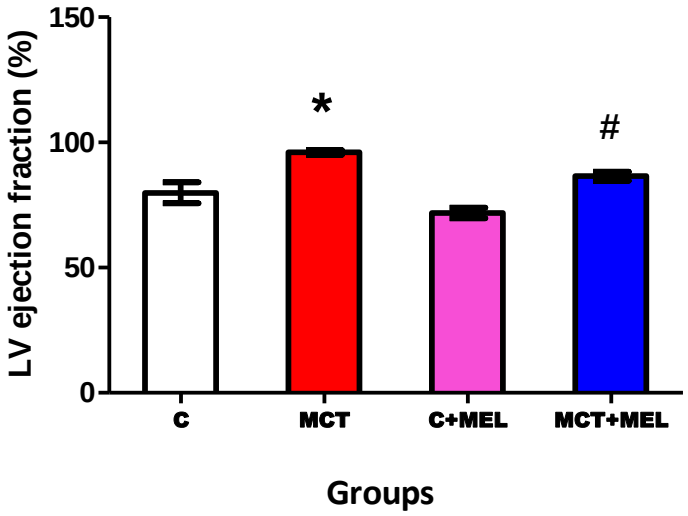


Figure 108. Effect of a preventive treatment with melatonin (6mg/kg) on left ventricular ejection fraction in a rat model of MCT-induced PH, ***p**<0.0001 (MCT vs. C), #p<0.003 (MCT+MEL vs. MCT), n≥6 per group.

5.3. Isolated heart perfusions: Left ventricular function

Isolated heart perfusions showed that melatonin treated MCT rats had similar LV developed pressures compared to MCT rats (108.80 ± 3.32 vs. 102.30 ± 7.86 mmHg) (Figure 109). Melatonin treated control rats had similar LV developed pressures compared to controls (115.60 ± 3.06 vs. 104.20 ± 5.08 mmHg) (Figure 109).

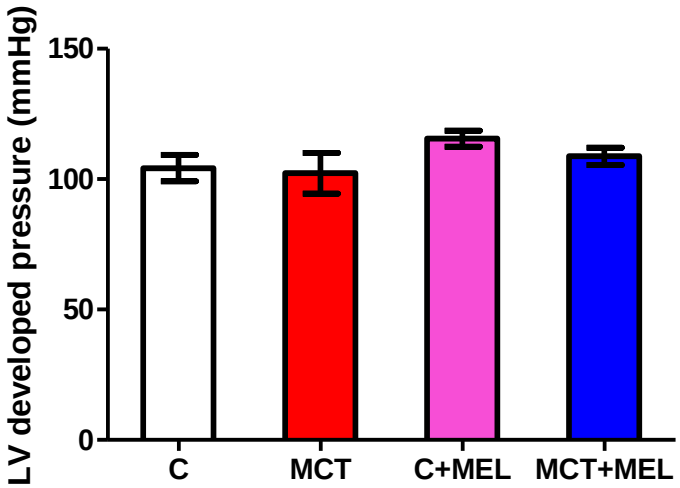


Figure 109. Effect of a preventive treatment with melatonin (6mg/kg) on left ventricular developed pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV systolic pressure was similar for melatonin treated MCT rats compared to controls (99.19 ± 6.63 vs. 103.80 ± 4.11 mmHg) (Figure 110). Melatonin treated control rats had similar LV systolic pressures compared to controls (106.20 ± 1.84 vs. 110.00 ± 3.51 mmHg) (Figure 110).

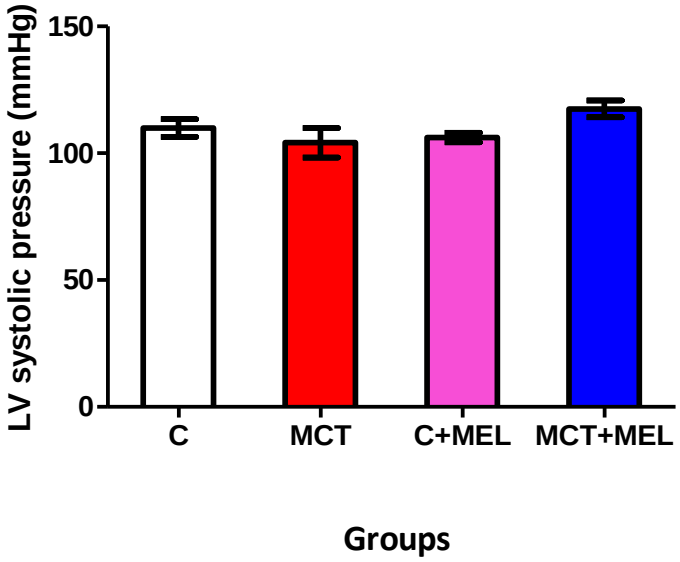


Figure 110. Effect of a preventive treatment with melatonin (6mg/kg) on left ventricular systolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The LV diastolic pressure was similar for melatonin treated MCT rats compared to controls (8.68 ± 0.10 vs. 8.42 ± 0.12 mmHg) (Figure 111). Melatonin treated control rats had similar LV diastolic pressures compared to controls (8.50 ± 0.09 vs. 8.42 ± 0.12 mmHg) (Figure 111).

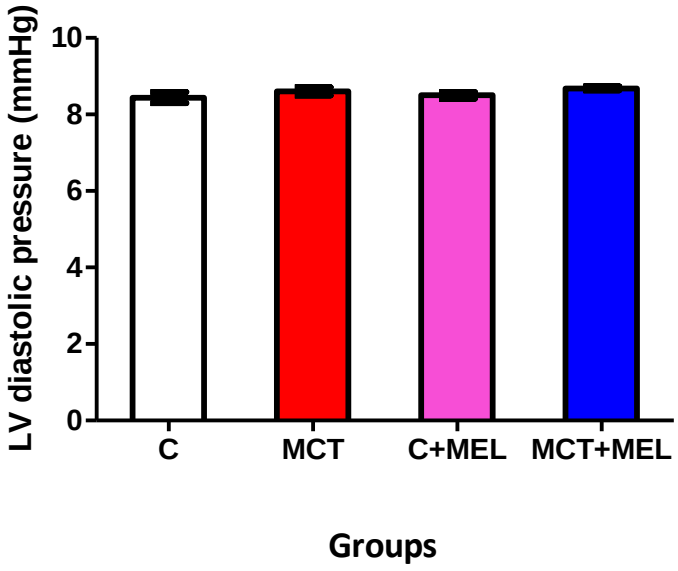


Figure 111. Effect of a preventive treatment with melatonin (6mg/kg) on left ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

The heart rate was similar for melatonin treated MCT rats compared to controls (318.70 ± 9.29 vs. 303.30 ± 18.16 bpm) (Figure 112). Melatonin treated control rats had similar heart rates compared to controls (302.60 ± 14.87 vs. 303.20 ± 14.77 bpm) (Figure 112).

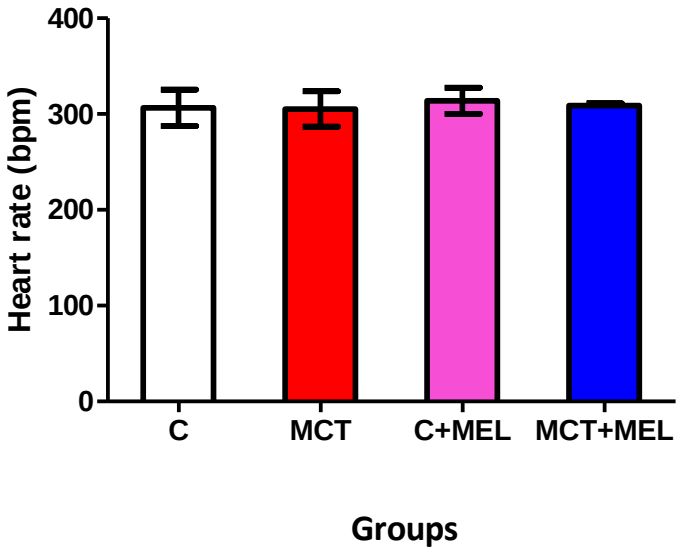


Figure 112. Effect of a preventive treatment with melatonin (6mg/kg) on heart rate in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

5.4. Isolated heart perfusions: Right ventricular function

The RV developed pressure was lower in the melatonin treated MCT rats compared to MCT rats (59.85 ± 3.77 vs. 81.22 ± 2.75 mmHg, $p<0.005$) (Figure 113) Control rats treated with melatonin had similar RV developed pressures compared to normal controls (49.04 ± 3.34 vs. 45.87 ± 2.11 mmHg) (Figures 113).

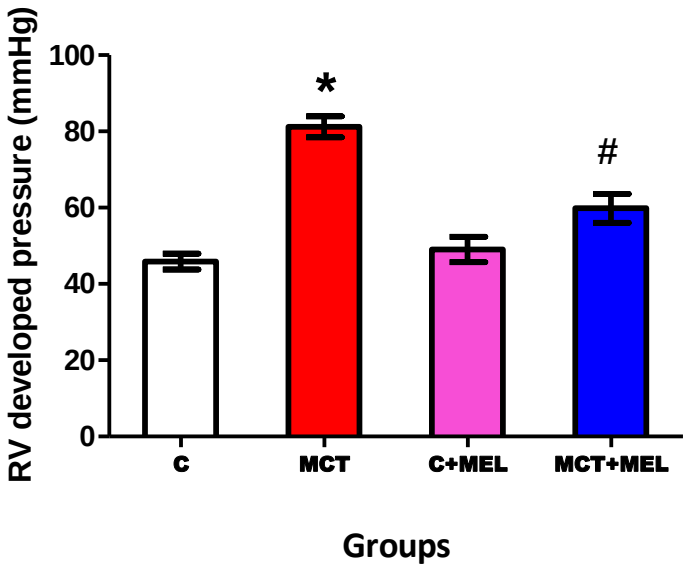


Figure 113. Effect of a preventive treatment with melatonin (6mg/kg) on right ventricular developed pressure in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.005 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had lower RV systolic pressures compared to the MCT rats (68.65 ± 3.82 vs. 87.91 ± 5.48 mmHg, $p<0.03$) (Figure 114). Control rats treated with melatonin had similar RV developed pressures compared to normal controls (57.80 ± 3.51 vs. 54.35 ± 2.28 mmHg) (Figure 114).

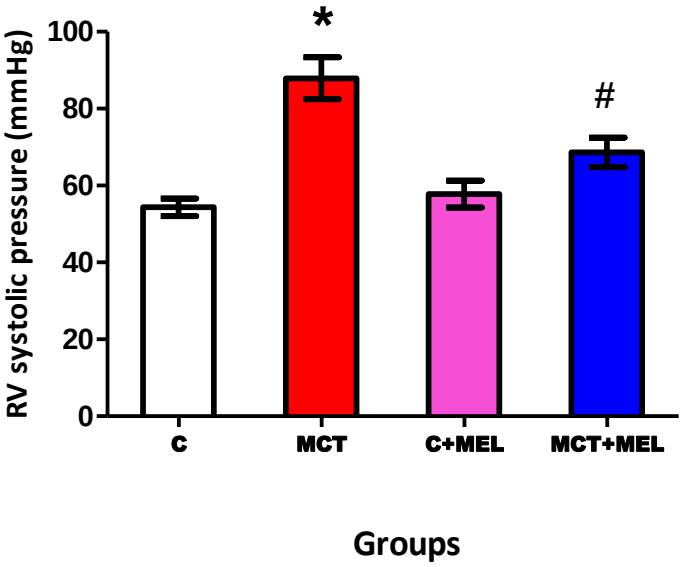


Figure 114. Effect of a preventive treatment with melatonin (6mg/kg) on right ventricular systolic pressure in a rat model of MCT-induced PH, ***p*<0.0001 (MCT vs. C), #*p*<0.03 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had similar RV diastolic pressures compared to the MCT rats (8.80 ± 0.08 vs. 8.73 ± 0.08 mmHg) (Figure 115). Control rats treated with melatonin had similar RV diastolic pressures compared to normal controls (8.60 ± 0.33 vs. 8.55 ± 0.10 mmHg) (Figure 115).

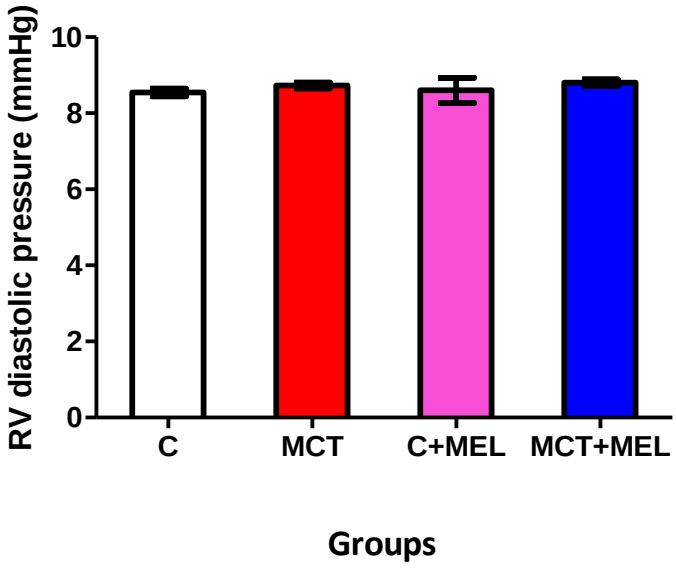


Figure 115. Effect of a preventive treatment with melatonin (6mg/kg) on right ventricular diastolic pressure in a rat model of MCT-induced PH, (MCT vs. C and MCT+MEL vs. MCT), n≥6 per group.

5.5. Plasma oxidative stress and antioxidant capacity

Levels of blood plasma were measured by assessing the thiobarbituric acid reactive substances (TBARS) all treated groups. Melatonin treated MCT rats had significantly lower levels of plasma lipid peroxidation compared to MCT rats (1.66 ± 0.09 vs. 2.27 ± 0.22 µmol/mL, p<0.002) (Figure 116). Melatonin treated controls had similar levels of plasma lipid peroxidation compared to normal controls (0.95 ± 0.06 vs. 0.83 ± 0.05 µmol/mL) (Figure 116).

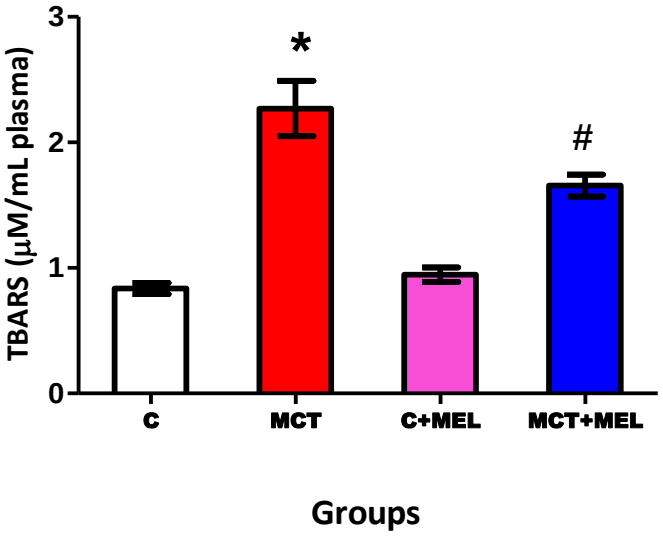


Figure 116. Effect of a preventive treatment with melatonin (6mg/kg) on plasma lipid peroxidation in a rat model of MCT-induced PH, *p<0.0001 (MCT vs. C), #p<0.002 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a higher antioxidant capacity compared to MCT rats (44.67 ± 2.33 vs. 18.40 ± 2.29 nmol/L trolox equivalents, $p<0.0001$) (Figure 117). Melatonin treated controls had a higher antioxidant capacity compared to normal controls (61.42 ± 2.63 vs. 30.67± 3.88 nmol/L trolox equivalents, $p<0.0001$) (Figure 117).

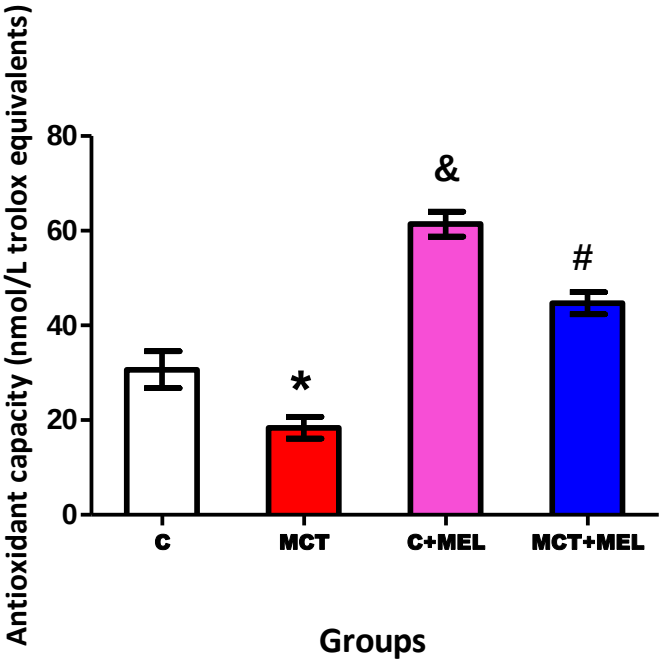


Figure 117. Effect of a preventive treatment with melatonin (6mg/kg) on plasma antioxidant capacity in a rat model of MCT-induced PH, ***p*<0.02 (MCT vs. C), #*p*<0.0001 (MCT+MEL vs. MCT), n≥6 per group, (CON+MEL vs. CON), n≥6 per group. **&***p*<0.0001

Melatonin treated MCT rats had a significantly lower catalase activity compared to MCT (3.19 ± 0.73 vs. 12.40 ± 1.79 IU/mg protein, $p<0.0008$) (Figure 118). Melatonin treated controls had a similar catalase activity compared to controls (4.75 ± 0.65 vs. 3.72 ± 0.40 IU/mg protein) (Figure 118).

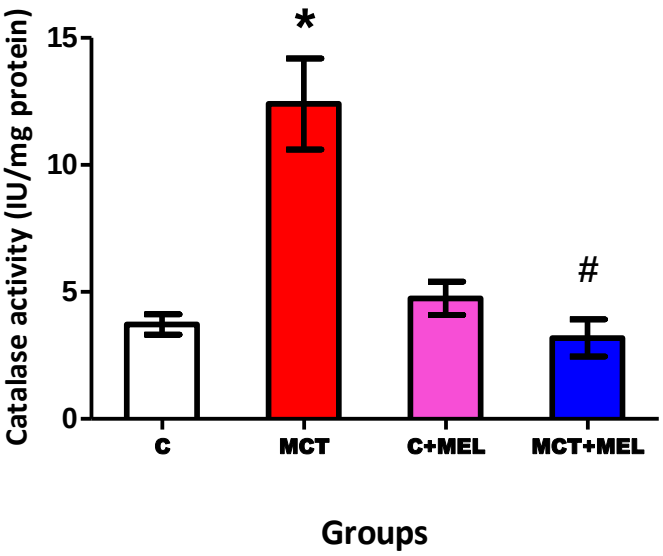


Figure 118. Effect of a preventive treatment with melatonin (6mg/kg) on plasma catalase activity in a rat model of MCT-induced PH, ***p*<0.0008 (MCT vs. C), #*p*<0.0008 (MCT+MEL vs. MCT), n≥6 per group.

Melatonin treated MCT rats had a significantly lower SOD activity compared to MCT rats (6.71 ± 0.48 vs. 9.97 ± 0.20 IU/mg protein, $p<0.001$) (Figure 119). Melatonin treated control rats had a similar SOD activity compared to controls (5.95 ± 0.34 vs. 6.57 ± 0.83 IU/mg protein) (Figure 119).

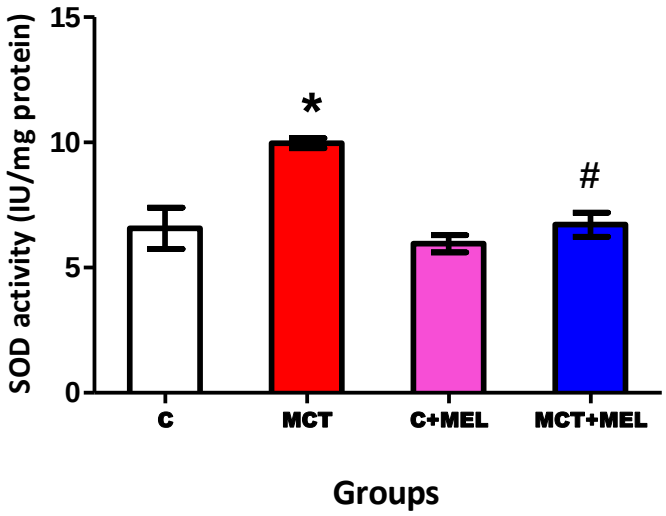


Figure 119. Effect of a preventive treatment with melatonin (6mg/kg) on plasma superoxide dismutase (SOD) activity in a rat model of MCT-induced PH, ***p*<0.003 (MCT vs. C), #*p*<0.001 (MCT+MEL vs. MCT), n≥6 per group.

5.6. Cardiac interstitial fibrosis: The effect of 6mg/kg preventive melatonin treatment

The percentage of collagen deposition in the RV sections of the MCT hearts were higher compared to the controls (4.68 ± 0.75 vs. 2.54 ± 0.36 % area, p<0.04) (Figure 120 A). The percentage of collagen deposition in the RV sections of the MCT+MEL hearts was significantly lower than the MCT rats (2.61 ± 0.5 vs. 4.68 ± 0.75 %, p<0.04) (Figure 120 A). Representative images of all groups are shown below (Figure 120 B).

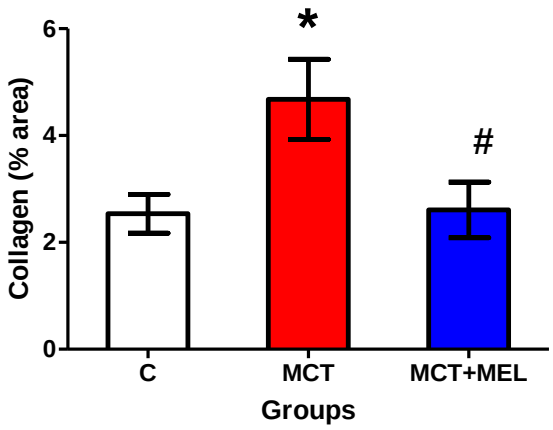


Figure 120 (A). Effect of a preventive treatment with melatonin (6mg/kg) on cardiac interstitial fibrosis in a rat model of MCT-induced PH, ***p**<0.04 (MCT vs. C), #p<0.04 (MCT+MEL vs. MCT), n=4 per group.

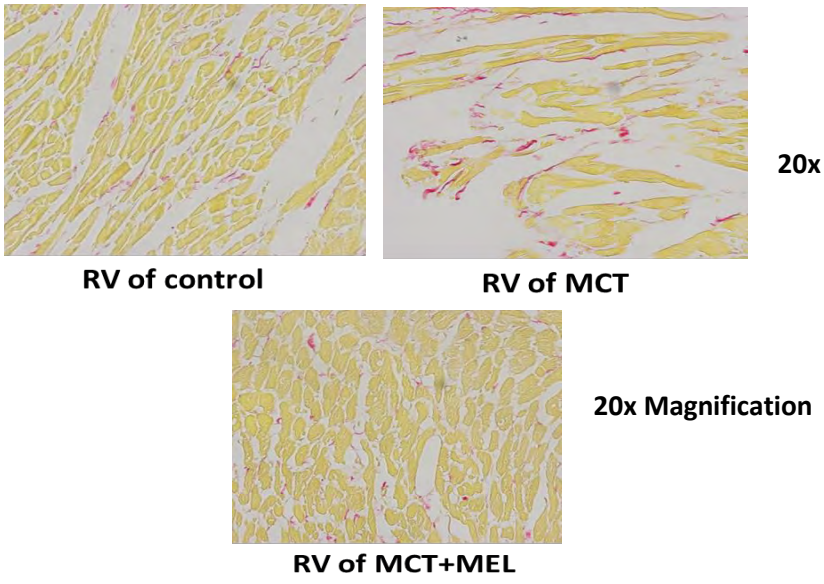


Figure 120 (B). Histology sections of hearts stained with picro-sirius red to assess interstitial fibrosis.

6. Discussion of the effects of melatonin as a therapeutic and preventive intervention

Our data demonstrate that a daily administration of melatonin either given as a therapeutic or preventive intervention attenuated the adverse effects of experimental PH on the heart. Hence, therapeutic and preventive melatonin treatment reduced RV hypertrophy, improved cardiac function, reduced oxidative stress, increased antioxidant capacity and normalised antioxidant enzyme activity in a rat model of MCT-induced PH.

Biometric measurements

The therapeutic approach has generated very important findings, as the rats were first injected with MCT, allowed to develop PH to a reasonably far progressed stage and then melatonin was administered. As seen from the biometric measurements, RV hypertrophy was already evident at day 14. Rats also displayed body weight loss and changes in heart weight, lung and liver weights. It has been shown that MCT rats have muscularization of the pulmonary arteries which leads to increased medial thickness and therefore PH, detectable as early as three to seven days after the MCT injection [350]. This pulmonary arterial remodelling and cardiac alterations are significantly increased between day ten and fourteen after MCT injection [350]. Therefore, this cardioprotective effect of melatonin seen in our study, means when melatonin was administered on day fourteen, it was able to stunt the RV hypertrophy, possibly by modulating pulmonary vascular pathology. In our study, the most striking effects of melatonin were seen on body weight, where it caused animals to not lose weight as those without treatment. This is striking, as even though melatonin was administered for a shorter period of time and only started when the disease is far progressed, it can still cease further body weight loss and reduce cachexia similar to the other melatonin treatment protocols. Similar effects were seen with the preventive melatonin treatment, where melatonin improved biometric measurements such as body weight, heart, lung and liver weight ratios as well as RV hypertrophy. There are a number studies that investigated the anti-hypertrophic effects of melatonin and possible mechanisms include the binding of melatonin to its receptors (MT-1 and 2), activation of anti-hypertrophic pathways and modulation of gene cardiac transcription [288].

Echocardiography and isolated heart perfusions: LV and RV function

Both the therapeutic and preventive treatments of melatonin improved cardiac function as seen with echocardiography and the isolated heart perfusions. These cardioprotective effects are attributed to the antioxidant properties of melatonin, which may have either attenuated PH or directly improved cardiac function. The direct effects of melatonin on cardiac function could have been achieved by either directly scavenging oxygen radicals in the heart or by binding to the melatonin receptors which have been shown to be present on ventricular cardiomyocytes [247]. These cardioprotective effects of melatonin were shown in hypertensive heart disease, ischemia/reperfusion, hyperthyroidism-induced cardiac hypertrophy and obesity associated myocardial dysfunction [273,294,347]. In a very interesting study by Ersahin et al, melatonin reduced oxidative stress and cardiac tissue, pathological remodelling and improve systolic function as assessed with echocardiography [351]. These desirable effects of melatonin on cardiac function were also ascribed to its antioxidant and anti-fibrotic properties. Melatonin may therefore, also employ similar functions in our study in order to improve cardiac function.

Blood plasma oxidative stress and antioxidant capacity

Both melatonin treatment protocols reduced plasma oxidative stress, increased antioxidant capacity and normalised antioxidant enzyme activities. A very important observation in the therapeutic protocol was that, at day fourteen, an increase in oxidative stress and a decrease in antioxidant capacity was already observed, while the catalase activity was increased but not the SOD activity. This is important, as the SOD activity does eventually increase at day 28 and the fact that only certain antioxidant enzymes increase their activity, may be the reason why oxidative damage far surpasses the antioxidant defence.

Furthermore, one need to acknowledge that other antioxidant enzymes such as glutathione peroxidase or non-enzymatic antioxidant molecules such as ascorbate, alpha-tocopherol and uric acid can also be employed to target ROS [352,353]. However, even if they did play a role in our study, oxidative stress is still increased which could mean that the antioxidant defence mechanisms of the body are not enough to counteract the damage caused by free radicals. Nevertheless, melatonin treatment reduced oxidative stress and normalized antioxidant status, despite the fact that the disease was far progressed.

Preventive melatonin treatment had similar effects on blood plasma oxidative stress and antioxidant status. A very interesting finding here was that preventive melatonin treatment also increased the blood plasma antioxidant capacity of the melatonin treated controls, to more than double the levels of control rats. This is an important observation, as it means that melatonin has the ability to increase the antioxidant capacity of the healthy blood plasma, which could potentially protect against future disease. Similar finding were generated by Piechota et al, who also administered 6mg of melatonin orally to healthy individuals [295]. These individuals, received melatonin treatment for two weeks after which blood samples were collected, plasma antioxidant ferric reducing ability was shown to be increased. These effects of melatonin were maintained even ten days after discontinuation of melatonin treatment [295]. On the other hand, this one should also be cautious about increasing antioxidant status too much, as this might also be detrimental [76,237].

As mentioned previously in this dissertation, Piantadosi et al, showed that ROS plays a crucial role in antioxidant-induced cardiac mitochondrial biogenesis by liberating, stabilizing and translocation of nuclear respiratory factor-2 to the nucleus [237]. Upon activation by ROS, nuclear respiratory factor-2 subsequently binds to multiple antioxidant-response-element motifs which bring about regulation of mitochondrial biogenesis, in conjunction with peroxisome proliferator-activated receptor-gamma coactivator-1-alpha [237]. Therefore suggesting that ROS may enable cardiomyocytes, to "sense" ROS-induced damage, employ its inherent antioxidant systems and counteract the damage as an adaptive response [76,237]. The maintenance of an intricate balance between scavenging detrimental free radicals and preserving some free radicals for physiological stimulus remains imperative. Nevertheless, in our study, preventive melatonin treatment eloquently reduced oxidative stress and normalized antioxidant capacity and antioxidant enzyme activity in MCT rats.

Cardiac interstitial fibrosis: The effect of therapeutic and preventive melatonin treatment

Both therapeutic and preventive melatonin reduced cardiac interstitial fibrosis compared to the MCT rats without treatment. Our data are supported by other who showed that melatonin can either reduce or prevent fibrosis in models of LV hypertrophy [271,354]. It is believed that the cardiac remodelling is dependent on the interaction between pro-proliferative neurohumoral factors such as catecholamines, angiotensin-II, endothelin, free radicals, cytokines or growths factors

and anti-proliferative factors including nitric oxide, prostacyclin, atrial natriuretic peptides or antioxidants [271]. In a recent publication it was shown that melatonin reduced interstitial fibrosis in a rat model of isoproterenol induced heart failure, where the mechanisms included increased nitric oxide levels and reduced angiotensin-II [271]. Another publication also showed that melatonin treatment reduced fibrosis in a rat model of L-arginine-methyl-ester (L-NAME) induced hypertensive heart disease [354]. Here, melatonin did not affect the nitric oxide synthase expression and activity but completely prevented oxidative stress in the form of conjugated dienes in the LV. Exploring the effects of melatonin on the nitric oxide pathway in our model would be of interest to understand the mechanisms involved in the melatonin-induced modulation of fibrosis in our model.

All the different melatonin treatments improved cardiac function with a concomitant reduction of oxidative stress. However, only the therapeutic and preventive melatonin treatments reduced interstitial fibrosis. Therefore, one cannot say that reduction of fibrosis was the main reason for the improved cardiac function but this reduction together with the reduction of oxidative stress may be the reason why we see better cardiac function and health benefit with the therapeutic and preventive treatments. We did not measure the cardiac interstitial fibrosis for all the melatonin treated MCT and control groups. This we will consider for investigation at a later stage.

7. Conclusions

In conclusion our data strongly sugggest that melatonin treatment is cardioprotective in the experimental model of PH by reducing oxidative stress and increasing antioxidant capacity. From these experiments it is clear that all three doses of melatonin conferred cardioprotection and 6mg/kg appeared to be the most efficacious dose. Furthermore, both therapeutic and preventive treatment with melatonin conferred cardioprotection, reduced oxidative stress and normalized antioxidant capacity and antioxidant enzyme activities. Lastly, melatonin treatment also reduced cardiac interstitial fibrosis, which together with reduced oxidative stress may be the mechanism for greater benefit seen with the therapeutic and preventive interventions. The data from the therapeutic melatonin treatment is of particular interest as this better reflects the clinical setting where patients usually present with PH and RV remodelling/dysfunction. Thus, our data suggest that melatonin could be a possible therapy for patients who already have cardiac pathology.

SECTION H

CONCLUSIONS

1. Summary - Conclusions

In this present dissertation we set out to establish the presence of oxidative stress and assessed antioxidant capacity in patients with PH and compared these to healthy controls. We then validated an experimental model of PH in the Long Evans rat strain and assessed RV hypertrophy with biometric measurements and cardiac function with echocardiography and the isolated heart perfusion apparatus. Furthermore, we assessed and established blood plasma levels of oxidative stress and antioxidant capacity. Hereafter, we tested whether melatonin, a powerful antioxidant, is cardioprotective in this model of PH by testing three different doses of melatonin (75ng/L, 4mg/kg and 6mg/kg). Upon completion of these experiments, we administered melatonin either fourteen days after the induction of experimental PH (therapeutic approach) or five days before the induction (preventive approach). In all these experiments, we assessed biometric measurements, cardiac function with echocardiography or isolated heart perfusions and oxidative stress and antioxidative capacity with blood plasma assays.

The main findings of our study:

Section D:

Oxidative stress in the form of lipid peroxidation was increased in the blood plasma of patients with PH compared to healthy controls. The antioxidant capacity of the plasma showed a trend towards a decrease and the antioxidant enzyme (catalase and SOD) activities were increased in response to the oxidative stress (Figure 121).

Section E:

We validated the MCT model of experimental PH in the Long Evans rat strain and showed the presence of both LV and RV dysfunction, RV hypertrophy and plasma oxidative stress. Furthermore, we showed this model displays plasma reduced antioxidant capacity and increased antioxidant enzyme (catalase and SOD) activities in response to the oxidative stress. These findings were the same as in the blood plasma of the PH patients (Figure 121).

Section F:

In this section of the study, we tested the three different doses of melatonin and showed that all doses were cardioprotective by reducing RV hypertrophy, cardiac function, reducing plasma oxidative stress, improving antioxidant capacity and normalising antioxidant enzyme activities. Here we also ascertained the 6mg/kg dose as the most efficacious dose and continued further experimentation with it (Figure 121).

Section G:

In this last section of the study, we administered melatonin (6mg/kg) as a therapeutic and preventive treatment. Melatonin conferred cardioprotection, reduced oxidative stress, improved antioxidant capacity and normalised antioxidant enzyme activities (Figure 121).

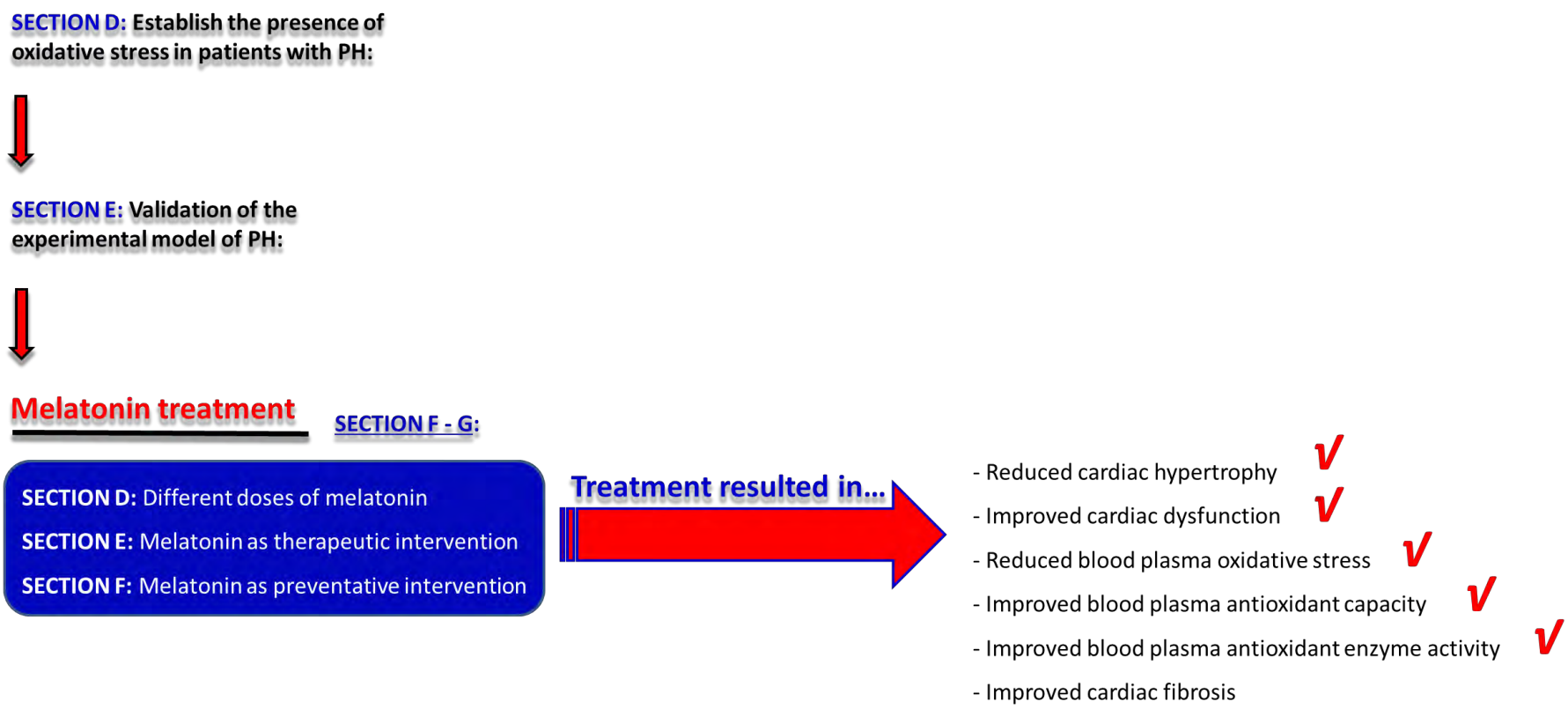


Figure 121. Conclusion of the effects of melatonin in PH, in this study. A graphical representation of our hypothesis that melatonin may be cardioprotective in PH. **PH:** pulmonary hypertension.

2. Limitations of the study

Although commonly used, the MCT model of PH has been widely criticised in the literature and it has been questioned whether it truly fully models PH as observed in patients [355] [324]. This is due to the fact that the MCT toxin has other effects on the myocardium which often resembles myocarditis and does not develop plexogenic vasculopathy as humans with PH do. According to the literature, other models of PH are better options which include the rat model of sugen-5416 plus either MCT or chronic hypoxia [333]. Nevertheless, these models are not available or established in South Africa and because the MCT model is highly reproducible and does not require technical skill, it provides a perfect platform for "proof of concept" studies.

A major limitation in our study was the low sensitivity of the echocardiography probe. We were not able to ascertain sufficient acoustic window to either visualize the RV properly or determine RV wall dimensions and filling pressures, as an echocardiography machine dedicated for animal use such as the Vevo 2100 (Visual Sonics) is not yet available in South Africa. Due to this, we could also not measure the haemodynamic measurements that are required to diagnose PH. These include mean pulmonary artery pressure and tricuspid valve annular plane excursion (TAPSE) [329].

The use of the isolated rat heart perfusion system gave valuable information regarding the contractility of the heart but does not reflect changes obtained with echocardiography. This technique is perhaps more relevant to pathologies such as ischemia/reperfusion but not in RV hypertrophy due to PH.

Another limitation in our study was that we only measured oxidative stress and antioxidant capacity in the blood plasma and not in the heart and lung tissue. These measurements may have given much more crucial information regarding the cardioprotective effects of melatonin. Also, we did not assess classic signalling pathways either involved in the cardiac hypertrophic response or pulmonary vascular remodelling that underline PH.

3. Future directions

We would like to complete further investigations into the mechanisms underlining the cardioprotective effects of melatonin in this model. We want to treatment more animals with MCT and melatonin and collect heart and lung samples for immunohistochemistry analyses of pro-hypertrophic and prosurvival signalling pathways such as calcineurin, calmodulin, NFAT, STAT-3, Akt, ERK, eNOS, smad-1 – 5 and smad-8. Furthermore, we are in the process of purchasing a more sensitive probe for the echocardiography machine and will conduct experiments to assess the necessary pulmonary and RV parameters. Lastly, we would also like to test melatonin in the pulmonary artery banding model of RV hypertrophy. With this model, PH is not present but increased RV afterload is created by placing a hemoclip around the main pulmonary artery in the heart. These rats develop RV hypertrophy in three to four weeks and it will allow us to assess the direct effects of melatonin on the heart, independent of the pulmonary system. These experiments will allow us to further characterize the cardioprotective effects of melatonin treatment, especially on the RV.

In conclusion, there are a number of treatments currently developed for PH in European countries but unfortunately, none of these treatments are currently available in South Africa, with the exception of the vasodilator sildenafil. PH is a terrible disease, with symptoms ranging from shortness of breath, fatigue, syncope and inability to do daily tasks such as climbing stairs or carrying heavy objects. Most patients in developing countries such as South Africa, are poor, have low income and cannot afford expensive treatments such as sildenafil and supplementary oxygen therapy to ease the management of the disease. Most patients, die rapidly due to the current absence of an effective treatment. There is thus a desperate need for new treatments for PH, especially treatments which are affordable and effective. Based on data from our study, melatonin could be used as an effective treatment for PH, if confirmed in humans/patients, as it evidently has a powerful cardioprotective effect and it presents the advantage of no reported side effects.

SECTION I

LIST OF PUBLICATIONS

1. Publications

Maarman G, Marais E, Lochner A, Du Toit EF, Effect of Chronic CPT-1 Inhibition on Myocardial Ischemia-Reperfusion Injury (I/R) in a Model of Diet-Induced Obesity, Cardiovascular Drugs and Therapy 2012; Number 3:205-216

Kelly-Laubscher R.F, King J.C, Hacking D, Somers S, Pedretti S, **Maarman G** and Lecour S, Preconditioning with sphingosine-1-phosphate requires activation of signal transducer and activator of transcription-3, Cardiovascular Journal of Africa; 2014 - Accepted for publication

Maarman G, Lecour S, Butrous G, Thienemann F and Sliwa K, A comprehensive review: the evolution of animal models in pulmonary hypertension research; are we there yet? *Pulmonary Circulation*, Vol. 3, No. 4 (December 2013), pp. 739-756.

Maarman G, Sliwa K, Lecour S, Cardioprotection in pulmonary hypertension: Possible therapeutic targets to protect the right ventricle, Final draft prepared for submission to *Cardiovascular Journal of Africa*, 2014.

2. Published abstracts

Maarman G, Blauwet L, Lecour S and Sliwa K, Can melatonin improve cardiac function in pulmonary arterial hypertension? European Society of Cardiology (ESC) annual congress 2013, *European Heart Journal* 2013; Vol 34(1):476.

Maarman G, Blauwet L, Sliwa K and Lecour S, Effects of melatonin treatment on cardiac function in a model of pulmonary arterial hypertension, Pan-African Society of Cardiology Congress 2013.

3. Conference outputs:

Maarman G, Blauwet L, Blackhurst D, Sliwa K and Lecour S, Cardioprotective effects of melatonin in an experimental model of pulmonary arterial hypertension, Spier Stellenbosch, 11 July – 12 July 2014.

Maarman G, Blauwet L, Blackhurst D, Sliwa K and Lecour S, Cardioprotective effects of melatonin on cardiac function and oxidative stress in pulmonary arterial hypertension, Frontiers in Cardiovascular Biology, Barcelona Spain, 1 July – 7 July 2014.

Thienemann F, Blauwet L, Burton R, Tibazarwa K, **Maarman G**, Dzudie A, Mocumbi AO, Sliwa K, HIV-associated pulmonary hypertension in a densely populated, peri-urban township in Cape Town, South Africa: clinical presentation and survival, To be presented at the European Society of Cardiology (ESC) Annual meeting in Barcelona Spain, 30th August until 3rd of September 2014.

Maarman G, Blauwet L, Sliwa K and Lecour S, Effects of melatonin treatment on cardiac function in a model of pulmonary arterial hypertension, Pan-African Society of Cardiology Congress 2013.

Maarman G, Blauwet L, Sliwa K, Lecour S, The effects of melatonin on cardiac function in a model of pulmonary arterial hypertension, The World Congress on Paediatric Cardiology & Cardiac Surgery 2013, Cape Town, South Africa.

SECTION J

REFERENCES

1. Mendis S, Chestnov O. The global burden of cardiovascular diseases: a challenge to improve. Curr Cardiol Rep 2014;16:486.

2. Alwan A, Maclean DR, Riley LM, et al. Monitoring and surveillance of chronic non-communicable diseases: progress and capacity in high-burden countries. Lancet 2010;376:1861-8.

3. WHO launches the World Health Statistics 2012. Euro Surveill 2012;17.

4. Badesch DB, Raskob GE, Elliott CG, et al. Pulmonary arterial hypertension: baseline characteristics from the REVEAL Registry. Chest 2010;137:376-87.

5. Simonneau G. (A new clinical classification of pulmonary hypertension). Bull Acad Natl Med 2009;193:1897-909.

6. Bogaard HJ, Abe K, Vonk Noordegraaf A, Voelkel NF. The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. Chest 2009;135:794-804.

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;114:1883-91.

8. Rich S. Right ventricular adaptation and maladaptation in chronic pulmonary arterial hypertension. Cardiol Clin 2012;30:257-69.

9. Gabler NB, French B, Strom BL, et al. Race and sex differences in response to endothelin receptor antagonists for pulmonary arterial hypertension. Chest 2012;141:20-6.

10. Rich S, Dantzker DR, Ayres SM, et al. Primary pulmonary hypertension. A national prospective study. Ann Intern Med 1987;107:216-23.

11. Argiento P, Vanderpool RR, Mule M, et al. Exercise Stress Echocardiography of the Pulmonary Circulation: Limits of Normal and Gender Differences. Chest 2012.

12. Campian ME, Verberne HJ, Hardziyenka M, et al. Serial noninvasive assessment of apoptosis during right ventricular disease progression in rats. J Nucl Med 2009;50:1371-7.

13. Minai OA, Chaouat A, Adnot S. Pulmonary hypertension in COPD: epidemiology, significance, and management: pulmonary vascular disease: the global perspective. Chest 2010;137:39S-51S.

14. Budev MM, Arroliga AC, Jennings CA. Diagnosis and evaluation of pulmonary hypertension. Cleve Clin J Med 2003;70 Suppl 1:S9-17.

15. Howard LS, Grapsa J, Dawson D, et al. Echocardiographic assessment of pulmonary hypertension: standard operating procedure. Eur Respir Rev 2012;21:239-48.

16. Galie N, Brundage BH, Ghofrani HA, et al. Tadalafil therapy for pulmonary arterial hypertension. Circulation 2009;119:2894-903.

17. Prudden HJ, Watts CH, Vickerman P, et al. Can the UNAIDS modes of transmission model be improved? A comparison of the original and revised model projections using data from a setting in west Africa. AIDS 2013;27:2623-35.

18. Price LC, Wort SJ, Perros F, et al. Inflammation in pulmonary arterial hypertension. Chest 2012;141:210-21.

19. Almodovar S, Cicalini S, Petrosillo N, Flores SC. Pulmonary hypertension associated with HIV infection: pulmonary vascular disease: the global perspective. Chest 2010;137:6S-12S.

20. Cicalini S, Almodovar S, Grilli E, Flores S. Pulmonary hypertension and human immunodeficiency virus infection: epidemiology, pathogenesis, and clinical approach. Clin Microbiol Infect 2011;17:25-33.

21. Adatia I, Kothari SS, Feinstein JA. Pulmonary hypertension associated with congenital heart disease: pulmonary vascular disease: the global perspective. Chest 2010;137:52S-61S.

22. Diller GP, van Eijl S, Okonko DO, et al. Circulating endothelial progenitor cells in patients with Eisenmenger syndrome and idiopathic pulmonary arterial hypertension. Circulation 2008;117:3020-30.

23. Lowe BS, Therrien J, Ionescu-Ittu R, Pilote L, Martucci G, Marelli AJ. Diagnosis of pulmonary hypertension in the congenital heart disease adult population impact on outcomes. J Am Coll Cardiol 2011;58:538-46.

24. Chaouat A, Naeije R, Weitzenblum E. Pulmonary hypertension in COPD. Eur Respir J 2008;32:1371-85.

25. Scharf SM, Iqbal M, Keller C, Criner G, Lee S, Fessler HE. Hemodynamic characterization of patients with severe emphysema. Am J Respir Crit Care Med 2002;166:314-22.

26. Humbert M, Sitbon O, Chaouat A, et al. Pulmonary arterial hypertension in France: results from a national registry. Am J Respir Crit Care Med 2006;173:1023-30.

27. Stewart S, Wilkinson D, Hansen C, et al. Predominance of heart failure in the Heart of Soweto Study cohort: emerging challenges for urban African communities. Circulation 2008;118:2360-7.

28. Sliwa K, Wilkinson D, Hansen C, et al. Spectrum of heart disease and risk factors in a black urban population in South Africa (the Heart of Soweto Study): a cohort study. Lancet 2008;371:915-22.

29. Summer R, Walsh K, Medoff BD. Obesity and pulmonary arterial hypertension: Is adiponectin the molecular link between these conditions? Pulm Circ 2011;1:440-7.

30. Farber HW, Loscalzo J. Pulmonary arterial hypertension. N Engl J Med 2004;351:1655-65.

31. Jurasz P, Courtman D, Babaie S, Stewart DJ. Role of apoptosis in pulmonary hypertension: from experimental models to clinical trials. Pharmacol Ther 2010;126:1-8.

32. Ryan J, Bloch K, Archer SL. Rodent models of pulmonary hypertension: harmonisation with the world health organisation's categorisation of human PH. Int J Clin Pract Suppl 2011:15-34.

33. Wright JL, Levy RD, Churg A. Pulmonary hypertension in chronic obstructive pulmonary disease: current theories of pathogenesis and their implications for treatment. Thorax 2005;60:605-9.

34. Rabinovitch M. Pathobiology of pulmonary hypertension. Annu Rev Pathol 2007;2:369-99.

35. Ogawa A, Matsubara H. How long can we leave patients with pulmonary arterial hypertension on oral drug monotreatment? Circ J 2012;76:1089-90.

36. Ogawa A, Firth AL, Ariyasu S, et al. Thrombin-mediated activation of Akt signaling contributes to pulmonary vascular remodeling in pulmonary hypertension. Physiol Rep 2013;1:e00190.

37. Todorovich-Hunter L, Dodo H, Ye C, McCready L, Keeley FW, Rabinovitch M. Increased pulmonary artery elastolytic activity in adult rats with monocrotaline-induced progressive hypertensive pulmonary vascular disease compared with infant rats with nonprogressive disease. Am Rev Respir Dis 1992;146:213-23.

38. Ye CL, Rabinovitch M. Inhibition of elastolysis by SC-37698 reduces development and progression of monocrotaline pulmonary hypertension. Am J Physiol 1991;261:H1255-67.

39. Maruyama K, Ye CL, Woo M, et al. Chronic hypoxic pulmonary hypertension in rats and increased elastolytic activity. Am J Physiol 1991;261:H1716-26.

40. Rabinovitch M. EVE and beyond, retro and prospective insights. Am J Physiol 1999;277:L5-12.

41. Rabinovitch M. Elastase and the pathobiology of unexplained pulmonary hypertension. Chest 1998;114:213S-24S.

42. Antunes MA, Rocco PR. Elastase-induced pulmonary emphysema: insights from experimental models. An Acad Bras Cienc 2011;83:1385-96.

43. Wright JL, Farmer SG, Churg A. A neutrophil elastase inhibitor reduces cigarette smoke-induced remodelling of lung vessels. Eur Respir J 2003;22:77-81.

44. Wilkins MR. Pulmonary hypertension: the science behind the disease spectrum. Eur Respir Rev 2012;21:19-26.

45. Christman BW, McPherson CD, Newman JH, et al. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med 1992;327:70-5.

46. Giaid A, Stewart DJ, Michel RP. Endothelin-1-like immunoreactivity in postobstructive pulmonary vasculopathy. J Vasc Res 1993;30:333-8.

47. Bauer M, Wilkens H, Langer F, Schneider SO, Lausberg H, Schafers HJ. Selective upregulation of endothelin B receptor gene expression in severe pulmonary hypertension. Circulation 2002;105:1034-6.

48. Behr J, Ryu JH. Pulmonary hypertension in interstitial lung disease. Eur Respir J 2008;31:1357-67.

49. Duong HT, Comhair SA, Aldred MA, et al. Pulmonary artery endothelium resident endothelial colony-forming cells in pulmonary arterial hypertension. Pulm Circ 2011;1:475-86.

50. Xia XD, Xu ZJ, Hu XG, Wu CY, Dai YR, Yang L. Impaired iNOS-sGC-cGMP signalling contributes to chronic hypoxic and hypercapnic pulmonary hypertension in rat. Cell Biochem Funct 2012.

51. Moncada S. The L-arginine: nitric oxide pathway, cellular transduction and immunological roles. Adv Second Messenger Phosphoprotein Res 1993;28:97-9.

52. Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N Engl J Med 1995;333:214-21.

53. Gunaydin S, Imai Y, Takanashi Y, et al. The effects of vasoactive intestinal peptide on monocrotaline induced pulmonary hypertensive rabbits following cardiopulmonary bypass: a comparative study with isoproteronol and nitroglycerine. Cardiovasc Surg 2002;10:138-45.

54. Leuchte HH, Baezner C, Baumgartner RA, et al. Inhalation of vasoactive intestinal peptide in pulmonary hypertension. Eur Respir J 2008;32:1289-94.

55. Said SI, Hamidi SA, Dickman KG, et al. Moderate pulmonary arterial hypertension in male mice lacking the vasoactive intestinal peptide gene. Circulation 2007;115:1260-8.

56. Said SI. Vasoactive intestinal peptide in pulmonary arterial hypertension. Am J Respir Crit Care Med 2012;185:786; author reply

57. Cogan JD, Vnencak-Jones CL, Phillips JA, 3rd, et al. Gross BMPR2 gene rearrangements constitute a new cause for primary pulmonary hypertension. Genet Med 2005;7:169-74.

58. Morrell NW. Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling? Proc Am Thorac Soc 2006;3:680-6.

59. Atkinson C, Stewart S, Upton PD, et al. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 2002;105:1672-8.

60. Talati M, West J, Blackwell TR, Loyd JE, Meyrick B. BMPR2 mutation alters the lung macrophage endothelin-1 cascade in a mouse model and patients with heritable pulmonary artery hypertension. Am J Physiol Lung Cell Mol Physiol 2010;299:L363-73.

61. Humbert M, Labrune P, Sitbon O, et al. Pulmonary arterial hypertension and type-I glycogen-storage disease: the serotonin hypothesis. Eur Respir J 2002;20:59-65.

62. Zakrzewicz A, Kouri FM, Nejman B, et al. The transforming growth factor-beta/Smad2,3 signalling axis is impaired in experimental pulmonary hypertension. Eur Respir J 2007;29:1094-104.

63. Zaiman AL, Podowski M, Medicherla S, et al. Role of the TGF-beta/Alk5 signaling pathway in monocrotaline-induced pulmonary hypertension. Am J Respir Crit Care Med 2008;177:896-905.

64. Ramos MF, Lame MW, Segall HJ, Wilson DW. Smad signaling in the rat model of monocrotaline pulmonary hypertension. Toxicol Pathol 2008;36:311-20.

65. Santibanez JF, Quintanilla M, Bernabeu C. TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. Clin Sci (Lond) 2011;121:233-51.

66. Vonk Noordegraaf A, Galie N. The role of the right ventricle in pulmonary arterial hypertension. Eur Respir Rev 2011;20:243-53.

67. Walker LA, Buttrick PM. The right ventricle: biologic insights and response to disease. Curr Cardiol Rev 2009;5:22-8.

68. Greyson CR. The right ventricle and pulmonary circulation: basic concepts. Rev Esp Cardiol 2010;63:81-95.

69. Handoko ML, de Man FS, Allaart CP, Paulus WJ, Westerhof N, Vonk-Noordegraaf A. Perspectives on novel therapeutic strategies for right heart failure in pulmonary arterial hypertension: lessons from the left heart. Eur Respir Rev 2010;19:72-82.

70. Banerjee D, Haddad F, Zamanian RT, Nagendran J. Right ventricular failure: a novel era of targeted therapy. Curr Heart Fail Rep 2010;7:202-11.

71. Leichsenring-Silva F, Tavares AM, Mosele F, Berger B, Llesuy S, Bello-Klein A. Association of the time course of pulmonary arterial hypertension with changes in oxidative stress in the left ventricle. Clin Exp Pharmacol Physiol 2011;38:804-10.

72. Maron BA. Targeting neurohumoral signaling to treat pulmonary hypertension: the right ventricle coming into focus. Circulation 2012;126:2806-8.

73. Braun MU, Szalai P, Strasser RH, Borst MM. Right ventricular hypertrophy and apoptosis after pulmonary artery banding: regulation of PKC isozymes. Cardiovascular research 2003;59:658-67.

74. Rouleau JL, Kapuku G, Pelletier S, et al. Cardioprotective effects of ramipril and losartan in right ventricular pressure overload in the rabbit: importance of kinins and influence on angiotensin II type 1 receptor signaling pathway. Circulation 2001;104:939-44.

75. Voelkel NF, Bogaard HJ, Al Husseini A, Farkas L, Gomez-Arroyo J, Natarajan R. Antioxidants for the treatment of patients with severe angioproliferative pulmonary hypertension? Antioxid Redox Signal 2013;18:1810-7.

76. Wong CM, Bansal G, Pavlickova L, Marcocci L, Suzuki YJ. Reactive oxygen species and antioxidants in pulmonary hypertension. Antioxid Redox Signal 2013;18:1789-96.

77. Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and heart failure. Am J Physiol Heart Circ Physiol 2011;301:H2181-90.

78. Hadi AM, Mouchaers KT, Schalij I, et al. Rapid quantification of myocardial fibrosis: a new macro-based automated analysis. Cell Oncol (Dordr) 2011;34:343-54.

79. Overbeek MJ, Mouchaers KT, Niessen HM, et al. Characteristics of interstitial fibrosis and inflammatory cell infiltration in right ventricles of systemic sclerosis-associated pulmonary arterial hypertension. Int J Rheumatol 2010;2010.

80. Shehata ML, Harouni AA, Skrok J, et al. Regional and global biventricular function in pulmonary arterial hypertension: a cardiac MR imaging study. Radiology 2013;266:114-22.

81. Stessel H, Brunner F. Effect of endothelin antagonism on contractility, intracellular calcium regulation and calcium regulatory protein expression in right ventricular hypertrophy of the rat. Basic Clin Pharmacol Toxicol 2004;94:37-45.

82. Zhang TT, Cui B, Dai DZ. Downregulation of Kv4.2 and Kv4.3 channel gene expression in right ventricular hypertrophy induced by monocrotaline in rat. Acta pharmacologica Sinica 2004;25:226-30.

83. Lee JK, Kodama I, Honjo H, Anno T, Kamiya K, Toyama J. Stage-dependent changes in membrane currents in rats with monocrotaline-induced right ventricular hypertrophy. Am J Physiol 1997;272:H2833-42.

84. Chen L, Gan XT, Haist JV, et al. Attenuation of compensatory right ventricular hypertrophy and heart failure following monocrotaline-induced pulmonary vascular injury by the Na+-H+ exchange inhibitor cariporide. J Pharmacol Exp Ther 2001;298:469-76.

85. Karmazyn M. The role of the myocardial sodium-hydrogen exchanger in mediating ischemic and reperfusion injury. From amiloride to cariporide. Ann N Y Acad Sci 1999;874:326-34.

86. Cingolani HE, Ennis IL. Sodium-hydrogen exchanger, cardiac overload, and myocardial hypertrophy. Circulation 2007;115:1090-100.

87. Baandrup JD, Markvardsen LH, Peters CD, et al. Pressure load: the main factor for altered gene expression in right ventricular hypertrophy in chronic hypoxic rats. PLoS One 2011;6:e15859.

88. Piao L, Fang YH, Cadete VJ, et al. The inhibition of pyruvate dehydrogenase kinase improves impaired cardiac function and electrical remodeling in two models of right ventricular hypertrophy: resuscitating the hibernating right ventricle. J Mol Med (Berl) 2010;88:47-60.

89. Piao L, Marsboom G, Archer SL. Mitochondrial metabolic adaptation in right ventricular hypertrophy and failure. J Mol Med (Berl) 2010;88:1011-20.

90. Galie N, Hoeper MM, Humbert M, et al. Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). Eur Heart J 2009;30:2493-537.

91. Raja SG, Raja SM. Treating pulmonary arterial hypertension: current treatments and future prospects. Ther Adv Chronic Dis 2011;2:359-70.

92. Nauser TD, Stites SW. Diagnosis and treatment of pulmonary hypertension. Am Fam Physician 2001;63:1789-98.

93. Rich S, Seidlitz M, Dodin E, et al. The short-term effects of digoxin in patients with right ventricular dysfunction from pulmonary hypertension. Chest 1998;114:787-92.

94. Mathur PN, Powles P, Pugsley SO, McEwan MP, Campbell EJ. Effect of digoxin on right ventricular function in severe chronic airflow obstruction. A controlled clinical trial. Ann Intern Med 1981;95:283-8.

95. Timms RM, Khaja FU, Williams GW. Hemodynamic response to oxygen therapy in chronic obstructive pulmonary disease. Ann Intern Med 1985;102:29-36.

96. Weitzenblum E, Sautegeau A, Ehrhart M, Mammosser M, Pelletier A. Long-term oxygen therapy can reverse the progression of pulmonary hypertension in patients with chronic obstructive pulmonary disease. Am Rev Respir Dis 1985;131:493-8.

97. Leuchte HH, Baezner CJ, Baumgartner RA, Mernitz P, Neurohr C, Behr J. Acute Hemodynamic Responses to Supplemental Oxygen and Their Prognostic Implications in Pulmonary Hypertension. Respiration 2012.

98. Shujaat A, Minkin R, Eden E. Pulmonary hypertension and chronic cor pulmonale in COPD. Int J Chron Obstruct Pulmon Dis 2007;2:273-82.

99. Humbert M, Barst RJ, Robbins IM, et al. Combination of bosentan with epoprostenol in pulmonary arterial hypertension: BREATHE-2. Eur Respir J 2004;24:353-9.

100. Humbert M, Sitbon O, Chaouat A, et al. Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era. Circulation 2010;122:156-63.

101. Rubin LJ. Primary pulmonary hypertension. N Engl J Med 1997;336:111-7.

102. Freund-Michel V, Guibert C, Dubois M, et al. Reactive oxygen species as therapeutic targets in pulmonary hypertension. Ther Adv Respir Dis 2013;7:175-200.

103. Rubin LJ, Badesch DB, Barst RJ, et al. Bosentan therapy for pulmonary arterial hypertension. N Engl J Med 2002;346:896-903.

104. Galie N, Olschewski H, Oudiz RJ, et al. Ambrisentan for the treatment of pulmonary arterial hypertension: results of the ambrisentan in pulmonary arterial hypertension, randomized, double-blind, placebo-controlled, multicenter, efficacy (ARIES) study 1 and 2. Circulation 2008;117:3010-9.

105. Hefke T, Zittermann A, Fuchs U, Schulte-Eistrup S, Gummert JF, Schulz U. Bosentan effects on hemodynamics and clinical outcome in heart failure patients with pulmonary hypertension awaiting cardiac transplantation. Thorac Cardiovasc Surg 2012;60:26-34.

106. Channick RN, Rubin LJ. New and experimental therapies for pulmonary hypertension. Clin Chest Med 2001;22:539-45.

107. Sitbon O, Humbert M, Nunes H, et al. Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival. J Am Coll Cardiol 2002;40:780-8.

108. Baptista R, Castro G, da Silva AM, Monteiro P, Providencia LA. Long-term effect of bosentan in pulmonary hypertension associated with complex congenital heart disease. Rev Port Cardiol 2013.

109. Badesch DB, Feldman J, Keogh A, et al. ARIES-3: ambrisentan therapy in a diverse population of patients with pulmonary hypertension. Cardiovasc Ther 2012;30:93-9.

110. Zuckerman WA, Leaderer D, Rowan CA, Mituniewicz JD, Rosenzweig EB. Ambrisentan for pulmonary arterial hypertension due to congenital heart disease. Am J Cardiol 2011;107:1381-5.

111. D'Alto M, Romeo E, Argiento P, et al. Ambrisentan for pulmonary arterial hypertension: long term effects on clinical status, exercise capacity and haemodynamics. Int J Cardiol 2012;156:244-5.

112. Wu C, Chan MF, Stavros F, et al. Discovery of TBC11251, a potent, long acting, orally active endothelin receptor-A selective antagonist. J Med Chem 1997;40:1690-7.

113. Barst RJ, Langleben D, Frost A, et al. Sitaxsentan therapy for pulmonary arterial hypertension. Am J Respir Crit Care Med 2004;169:441-7.

114. Barst RJ, Rich S, Widlitz A, Horn EM, McLaughlin V, McFarlin J. Clinical efficacy of sitaxsentan, an endothelin-A receptor antagonist, in patients with pulmonary arterial hypertension: open-label pilot study. Chest 2002;121:1860-8.

115. Wittbrodt ET, Abubakar A. Sitaxsentan for treatment of pulmonary hypertension. Ann Pharmacother 2007;41:100-5.

116. Girgis RE, Dixon RA. Sitaxsentan for the treatment of pulmonary arterial hypertension. Future Cardiol 2007;3:377-85.

117. Girgis RE, Frost AE, Hill NS, et al. Selective endothelin A receptor antagonism with sitaxsentan for pulmonary arterial hypertension associated with connective tissue disease. Ann Rheum Dis 2007;66:1467-72.

118. Apostolopoulou SC, Rammos S, Kyriakides ZS, et al. Acute endothelin A receptor antagonism improves pulmonary and systemic haemodynamics in patients with pulmonary arterial hypertension that is primary or autoimmune and related to congenital heart disease. Heart 2003;89:1221-6.

119. Apostolopoulou SC, Rammos S. Sitaxsentan in pulmonary arterial hypertension. Chest 2003;123:1772; author reply -3.

120. Sandoval J, Torbicki A, Souza R, et al. Safety and efficacy of sitaxsentan 50 and 100 mg in patients with pulmonary arterial hypertension. Pulm Pharmacol Ther 2012;25:33-9.

121. Barst RJ, Langleben D, Badesch D, et al. Treatment of pulmonary arterial hypertension with the selective endothelin-A receptor antagonist sitaxsentan. J Am Coll Cardiol 2006;47:2049-56.

122. Chin M, Levy RD, Yoshida EM, Byrne MF. Sitaxsentan-induced acute severe hepatitis treated with glucocorticoid therapy. Can Respir J 2012;19:e1-2.

123. Hoeper MM, Olsson KM, Schneider A, Golpon H. Severe hepatitis associated with sitaxentan and response to glucocorticoid therapy. Eur Respir J 2009;33:1518-9.

124. Lee WT, Kirkham N, Johnson MK, Lordan JL, Fisher AJ, Peacock AJ. Sitaxentan-related acute liver failure in a patient with pulmonary arterial hypertension. Eur Respir J 2011;37:472-4.

125. Lavelle A, Sugrue R, Lawler G, et al. Sitaxentan-induced hepatic failure in two patients with pulmonary arterial hypertension. Eur Respir J 2009;34:770-1.

126. Galie N, Hoeper MM, Gibbs JS, Simonneau G. Liver toxicity of sitaxentan in pulmonary arterial hypertension. Eur Respir J 2011;37:475-6.

127. Waxman AB, Zamanian RT. Pulmonary arterial hypertension: new insights into the optimal role of current and emerging prostacyclin therapies. Am J Cardiol 2013;111:1A-16A; quiz 7A-9A.

128. Badesch DB, Tapson VF, McGoon MD, et al. Continuous intravenous epoprostenol for pulmonary hypertension due to the scleroderma spectrum of disease. A randomized, controlled trial. Ann Intern Med 2000;132:425-34.

129. Gomberg-Maitland M, McLaughlin V, Gulati M, Rich S. Efficacy and safety of sildenafil added to treprostinil in pulmonary hypertension. Am J Cardiol 2005;96:1334-6.

130. Jing ZC, Parikh K, Pulido T, et al. Efficacy and Safety of Oral Treprostinil Monotherapy for the Treatment of Pulmonary Arterial Hypertension: A Randomized Controlled Trial. Circulation 2013.

131. Galie N, Humbert M, Vachiery JL, et al. Effects of beraprost sodium, an oral prostacyclin analogue, in patients with pulmonary arterial hypertension: a randomized, double-blind, placebo-controlled trial. J Am Coll Cardiol 2002;39:1496-502.

132. Olschewski H, Simonneau G, Galie N, et al. Inhaled iloprost for severe pulmonary hypertension. N Engl J Med 2002;347:322-9.

133. Wilkins MR, Wharton J, Grimminger F, Ghofrani HA. Phosphodiesterase inhibitors for the treatment of pulmonary hypertension. Eur Respir J 2008;32:198-209.

134. Mikhail GW, Prasad SK, Li W, et al. Clinical and haemodynamic effects of sildenafil in pulmonary hypertension: acute and mid-term effects. Eur Heart J 2004;25:431-6.

135. Galie N, Ghofrani HA, Torbicki A, et al. Sildenafil citrate therapy for pulmonary arterial hypertension. N Engl J Med 2005;353:2148-57.

136. Humpl T, Reyes JT, Holtby H, Stephens D, Adatia I. Beneficial effect of oral sildenafil therapy on childhood pulmonary arterial hypertension: twelve-month clinical trial of a single-drug, open-label, pilot study. Circulation 2005;111:3274-80.

137. Lu XL, Xiong CM, Shan GL, et al. Impact of sildenafil therapy on pulmonary arterial hypertension in adults with congenital heart disease. Cardiovasc Ther 2010;28:350-5.

138. Oudiz RJ, Brundage BH, Galie N, et al. Tadalafil for the treatment of pulmonary arterial hypertension: a double-blind 52-week uncontrolled extension study. J Am Coll Cardiol 2012;60:768-74.

139. Aggarwal P, Patial RK, Negi PC, Marwaha R. Oral tadalafil in pulmonary artery hypertension: a prospective study. Indian Heart J 2007;59:329-35.

140. Udeoji DU, Schwarz ER. Tadalafil as monotherapy and in combination regimens for the treatment of pulmonary arterial hypertension. Ther Adv Respir Dis 2012.

141. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. Am J Physiol Lung Cell Mol Physiol 2009;297:L1013-32.

142. Sztrymf B, Souza R, Bertoletti L, et al. Prognostic factors of acute heart failure in patients with pulmonary arterial hypertension. Eur Respir J 2010;35:1286-93.

143. Gomberg-Maitland M, Dufton C, Oudiz RJ, Benza RL. Compelling evidence of long-term outcomes in pulmonary arterial hypertension? A clinical perspective. J Am Coll Cardiol 2011;57:1053-61.

144. Archer SL, Marsboom G, Kim GH, et al. Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. Circulation 2010;121:2661-71.

145. Essop MR. Contemporary insights into the pathogenesis, diagnosis and therapy of pulmonary arterial hypertension. Cardiovasc J Afr 2010;21:334-7.

146. Rich S, Pogoriler J, Husain AN, Toth PT, Gomberg-Maitland M, Archer SL. Long-term effects of epoprostenol on the pulmonary vasculature in idiopathic pulmonary arterial hypertension. Chest 2010;138:1234-9.

147. O'Callaghan DS, Dorfmuller P, Jais X, et al. Pulmonary veno-occlusive disease: the bete noire of pulmonary hypertension in connective tissue diseases? Presse Med 2011;40:e65-78.

148. Bogaard HJ, Mizuno S, Hussaini AA, et al. Suppression of histone deacetylases worsens right ventricular dysfunction after pulmonary artery banding in rats. Am J Respir Crit Care Med 2011;183:1402-10.

149. McLaughlin VV. Medical management of primary pulmonary hypertension. Expert Opin Pharmacother 2002;3:159-65.

150. Antoniou T, Koletsis EN, Prokakis C, et al. Hemodynamic Effects of Combination Therapy With Inhaled Nitric Oxide and Iloprost in Patients With Pulmonary Hypertension and Right Ventricular Dysfunction After High-Risk Cardiac Surgery. J Cardiothorac Vasc Anesth 2012.

151. Hoeper MM, Faulenbach C, Golpon H, Winkler J, Welte T, Niedermeyer J. Combination therapy with bosentan and sildenafil in idiopathic pulmonary arterial hypertension. Eur Respir J 2004;24:1007-10.

152. Hoeper MM, Dinh-Xuan AT. Combination therapy for pulmonary arterial hypertension: still more questions than answers. Eur Respir J 2004;24:339-40.

153. Bendayan D, Shitrit D, Kramer MR. Combination therapy with prostacyclin and tadalafil for severe pulmonary arterial hypertension: a pilot study. Respirology 2008;13:916-8.

154. Kim SY, Shim JK, Shim YH, Hong SW, Choi KH, Kwak YL. Sildenafil and beraprost combination therapy in patients with pulmonary hypertension undergoing valvular heart surgery. J Heart Valve Dis 2010;19:333-40.

155. Mukherjee B, Howard L. Combination therapy in pulmonary arterial hypertension: do we have the right strategy? Expert Rev Respir Med 2011;5:191-205.

156. Sulica R, Poon M. Current medical treatment of pulmonary arterial hypertension. Mt Sinai J Med 2004;71:103-14.

157. Vachiery JL, Gaine S. Challenges in the diagnosis and treatment of pulmonary arterial hypertension. Eur Respir Rev 2012;21:313-20.

158. Ventetuolo CE, Klinger JR. WHO Group 1 pulmonary arterial hypertension: Current and investigative therapies. Prog Cardiovasc Dis 2012;55:89-103.

159. Stanley WC, Sabbah HN. Metabolic therapy for ischemic heart disease: the rationale for inhibition of fatty acid oxidation. Heart Fail Rev 2005;10:275-9.

160. Doggrell SA, Brown L. Rat models of hypertension, cardiac hypertrophy and failure. Cardiovascular research 1998;39:89-105.

161. Choudhury L, Guzzetti S, Lefroy DC, et al. Myocardial beta adrenoceptors and left ventricular function in hypertrophic cardiomyopathy. Heart 1996;75:50-4.

162. Ecarnot-Laubriet A, Assem M, Poirson-Bichat F, et al. Stage-dependent activation of cell cycle and apoptosis mechanisms in the right ventricle by pressure overload. Biochim Biophys Acta 2002;1586:233-42.

163. Martin-Puig S, Fuster V, Torres M. Heart repair: from natural mechanisms of cardiomyocyte production to the design of new cardiac therapies. Ann N Y Acad Sci 2012;1254:71-81.

164. Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA. Controversies in ventricular remodelling. Lancet 2006;367:356-67.

165. Shah AM, Mann DL. In search of new therapeutic targets and strategies for heart failure: recent advances in basic science. Lancet 2011;378:704-12.

166. Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. Circulation 1998;97:276-81.

167. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999;6:99-104.

168. Zhao ZQ, Morris CD, Budde JM, et al. Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction during reperfusion. Cardiovascular research 2003;59:132-42.

169. Das M. Apoptosis as a therapeutic target in heart failure. Am J Physiol Heart Circ Physiol 2007;293:H1322-3.

170. Shen T, Zheng M, Cao C, et al. Mitofusin-2 is a major determinant of oxidative stress-mediated heart muscle cell apoptosis. J Biol Chem 2007;282:23354-61.

171. Umar S, Lee JH, de Lange E, et al. Spontaneous ventricular fibrillation in right ventricular failure secondary to chronic pulmonary hypertension. Circ Arrhythm Electrophysiol 2012;5:181-90.

172. McKinsey TA. Therapeutic potential for HDAC inhibitors in the heart. Annu Rev Pharmacol Toxicol 2012;52:303-19.

173. Cavasin MA, Demos-Davies K, Horn TR, et al. Selective class I histone deacetylase inhibition suppresses hypoxia-induced cardiopulmonary remodeling through an antiproliferative mechanism. Circ Res 2012;110:739-48.

174. McKinsey TA. The biology and therapeutic implications of HDACs in the heart. Handb Exp Pharmacol 2011;206:57-78.

175. Cao DJ, Wang ZV, Battiprolu PK, et al. Histone deacetylase (HDAC) inhibitors attenuate cardiac hypertrophy by suppressing autophagy. Proc Natl Acad Sci U S A 2011;108:4123-8.

176. Cho YK, Eom GH, Kee HJ, et al. Sodium valproate, a histone deacetylase inhibitor, but not captopril, prevents right ventricular hypertrophy in rats. Circ J 2010;74:760-70.

177. McKinsey TA. Targeting inflammation in heart failure with histone deacetylase inhibitors. Mol Med 2011;17:434-41.

178. Latronico MV, Condorelli G. microRNAs in hypertrophy and heart failure. Exp Biol Med (Maywood) 2011;236:125-31.

179. van Rooij E, Marshall WS, Olson EN. Toward microRNA-based therapeutics for heart disease: the sense in antisense. Circ Res 2008;103:919-28.

180. van Rooij E, Sutherland LB, Thatcher JE, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A 2008;105:13027-32.

181. Wang N, Zhou Z, Liao X, Zhang T. Role of microRNAs in cardiac hypertrophy and heart failure. IUBMB Life 2009;61:566-71.

182. Pullamsetti SS, Doebele C, Fischer A, et al. Inhibition of microRNA-17 improves lung and heart function in experimental pulmonary hypertension. Am J Respir Crit Care Med 2012;185:409-19.

183. Naga Prasad SV, Duan ZH, Gupta MK, et al. Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. J Biol Chem 2009;284:27487-99.

184. Care A, Catalucci D, Felicetti F, et al. MicroRNA-133 controls cardiac hypertrophy. Nat Med 2007;13:613-8.

185. Li RC, Tao J, Guo YB, et al. In vivo suppression of microRNA-24 prevents the transition toward decompensated hypertrophy in aortic-constricted mice. Circ Res 2013;112:601-5.

186. Brock M, Samillan VJ, Trenkmann M, et al. AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension. Eur Heart J 2012.

187. Kumar S, Wei C, Thomas CM, et al. Cardiac-specific genetic inhibition of nuclear factor-kappaB prevents right ventricular hypertrophy induced by monocrotaline. Am J Physiol Heart Circ Physiol 2012;302:H1655-66.

188. Rusanescu G, Weissleder R, Aikawa E. Notch signaling in cardiovascular disease and calcification. Curr Cardiol Rev 2008;4:148-56.

189. Niessen K, Karsan A. Notch signaling in cardiac development. Circ Res 2008;102:1169-81.

190. Gude NA, Emmanuel G, Wu W, et al. Activation of Notch-mediated protective signaling in the myocardium. Circ Res 2008;102:1025-35.

191. Rizzo P, Miele L, Ferrari R. The Notch pathway: a crossroad between the life and death of the endothelium. Eur Heart J 2013;34:2504-9.

192. Stanley WC, Lopaschuk GD, Hall JL, McCormack JG. Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. Cardiovascular research 1997;33:243-57.

193. Taegtmeyer H. Metabolism--the lost child of cardiology. J Am Coll Cardiol 2000;36:1386-8.

194. Taegtmeyer H. Cardiac metabolism as a target for the treatment of heart failure. Circulation 2004;110:894-6.

195. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. Physiol Rev 2010;90:207-58.

196. Tuder RM, Davis LA, Graham BB. Targeting energetic metabolism: a new frontier in the pathogenesis and treatment of pulmonary hypertension. Am J Respir Crit Care Med 2012;185:260-6.

197. Wambolt RB, Lopaschuk GD, Brownsey RW, Allard MF. Dichloroacetate improves postischemic function of hypertrophied rat hearts. J Am Coll Cardiol 2000;36:1378-85.

198. Aad G, Abajyan T, Abbott B, et al. Search for magnetic monopoles in sqrt(s)=7 TeV pp collisions with the ATLAS detector. Phys Rev Lett 2012;109:261803.

199. Karmazyn M, Kilic A, Javadov S. The role of NHE-1 in myocardial hypertrophy and remodelling. J Mol Cell Cardiol 2008;44:647-53.

200. Moor S, Sharrock G, Scott J, et al. Evaluation of a teaching package designed to improve teachers' recognition of depressed pupils-a pilot study. J Adolesc 2000;23:331-42.

201. Snabaitis AK, D'Mello R, Dashnyam S, Avkiran M. A novel role for protein phosphatase 2A in receptor-mediated regulation of the cardiac sarcolemmal Na+/H+ exchanger NHE1. J Biol Chem 2006;281:20252-62.

202. Knight DR, Smith AH, Flynn DM, et al. A novel sodium-hydrogen exchanger isoform-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. J Pharmacol Exp Ther 2001;297:254-9.

203. Cheitlin MD, Byrd R, Benowitz N, Liu E, Modin G. Amiloride improves hemodynamics in patients with chronic congestive heart failure treated with chronic digoxin and diuretics. Cardiovasc Drugs Ther 1991;5:719-25.

204. Loennechen JP, Wisloff U, Falck G, Ellingsen O. Effects of cariporide and losartan on hypertrophy, calcium transients, contractility, and gene expression in congestive heart failure. Circulation 2002;105:1380-6.

205. Rungwerth K, Schindler U, Gerl M, et al. Inhibition of Na+-H+ exchange by cariporide reduces inflammation and heart failure in rabbits with myocardial infarction. Br J Pharmacol 2004;142:1147-54.

206. Marano G, Vergari A, Catalano L, et al. Na+/H+ exchange inhibition attenuates left ventricular remodeling and preserves systolic function in pressure-overloaded hearts. Br J Pharmacol 2004;141:526-32.

207. Garciarena CD, Caldiz CI, Portiansky EL, Chiappe de Cingolani GE, Ennis IL. Chronic NHE-1 blockade induces an antiapoptotic effect in the hypertrophied heart. J Appl Physiol (1985) 2009;106:1325-31.

208. Handoko ML, Lamberts RR, Redout EM, et al. Right ventricular pacing improves right heart function in experimental pulmonary arterial hypertension: a study in the isolated heart. Am J Physiol Heart Circ Physiol 2009;297:H1752-9.

209. McAlister FA, Ezekowitz J, Hooton N, et al. Cardiac resynchronization therapy for patients with left ventricular systolic dysfunction: a systematic review. JAMA 2007;297:2502-14.

210. Bowers R, Cool C, Murphy RC, et al. Oxidative stress in severe pulmonary hypertension. Am J Respir Crit Care Med 2004;169:764-9.

211. Demarco VG, Whaley-Connell AT, Sowers JR, Habibi J, Dellsperger KC. Contribution of oxidative stress to pulmonary arterial hypertension. World J Cardiol 2010;2:316-24.

212. Farley KS, Wang L, Mehta S. Septic pulmonary microvascular endothelial cell injury: role of alveolar macrophage NADPH oxidase. Am J Physiol Lung Cell Mol Physiol 2009;296:L480-8.

213. Irodova NL, Lankin VZ, Konovalova GK, Kochetov AG, Chazova IE. Oxidative stress in patients with primary pulmonary hypertension. Bull Exp Biol Med 2002;133:580-2.

214. Iqbal M, Cawthon D, Wideman RF, Jr., Bottje WG. Lung mitochondrial dysfunction in pulmonary hypertension syndrome. II. Oxidative stress and inability to improve function with repeated additions of adenosine diphosphate. Poult Sci 2001;80:656-65.

215. Grobe AC, Wells SM, Benavidez E, et al. Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. Am J Physiol Lung Cell Mol Physiol 2006;290:L1069-77.

216. Crosswhite P, Sun Z. Nitric oxide, oxidative stress and inflammation in pulmonary arterial hypertension. J Hypertens 2010;28:201-12.

217. Dorfmuller P, Chaumais MC, Giannakouli M, et al. Increased oxidative stress and severe arterial remodeling induced by permanent high-flow challenge in experimental pulmonary hypertension. Respir Res 2011;12:119.

218. Mohammadi S, Najafi M, Hamzeiy H, et al. Protective effects of methylsulfonylmethane on hemodynamics and oxidative stress in monocrotaline-induced pulmonary hypertensive rats. Adv Pharmacol Sci 2012;2012:507278.

219. Redout EM, van der Toorn A, Zuidwijk MJ, et al. Antioxidant treatment attenuates pulmonary arterial hypertension-induced heart failure. Am J Physiol Heart Circ Physiol 2010;298:H1038-47.

220. Vonk Noordegraaf A, Marcus JT, Roseboom B, Postmus PE, Faes TJ, de Vries PM. The effect of right ventricular hypertrophy on left ventricular ejection fraction in pulmonary emphysema. Chest 1997;112:640-5.

221. Qipshidze N, Tyagi N, Metreveli N, Lominadze D, Tyagi SC. Autophagy mechanism of right ventricular remodeling in murine model of pulmonary artery constriction. Am J Physiol Heart Circ Physiol 2012;302:H688-96.

222. Tang Z, Iqbal M, Cawthon D, Bottje WG. Heart and breast muscle mitochondrial dysfunction in pulmonary hypertension syndrome in broilers (Gallus domesticus). Comp Biochem Physiol A Mol Integr Physiol 2002;132:527-40.

223. Toporsian M, Jerkic M, Zhou YQ, et al. Spontaneous adult-onset pulmonary arterial hypertension attributable to increased endothelial oxidative stress in a murine model of hereditary hemorrhagic telangiectasia. Arterioscler Thromb Vasc Biol 2010;30:509-17.

224. Masri FA, Comhair SA, Dostanic-Larson I, et al. Deficiency of lung antioxidants in idiopathic pulmonary arterial hypertension. Clin Transl Sci 2008;1:99-106.

225. Spiekermann S, Landmesser U, Dikalov S, et al. Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 2003;107:1383-9.

226. Spiekermann S, Schenk K, Hoeper MM. Increased xanthine oxidase activity in idiopathic pulmonary arterial hypertension. Eur Respir J 2009;34:276.

227. Cracowski JL, Cracowski C, Bessard G, et al. Increased lipid peroxidation in patients with pulmonary hypertension. Am J Respir Crit Care Med 2001;164:1038-42.

228. Preston IR, Klinger JR, Houtches J, Nelson D, Farber HW, Hill NS. Acute and chronic effects of sildenafil in patients with pulmonary arterial hypertension. Respir Med 2005;99:1501-10.

229. Odhiambo A, Perlman DH, Huang H, et al. Identification of oxidative post-translational modification of serum albumin in patients with idiopathic pulmonary arterial hypertension and pulmonary hypertension of sickle cell anemia. Rapid Commun Mass Spectrom 2007;21:2195-203.

230. Joppa P, Petrasova D, Stancak B, Dorkova Z, Tkacova R. Oxidative stress in patients with COPD and pulmonary hypertension. Wien Klin Wochenschr 2007;119:428-34.

231. Ahmed MN, Codipilly C, Hogg N, Auten RL. The protective effect of overexpression of extracellular superoxide dismutase on nitric oxide bioavailability in the lung after exposure to hyperoxia stress. Exp Lung Res 2011;37:10-7.

232. Csiszar A, Labinskyy N, Olson S, et al. Resveratrol prevents monocrotaline-induced pulmonary hypertension in rats. Hypertension 2009;54:668-75.

233. Yang DL, Zhang HG, Xu YL, et al. Resveratrol inhibits right ventricular hypertrophy induced by monocrotaline in rats. Clin Exp Pharmacol Physiol 2010;37:150-5.

234. Paffett ML, Lucas SN, Campen MJ. Resveratrol reverses monocrotaline-induced pulmonary vascular and cardiac dysfunction: a potential role for atrogin-1 in smooth muscle. Vascul Pharmacol 2012;56:64-73.

235. Jankov RP, Kantores C, Pan J, Belik J. Contribution of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats. Am J Physiol Lung Cell Mol Physiol 2008;294:L233-45.

236. Ekelund UE, Harrison RW, Shokek O, et al. Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. Circ Res 1999;85:437-45.

237. Piantadosi CA, Carraway MS, Babiker A, Suliman HB. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. Circ Res 2008;103:1232-40.

238. Deora AA, Win T, Vanhaesebroeck B, Lander HM. A redox-triggered ras-effector interaction. Recruitment of phosphatidylinositol 3'-kinase to Ras by redox stress. J Biol Chem 1998;273:29923-8.

239. O'Connor PM, Gutterman DD. Resurrecting hope for antioxidant treatment of cardiovascular disease: focus on mitochondria. Circ Res 2010;107:9-11.

240. Heart Protection Study Collaborative G. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 2002;360:7-22.

241. Palumbo G, Avanzini F, Alli C, et al. Effects of vitamin E on clinic and ambulatory blood pressure in treated hypertensive patients. Collaborative Group of the Primary Prevention Project (PPP)--Hypertension study. Am J Hypertens 2000;13:564-7.

242. Kim MK, Sasaki S, Sasazuki S, Okubo S, Hayashi M, Tsugane S. Lack of long-term effect of vitamin C supplementation on blood pressure. Hypertension 2002;40:797-803.

243. Dikalova AE, Bikineyeva AT, Budzyn K, et al. Therapeutic targeting of mitochondrial superoxide in hypertension. Circ Res 2010;107:106-16.

244. Reiter RJ, Manchester LC, Fuentes-Broto L, Tan DX. Cardiac hypertrophy and remodelling: pathophysiological consequences and protective effects of melatonin. J Hypertens 2010;28 Suppl 1:S7-12.

245. Reiter RJ, Tan DX, Fuentes-Broto L. Melatonin: a multitasking molecule. Prog Brain Res 2010;181:127-51.

246. Reiter RJ, Tan DX, Paredes SD, Fuentes-Broto L. Beneficial effects of melatonin in cardiovascular disease. Ann Med 2010;42:276-85.

247. Boutin JA, Audinot V, Ferry G, Delagrange P. Molecular tools to study melatonin pathways and actions. Trends Pharmacol Sci 2005;26:412-9.

248. Konturek SJ, Konturek PC, Brzozowski T, Bubenik GA. Role of melatonin in upper gastrointestinal tract. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 2007;58 Suppl 6:23-52.

249. Yorgancioglu A, Cruz AA, Bousquet J, et al. The Global Alliance against Respiratory Diseases (GARD) Country Report. Prim Care Respir J 2014;23:98-101.

250. Lamont KT, Somers S, Lacerda L, Opie LH, Lecour S. Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol- and melatonin-induced cardioprotection. Journal of pineal research 2011;50:374-80.

251. Paulis L, Simko F. Blood pressure modulation and cardiovascular protection by melatonin: potential mechanisms behind. Physiol Res 2007;56:671-84.

252. Paulis L, Vazan R, Simko F, et al. Morphological alterations and NO-synthase expression in the heart after continuous light exposure of rats. Physiol Res 2007;56 Suppl 2:S71-6.

253. Dominguez-Rodriguez A. Melatonin in cardiovascular disease. Expert Opin Investig Drugs 2012;21:1593-6.

254. Dominguez-Rodriguez A, Abreu-Gonzalez P, Reiter RJ. Melatonin and cardiovascular disease: myth or reality? Rev Esp Cardiol (Engl Ed) 2012;65:215-8.

255. Paclt I, Ptacek R, Kuzelova H, et al. Circadian rhythms of saliva melatonin in ADHD, anxious and normal children. Neuro Endocrinol Lett 2011;32:790-8.

256. Gringras P, Gamble C, Jones AP, et al. Melatonin for sleep problems in children with neurodevelopmental disorders: randomised double masked placebo controlled trial. BMJ 2012;345:e6664.

257. Lemoine P, Wade AG, Katz A, Nir T, Zisapel N. Efficacy and safety of prolonged-release melatonin for insomnia in middle-aged and elderly patients with hypertension: a combined analysis of controlled clinical trials. Integr Blood Press Control 2012;5:9-17.

258. Appleton RE, Jones AP, Gamble C, et al. The use of MElatonin in children with neurodevelopmental disorders and impaired sleep: a randomised, double-blind, placebo-controlled, parallel study (MENDS). Health technology assessment 2012;16:i-239.

259. DeMuro RL, Nafziger AN, Blask DE, Menhinick AM, Bertino JS, Jr. The absolute bioavailability of oral melatonin. Journal of clinical pharmacology 2000;40:781-4.

260. Bartoli A, De Gregori S, Molinaro M, Broglia M, Tinelli C, Imberti R. Bioavailability of a new oral spray melatonin emulsion compared with a standard oral formulation in healthy volunteers. J Bioequivalence & Bioavailability 2012;4:96-9.

261. Russcher M, Koch BC, Nagtegaal JE, et al. Long-term effects of melatonin on quality of life and sleep in haemodialysis patients (Melody study): a randomized controlled trial. British journal of clinical pharmacology 2013;76:668-79.

262. Pandi-Perumal SR, Verster JC, Kayumov L, et al. Sleep disorders, sleepiness and traffic safety: a public health menace. Brazilian journal of medical and biological research Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica et al 2006;39:863-71.

263. Tan DX, Manchester LC, Reiter RJ, et al. A novel melatonin metabolite, cyclic 3-hydroxymelatonin: a biomarker of in vivo hydroxyl radical generation. Biochemical and biophysical research communications 1998;253:614-20.

264. Mayo JC, Sainz RM, Tan DX, et al. Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. Journal of neuroimmunology 2005;165:139-49.

265. Kotliarova S, Jana NR, Sakamoto N, et al. Decreased expression of hypothalamic neuropeptides in Huntington disease transgenic mice with expanded polyglutamine-EGFP fluorescent aggregates. J Neurochem 2005;93:641-53.

266. Hardeland R. Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. Endocrine 2005;27:119-30.

267. Waldhauser F, Waldhauser M, Lieberman HR, Deng MH, Lynch HJ, Wurtman RJ. Bioavailability of oral melatonin in humans. Neuroendocrinology 1984;39:307-13.

268. Aldhous M, Franey C, Wright J, Arendt J. Plasma concentrations of melatonin in man following oral absorption of different preparations. British journal of clinical pharmacology 1985;19:517-21.

269. Mao S, Chen J, Wei Z, Liu H, Bi D. Intranasal administration of melatonin starch microspheres. International journal of pharmaceutics 2004;272:37-43.

270. Hickie IB, Rogers NL. Novel melatonin-based therapies: potential advances in the treatment of major depression. Lancet 2011;378:621-31.

271. Simko F, Bednarova KR, Krajcirovicova K, et al. Melatonin reduces cardiac remodeling and improves survival in rats with isoproterenol-induced heart failure. Journal of pineal research 2014.

272. Agil A, Rosado I, Ruiz R, Figueroa A, Zen N, Fernandez-Vazquez G. Melatonin improves glucose homeostasis in young Zucker diabetic fatty rats. Journal of pineal research 2012;52:203-10.

273. Nduhirabandi F, Du Toit EF, Blackhurst D, Marais D, Lochner A. Chronic melatonin consumption prevents obesity-related metabolic abnormalities and protects the heart

against myocardial ischemia and reperfusion injury in a prediabetic model of diet-induced obesity. Journal of pineal research 2011;50:171-82.

274. Di Bella G, Mascia F, Gualano L, Di Bella L. Melatonin anticancer effects: review. Int J Mol Sci 2013;14:2410-30.

275. Borazan H, Tuncer S, Yalcin N, Erol A, Otelcioglu S. Effects of preoperative oral melatonin medication on postoperative analgesia, sleep quality, and sedation in patients undergoing elective prostatectomy: a randomized clinical trial. J Anesth 2010;24:155-60.

276. Rosales-Corral SA, Lopez-Armas G, Cruz-Ramos J, et al. Alterations in Lipid Levels of Mitochondrial Membranes Induced by Amyloid-beta: A Protective Role of Melatonin. International journal of Alzheimer's disease 2012;2012:459806.

277. El-Missiry MA, Abd El-Aziz AF. Influence of melatonin on proliferation and antioxidant system in Ehrlich ascites carcinoma cells. Cancer letters 2000;151:119-25.

278. Tengattini S, Reiter RJ, Tan DX, Terron MP, Rodella LF, Rezzani R. Cardiovascular diseases: protective effects of melatonin. Journal of pineal research 2008;44:16-25.

279. Dominguez-Rodriguez A, Abreu-Gonzalez P, Garcia-Gonzalez MJ, Samimi-Fard S, Kaski JC. Relation of ischemia-modified albumin levels and left ventricular systolic function in patients with ST-segment elevation myocardial infarction treated with primary percutaneous coronary intervention. Clin Chim Acta 2008;388:196-9.

280. Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F. Melatonin: a peroxyl radical scavenger more effective than vitamin E. Life sciences 1994;55:PL271-6.

281. Marchiafava PL, Longoni B. Melatonin as an antioxidant in retinal photoreceptors. Journal of pineal research 1999;26:184-9.

282. Reiter RJ, Tan DX, Mayo JC, Sainz RM, Leon J, Czarnocki Z. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. Acta Biochim Pol 2003;50:1129-46.

283. Tan DX, Poeggeler B, Reiter RJ, et al. The pineal hormone melatonin inhibits DNA-adduct formation induced by the chemical carcinogen safrole in vivo. Cancer letters 1993;70:65-71.

284. Stasica P, Paneth P, Rosiak JM. Hydroxyl radical reaction with melatonin molecule: a computational study. Journal of pineal research 2000;29:125-7.

285. Tan DX, Manchester LC, Reiter RJ, et al. Melatonin directly scavenges hydrogen peroxide: a potentially new metabolic pathway of melatonin biotransformation. Free Radic Biol Med 2000;29:1177-85.

286. Tan DX, Hardeland R, Manchester LC, Galano A, Reiter RJ. Cyclic-3-hydroxymelatonin (C3HOM), a potent antioxidant, scavenges free radicals and suppresses oxidative reactions. Current medicinal chemistry 2014;21:1557-65.

287. Tan DX, Manchester LC, Burkhardt S, et al. N1-acetyl-N2-formyl-5-methoxykynuramine, a biogenic amine and melatonin metabolite, functions as a potent antioxidant. FASEB J 2001;15:2294-6.

288. Rodriguez C, Mayo JC, Sainz RM, et al. Regulation of antioxidant enzymes: a significant role for melatonin. Journal of pineal research 2004;36:1-9.

289. Pogan L, Bissonnette P, Parent L, Sauve R. The effects of melatonin on Ca(2+) homeostasis in endothelial cells. Journal of pineal research 2002;33:37-47.

290. Das R, Balonan L, Ballard HJ, Ho S. Chronic hypoxia inhibits the antihypertensive effect of melatonin on pulmonary artery. Int J Cardiol 2008;126:340-5.

291. Tain YL, Hsieh CS, Chen CC, Sheen JM, Lee CT, Huang LT. Melatonin prevents increased asymmetric dimethylarginine in young rats with bile duct ligation. Journal of pineal research 2010;48:212-21.

292. Ghosh G, De K, Maity S, et al. Melatonin protects against oxidative damage and restores expression of GLUT4 gene in the hyperthyroid rat heart. Journal of pineal research 2007;42:71-82.

293. Tain YL, Huang LT, Lin IC, Lau YT, Lin CY. Melatonin prevents hypertension and increased asymmetric dimethylarginine in young spontaneous hypertensive rats. Journal of pineal research 2010;49:390-8.

294. Girouard H, Chulak C, Lejossec M, Lamontagne D, de Champlain J. Vasorelaxant effects of the chronic treatment with melatonin on mesenteric artery and aorta of spontaneously hypertensive rats. J Hypertens 2001;19:1369-77.

295. Piechota A, Lipinska S, Szemraj J, Goraca A. Long-term melatonin administration enhances the antioxidant potential of human plasma maintained after discontinuation of the treatment. Gen Physiol Biophys 2010;29:144-50.

296. Okatani Y, Watanabe K, Hayashi K, Wakatsuki A, Sagara Y. Melatonin inhibits vasospastic action of hydrogen peroxide in human umbilical artery. Journal of pineal research 1997;22:163-8.

297. Scheer FA, Van Montfrans GA, van Someren EJ, Mairuhu G, Buijs RM. Daily nighttime melatonin reduces blood pressure in male patients with essential hypertension. Hypertension 2004;43:192-7.

298. Cagnacci A, Cannoletta M, Renzi A, Baldassari F, Arangino S, Volpe A. Prolonged melatonin administration decreases nocturnal blood pressure in women. Am J Hypertens 2005;18:1614-8.

299. Mansoor GA. Ambulatory blood pressure monitoring in clinical trials in adults and children. Am J Hypertens 2002;15:38S-42S.

300. de Matos Cavalcante AG, de Bruin PF, de Bruin VM, et al. Melatonin reduces lung oxidative stress in patients with chronic obstructive pulmonary disease: a randomized, double-blind, placebo-controlled study. Journal of pineal research 2012;53:238-44.

301. Okutan H, Savas C, Delibas N. The antioxidant effect of melatonin in lung injury after aortic occlusion-reperfusion. Interact Cardiovasc Thorac Surg 2004;3:519-22.

302. Pablos MI, Reiter RJ, Chuang JI, et al. Acutely administered melatonin reduces oxidative damage in lung and brain induced by hyperbaric oxygen. J Appl Physiol (1985) 1997;83:354-8.

303. Unlu M, Fidan F, Sezer M, et al. Effects of melatonin on the oxidant/antioxidant status and lung histopathology in rabbits exposed to cigarette smoke. Respirology 2006;11:422-8.

304. Pedreira PR, Garcia-Prieto E, Parra D, et al. Effects of melatonin in an experimental model of ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 2008;295:L820-7.

305. Ozkan E, Yaman H, Cakir E, et al. Plasma melatonin and urinary 6-hydroxymelatonin levels in patients with pulmonary tuberculosis. Inflammation 2012;35:1429-34.

306. Gumral N, Naziroglu M, Ongel K, et al. Antioxidant enzymes and melatonin levels in patients with bronchial asthma and chronic obstructive pulmonary disease during stable and exacerbation periods. Cell Biochem Funct 2009;27:276-83.

307. Sakotnik A, Liebmann PM, Stoschitzky K, et al. Decreased melatonin synthesis in patients with coronary artery disease. Eur Heart J 1999;20:1314-7.

308. Brugger P, Marktl W, Herold M. Impaired nocturnal secretion of melatonin in coronary heart disease. Lancet 1995;345:1408.

309. Xu MF, Ho S, Qian ZM, Tang PL. Melatonin protects against cardiac toxicity of doxorubicin in rat. Journal of pineal research 2001;31:301-7.

310. Wagner GS, Marriott's Practical Electrocardiography, 11th Edition, Lippincott Williams & Wilkins, 2008.

311. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 1978;58:1072-83.

312. Dickstein K, Cohen-Solal A, Filippatos G, et al. ESC Guidelines for the Diagnosis And Treatment of Acute And Chronic Heart Failure 2008 The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Rev Esp Cardiol (Engl Ed) 2008;61:1329.

313. Jentzsch AM, Bachmann H, Furst P, Biesalski HK. Improved analysis of malondialdehyde in human body fluids. Free Radic Biol Med 1996;20:251-6.

314. Prior RL, Hoang H, Gu L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. J Agric Food Chem 2003;51:3273-9.

315. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 2002;50:4437-44.

316. Aebi H. Catalase in vitro. Methods in enzymology 1984;105:121-6.

317. Dalloz F, Maingon P, Cottin Y, Briot F, Horiot JC, Rochette L. Effects of combined irradiation and doxorubicin treatment on cardiac function and antioxidant defenses in the rat. Free Radic Biol Med 1999;26:785-800.

318. Heath D, Shaba J, Williams A, Smith P, Kombe A. A pulmonary hypertension-producing plant from Tanzania. Thorax 1975;30:399-404.

319. Kay JM, Harris P, Heath D. Pulmonary hypertension produced in rats by ingestion of Crotalaria spectabilis seeds. Thorax 1967;22:176-9.

320. Kay JM, Heath D, Smith P, Bras G, Summerell J. Hypertensive pulmonary vascular disease produced in rats by administration of the pyrrolizidine alkaloid fulvine. J Pathol 1971;103:P3.

321. Kay JM. Dietary pulmonary hypertension. Thorax 1994;49 Suppl:S33-8.

322. Chesney CF, Allen JR. Monocrotaline induced pulmonary vascular lesions in non-human primates. Cardiovascular research 1973;7:508-18.

323. Chesney CF, Allen JR. Animal model: pulmonary hypertension, cor pulmonale and endocardial fibroelastosis in monocrotaline-intoxicated nonhuman primates. Am J Pathol 1973;70:489-92.

324. 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:L363-9.

325. Shah M, Patel K, Sehgal PB. Monocrotaline pyrrole-induced endothelial cell megalocytosis involves a Golgi blockade mechanism. Am J Physiol Cell Physiol 2005;288:C850-62.

326. Hessel MH, Steendijk P, den Adel B, Schutte CI, van der Laarse A. Characterization of right ventricular function after monocrotaline-induced pulmonary hypertension in the intact rat. Am J Physiol Heart Circ Physiol 2006;291:H2424-30.

327. Le Pavec J, Perros F, Eddahibi S, et al. Cirrhosis ameliorates monocrotaline-induced pulmonary hypertension in rats. Eur Respir J 2009;34:731-9.

328. Maruyama J, Maruyama K, Mitani Y, Kitabatake M, Yamauchi T, Miyasaka K. Continuous low-dose NO inhalation does not prevent monocrotaline-induced pulmonary hypertension in rats. Am J Physiol 1997;272:H517-24.

329. Kosanovic D, Kojonazarov B, Luitel H, et al. Therapeutic efficacy of TBC3711 in monocrotaline-induced pulmonary hypertension. Respir Res 2011;12:87.

330. McGivern RF, Henschel D, Hutcheson M, Pangburn T. Sex difference in daily water consumption of rats: effect of housing and hormones. Physiol Behav 1996;59:653-8.

331. Preston IR, Tang G, Tilan JU, Hill NS, Suzuki YJ. Retinoids and pulmonary hypertension. Circulation 2005;111:782-90.

332. Ghasemzadeh N, Patel RS, Eapen DJ, et al. Oxidative stress is associated with increased pulmonary artery systolic pressure in humans. Hypertension 2014;63:1270-5.

333. Maarman G, Lecour S, Butrous G, Thienemann F, Sliwa K. A comprehensive review: the evolution of animal models in pulmonary hypertension research; are we there yet? Pulm Circ 2013;3:739-56.

334. Somers SJ, Frias M, Lacerda L, Opie LH, Lecour S. Interplay between SAFE and RISK pathways in sphingosine-1-phosphate-induced cardioprotection. Cardiovasc Drugs Ther 2012;26:227-37.

335. Stones R, Benoist D, Peckham M, White E. Microtubule proliferation in right ventricular myocytes of rats with monocrotaline-induced pulmonary hypertension. J Mol Cell Cardiol 2013;56:91-6.

336. Hardziyenka M, Campian ME, Verkerk AO, et al. Electrophysiologic remodeling of the left ventricle in pressure overload-induced right ventricular failure. J Am Coll Cardiol 2012;59:2193-202.

337. Akhavein F, St-Michel EJ, Seifert E, Rohlicek CV. Decreased left ventricular function, myocarditis, and coronary arteriolar medial thickening following monocrotaline administration in adult rats. J Appl Physiol (1985) 2007;103:287-95.

338. Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol 2009;47:32-40.

339. Steffen BT, Lees SJ, Booth FW. Anti-TNF treatment reduces rat skeletal muscle wasting in monocrotaline-induced cardiac cachexia. J Appl Physiol (1985) 2008;105:1950-8.

340. Cagnacci A, Arangino S, Angiolucci M, Maschio E, Longu G, Melis GB. Potentially beneficial cardiovascular effects of melatonin administration in women. Journal of pineal research 1997;22:16-9.

341. Cagnacci A, Arangino S, Angiolucci M, Maschio E, Melis GB. Influences of melatonin administration on the circulation of women. Am J Physiol 1998;274:R335-8.

342. Arangino S, Cagnacci A, Angiolucci M, et al. Effects of melatonin on vascular reactivity, catecholamine levels, and blood pressure in healthy men. Am J Cardiol 1999;83:1417-9.

343. Wahnschaffe A, Haedel S, Rodenbeck A, et al. Out of the lab and into the bathroom: evening short-term exposure to conventional light suppresses melatonin and increases alertness perception. Int J Mol Sci 2013;14:2573-89.

344. Xia CM, Shao CH, Xin L, et al. Effects of melatonin on blood pressure in stress-induced hypertension in rats. Clin Exp Pharmacol Physiol 2008;35:1258-64.

345. Pechanova O, Zicha J, Paulis L, et al. The effect of N-acetylcysteine and melatonin in adult spontaneously hypertensive rats with established hypertension. Eur J Pharmacol 2007;561:129-36.

346. Simko F, Pechanova O, Pelouch V, et al. Effect of melatonin, captopril, spironolactone and simvastatin on blood pressure and left ventricular remodelling in spontaneously hypertensive rats. J Hypertens Suppl 2009;27:S5-10.

347. Yeung HM, Hung MW, Fung ML. Melatonin ameliorates calcium homeostasis in myocardial and ischemia-reperfusion injury in chronically hypoxic rats. Journal of pineal research 2008;45:373-82.

348. Venegas C, Garcia JA, Escames G, et al. Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. Journal of pineal research 2012;52:217-27.

349. Simko F, Paulis L. Antifibrotic effect of melatonin--perspective protection in hypertensive heart disease. Int J Cardiol 2013;168:2876-7.

350. Meyrick B, Reid L. Development of pulmonary arterial changes in rats fed Crotalaria spectabilis. Am J Pathol 1979;94:37-50.

351. Ersahin M, Sehirli O, Toklu HZ, et al. Melatonin improves cardiovascular function and ameliorates renal, cardiac and cerebral damage in rats with renovascular hypertension. Journal of pineal research 2009;47:97-106.

352. Poljsak B, Suput D, Milisav I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid Med Cell Longev 2013;2013:956792.

353. Tinkel J, Hassanain H, Khouri SJ. Cardiovascular antioxidant therapy: a review of supplements, pharmacotherapies, and mechanisms. Cardiol Rev 2012;20:77-83.

354. Paulis L, Pechanova O, Zicha J, et al. Melatonin prevents fibrosis but not hypertrophy development in the left ventricle of NG-nitro-L-arginine-methyl ester hypertensive rats. J Hypertens Suppl 2009;27:S11-6.

355. Rocchetti M, Sala L, Rizzetto R, et al. Ranolazine prevents INaL enhancement and blunts myocardial remodelling in a model of pulmonary hypertension. Cardiovascular research 2014.