BIOMARKERS AND HISTOPATHOLOGIC CHANGES IN RATS WITH MONOCROTALINE-INDUCED PULMONARY HYPERTENSION FOLLOWING ADMINISTRATION OF ANTIRETROVIRAL MEDICATIONS

By

ADEKUNLE OLATAYO ADEOTI

MBBS, FMCP, FWACP

Submitted in partial fulfilment of the requirements for the degree of Master of Medical Science in the

Discipline of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

University of KwaZulu-Natal

2020

Supervisor

DR ANAND NADAR (PhD: Physiology)

PREFACE

I, Adekunle Olatayo Adeoti (Student number: 218058908) hereby declare that the thesis/dissertation entitled: *Biomarkers and histopathologic changes in rats with monocrotaline-induced pulmonary hypertension following administration of antiretroviral medications* is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. Where use was made of the work of others, it is duly acknowledged. The experimental studies carried out in this thesis were conducted in the Department of Human Physiology at the University of KwaZulu-Natal (Westville Campus) under the supervision of Dr Anand Nadar and co-supervision of Prof ML Channa.

8th December 2020

Student: Adekunle Olatayo Adeoti — Date

26/04/2021

Supervisor: Dr Anand Nadar — Date

Co – Supervisor: Prof ML Channa — Date

DECLARATION

I, Dr Adekunle Olatayo Adeoti declare as follows:

i. The research reported in this dissertation, except where otherwise indicated is my original work.
ii. This dissertation has not been submitted for any degree or examination at any other university.
iii. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
iv. This dissertation does not contain other persons writing, unless specifically acknowledged as being sourced from other researchers. Where other sources have been quoted, then:
v. Their words have been rewritten but the general information attributed by them has been referenced.
vi. Where their exact words have been used their writing had been placed inside quotation marks and referenced.
vii. Where I have reproduced a publication of which I am a co-author, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.
viii. This dissertation does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged and the source being detailed in the dissertation and the reference sections.

8th December 2020

_____ _____

Signed Date

DEDICATION

I dedicate this work to HIV/AIDS patients especially in Africa where the burden of HIV associated pulmonary hypertension is yet to be fully established.

FUNDING

This project was funded by College of Health Sciences (UKZN) South Africa.

ACKNOWLEDGEMENTS

I am grateful to God, the giver of life and knowledge who enabled me to complete this work.

I am thankful to Dr Anand Nadar for his guidance, encouragement, and understanding throughout the period of study. For the time of interact and practical experience gained in animal work.

I would like to specially acknowledge my co-supervisor Prof ML Channa, for his assistance, and guidance during the programme.

I am indebted to my close friends from Nigeria, Drs Alese and Omodon, whose help were immeasurable during the programme. This thesis would not have been possible without their constant support.

I wish to express my sincere appreciation to the members of our unit especially Kibwe Mwewa who went out of his way to put me through the nitty gritty of the laboratory work and Sibo, Tolu and Dare for their assistance during the work.

I want to say thank you to Mr Simeone Eche, Timi Adu, Akinnuga and Taiye for their support, and contributions to the success of this research project.

I would like to thank my family for their prayers, encouragement, and support especially my beloved wife, Oluwatomi, for her full support during this study.

PUBLICATIONS

The following manuscript have been submitted:

Respiratory Physiology & Neurobiology.

Adeoti AO, Nadar A, Channa ML (2020). Oxidative stress and gene expression of antioxidants enzymes in monocrotaline-induced pulmonary hypertension following the administration of antiretroviral medications in rats. (Under review).

Free Radical Biology and Medicine.

Adeoti AO, Nadar A, Channa ML (2020). Role of Apoptosis signal regulating kinase-1 (ASK-1) in monocrotaline-induced Pulmonary hypertension following administration of antiretroviral medications in rat model. (Under review).

ABBREVIATIONS AND SYMBOLS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
ASK-1	Apoptosis Signal-Regulating Kinase- 1
BNP	Brain Natriuretic Peptide
CRP	C-reactive protein
°C	Degrees Celsius
DCs	Dendritic Cells
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
Fig	Figure
g	Grams
gp	Glycoprotein
HIV	Human immunodeficiency virus
hrs	Hours
InSTIs	Integrase Strand Transfer Inhibitors
IPAH	Idiopathic pulmonary arterial hypertension
KZN	KwaZulu-Natal
LV	Left ventricle/left ventricular

MCT	Monocrotaline
MCTP	Dehydromonocrotaline
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NO	Nitric oxide
NRTIs	Nucleoside reverse transcriptase inhibitors
ns	Not significant
PA	Pulmonary artery
PAEC	Pulmonary artery endothelial cell
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PAP	Pulmonary arterial pressure
PCR	Polymerase chain reaction
PH	Pulmonary hypertension
PIs	Protease Inhibitors
PVR	Pulmonary vascular resistance
RHC	Right Heart Catherization
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RV	Right ventricle/right ventricular
SMC	Smooth muscle cell
Tat	Trans-activator of transcription

TNF	Tumour Necrosis Factor
WHO	World Health Organization
WSPW	World Symposium on Pulmonary Hypertension
ZDV	Zidovudine

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Pulmonary hypertension (PH) is a progressive life-threatening vasculopathy characterized by dysregulated pulmonary vascular remodelling that results in an increased pulmonary vascular resistance, right ventricular hypertrophy, right heart failure and untimely death. Human Immunodeficiency virus (HIV) is a recognized cause of PH with a relatively stable prevalence of HIV associated PH of 0.5% in most developed countries. One of the animal models of PH is comprises a once off monocrotaline (MCT) in rats, which leads to PH that mimics typical PH presentation observed in humans. Early administration of antiretroviral medication has been shown to prevent the development of PH in human subjects, however, in advanced cases no significant improvement was reported. The impact of antiretroviral medications is controversial; however, nucleoside reverse transcriptase inhibitors (NRTI) and protease inhibitors (PI) have been shown to improve outcome in PH animal models. A potential connection between combination antiretroviral and PH in human subjects has been established which was contrary the protective effects of solely administer NRTI. The study was conducted to test the hypothesis that antiretroviral medications could ameliorate MCT induced PH in rat models and identify potential biomarker for PH.

An approval was given by the Animal Research Ethics Committee of the institution (AREC/066/018M) of University of KwaZulu-Natal, Durban, South Africa, to conduct the study. Forty adult male Sprague-Dawley rats (body weight: 200-250 g) were randomly divided into five groups (n=8 per group). The treatment groups received a single intraperitoneal injection of MCT (60 mg kg^{-1}) while the control group received an equivalent volume of intraperitoneal saline injection. Zidovudine (100 mg kg^{-1}), ritonavir (30 mg kg^{-1}), or combination of both drugs (zidovudine 100 mg kg^{-1} and ritonavir 30 mg kg^{-1}) were administrated daily for the study period of 28 days to the rats in three of the four groups with MCT for 28 days respectively. On the twenty-eighth day of the study, rats were sacrificed, and the harvested lungs and hearts organ were analyzed. Gene expression was conducted using RT-PCR for the antioxidant's enzymes, ASK-1 and a laboratory assay for lipid peroxidation was performed.

A significantly higher mRNA gene expression of catalase, superoxide dismutase, and glutathione peroxidase in the heart tissue of the antiretroviral treated rats was observed and compared to the untreated groups. There was an increase in malondialdehyde (MDA) in the heart tissues of untreated rats (37.01±1.16 nmol/g, $p<0.0001$) compared to the control group (3.46±0.97 nmol/g) with an associated reduction in MDA by the antiretrovirals. Furthermore, an increase in the total antioxidant

capacity (TAC) in AZT (0.85±0.02 nmol/g, p<0.0001), RTV (0.63±0.03 nmol/g, p<0.0001) and combination of AZT/RTV (0.77±0.06 nmol/g, p<0.0001) compared to untreated (0.28±0.025) rats.

Furthermore, lower relative mRNA gene expression of ASK-1 was observed in the heart of the treated rats with zidovudine (2.67 ± 0.09, p < 0.0001), ritonavir (2.57 ±0.11, p < 0.0001) and a combination of both (2.75 ± 0.06, p < 0.0001) when compared to rats in the untreated group. An overexpressed mRNA gene of ASK-1 in the untreated rats (12.0 ± 0.90, p < 0.0001) when compared to the control.

This study shows evidence that zidovudine and ritonavir ameliorate MCT-induced PH in rats by suppressing oxidative stress. Also, ASK-1 is a potential biomarker for anti-apoptotic characteristics of PH. Our findings indicate the antioxidative role of antiretroviral medications in PH and the role of biomarkers in PH.

Keywords: Pulmonary Hypertension, Oxidative stress, Reactive oxygen species, Monocrotaline, ASK-1

UKUQALA

I-Pulmonary hypertension iyi-vasculopathy esongela impilo eqhubekayo ebonakala ngokuqina kokuqina kwemithambo yemithambo yegazi ephumela ekuphikeni kokuqina kwemithambo ye-pulmonary vascular, hypertrophy yesibindi, ukungaphumeleli kwenhliziyo nokuhluleka kokufa okungazelelwe. Igciwane lengculazi i-Human Immunodeficiency virus (i-HIV) liyimbangela eyaziwayo yomfutho we-pulmonary hypertension (PH) ngokuqina okuzinzile kwe-PH ehlobene ne-HIV ka-0.5% emazweni amaningi athuthukile. Imodeli yezilwane ye-PH yathuthukiswa ngokufaka amagundane ku-monocrotaline (MCT) okuvela obala okulingisa okutholakele okujwayelekile kubantu. Umthelela wemithi yezidambisigciwane uyimpikiswano; noma kunjalo, i-nucleoside reverse transcriptase inhibitors (NRTI) ne-proteinase inhibitors (PI) ikhonjisiwe ukuthuthukisa umphumela kumamodeli wezilwane. Ucwaningo lwenziwe ukuze kuvivinywe ukuthi imishanguzo yegciwane lengculaza ingakhulisa i-MCT ingene kumodeli yama-rat futhi ikhombe ongaba ngumthengisi we-biomarker we-PH.

Imvume yanikezwa yiKomidi Lezimilo Zokucwaninga Ngezilwane lesikhungo (AREC / 066 / 018M) saseNyuvesi yaKwaZulu-Natali (UKZN), eThekwini, South Africa, ukuthi lenze izifundo. Amagundane angamashumi amane amaduna akwaSprague-Dawley (isisindo somzimba: 200-250 g) ahlukaniswe ngezikhathi ngamaqembu amahlanu (n = 8 ngeqembu ngalinye). Amaqembu okwelashwa athole umjovo owodwa we-intraperitoneal we-MCT (60 mg Kg-1) ngenkathi iqembu elilawulayo lithola umthamo olinganayo womjovo we-saline we-intraperitoneal. I-Zidovudine (100mg Kg-1), i-ritonavir (30mg. Kg-1), noma inhlanganisela yazo zombili lezi zidakamizwa (i-zidovudine 100mg. Kg-1 kanye ne-ritonavir 30mg. Kg-1) yayilawulwa nsuku zonke esikhathini sokufunda sezinsuku ezingama-28 kuya amagundane ngamaqembu amathathu kulawa amane ane-MCT izinsuku ezingama-28 ngokulandelana. Ngosuku lwamashumi amabili nesishiyagalombili locwaningo, kwenziwa imihlatshelo, kwahlolwa amasitho. Isisho se-Gene sisebenzisa i-RT-PCR yama-antioxidants, ASK-1 kanye ne-laby assay ye-lipid peroxidation kwahlaziywa.

Inkulumo ephakeme kakhulu ye-mRNA gene ye-catalase, superoxide dismutase, kanye ne-glutathione peroxidase kumagundane aphathisiwe yabonwa ngokuqhathaniswa nokungalandelwanga. Kwakukhona ukwanda kwe-malondialdehyde (MDA) izicubu zenhliziyo zamagundwane angahlushwanga (37.01 ± 1.16nmol / g, p <0.0001) ngokuqhathaniswa neqembu lokulawula (3.46 ± 0.97 nmol / g) nokwehliswa okuhlobene kwe-MDA ngama-antiretrovirals . Ngaphezu kwalokho, ukukhuphuka kwenani eliphelele le-antioxidant umthamo (TAC) ku-AZT

(0.85 ± 0.02 nmol / g, p <0.0001), RTV (0.627 ± 0.03 nmol / g, p <0.0001) kanye nokuhlanganiswa kwe-AZT / RTV (0.765 ± 0.06 nmol / g, p <0.0001) ngokuqhathaniswa namagundane angaphendulwanga (0.28 ± 0.03).

Ngaphezu kwalokho, ukuveza okungaphansi kwe-mRNA uhlobo lwe-ASK-1 enhliziyweni yamagundane aphathwe nge-zidovudine (2.67 ± 0.09, p <0.0001), ritonavir (2,57 ± 0.11, p <0.0001) kanye nenhlanganisela yomibili (2.75 ± 0.06, p. <0.0001) uma uyiqhathanisa namagundane eqenjini elingalwanga. Uhlobo lwe-mRNA olu-overexpressed lwe-ASK-1 kumagundane angalwanga (12.0 ± 0.90, p <0.0001) uma luqhathaniswa nolawulo.

Lolu cwaningo lukhombisa ubufakazi bokuthi i-zidovudine ne-ritonavir ameliorate MCT-indised PH kumagundane ngokucindezela ukucindezela kwe-oxidative. Futhi, i-ASK-1 iyi-biomarker eyiqiniso yezimpawu zokulwa ne-apoptotic ze-PH. Ukuthola kwethu kuzothuthukisa uphenyo olusha ngendima yabanye ababekade bengabanikazi bezokwelapha ku-PH kanye nezinzuzo zokwelapha ezingaba khona zemithi yezidambisigciwane ekuvimbeleni i-PH.

Amagama angukhiye: I-Pulmonary Hypertension, ingcindezi ye-Oxidative, Izinhlobo ze-oxygen ezisebenzayo, iMonocrotaline, ASK-

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Pulmonary hypertension (PH) is a complex, progressive and life-threatening vasculopathy characterized by increased pulmonary vascular resistance and elevated pulmonary arterial pressure resulting in right ventricular hypertrophy and failure (Hoeper et al., 2013; Hadri et al., 2013).

In the 1960s, a sudden rise in PH cases was observed in some European countries which was attributed to the use of anorectic stimulant, aminorex for the treatment of obesity. This prompted the first World Symposium on PH (WSPH) in October 1973, which was organised by the World Health Organisation (WHO). Subsequently, the periodic symposia, for regular review of the classification, definitions and current research in PH with the view of improving the patients' management outcome (Condon et al., 2019).

Pulmonary hypertension is a major global health issue that interfaces both communicable and non-communicable diseases. Globally, left-sided heart failure is the leading cause of PH, probably affecting 5–10% of individuals aged 65 years or older. However, HIV infection, schistosomiasis, post-streptococcal rheumatic heart disease, and sickle cell disease, which are often endemic in sub-Saharan Africa, are frequent causes of PH, which are given less attention, in these countries (Hoeper et al., 2016). With the recent advancements in this field, and the development of new drugs, there has been increased survival and improved quality of life in individuals living with PH however, no therapy currently provides a cure (Studer and Gilkin, 2014).

Despite the progress in the subject, there is an obvious gap in the definition, understanding of the pathogenesis, treatment options, and late diagnosis. The adopted definition from the first WSPH in 1973, regarded PH as a mean pulmonary arterial pressure (mPAP) $\geqslant$25 mmHg at rest, measured by right heart catheterisation which is the gold standard for diagnosis (Simonneau et al., 2019). In the definition of PH during exercise, an arbitrary figure of mPAP >30 mmHg was earlier used, but currently under review, as it is not supported by published data and healthy individuals were found to have much higher values than this predetermined value (Galie et al., 2009). However, the latest review at the 6th WSPH, February 2018, the task force proposed to include pulmonary vascular resistance $\geqslant$3 Wood units in the definition of all forms of pre-capillary PH associated with mPAP >20 mmHg but exercise PH was not considered by the task force for the lack of convincing data (Galie et al., 2019).

Over the decades, preclinical/translational studies have played a major role in advancing the understanding of the pathogenesis and therapeutics in PH. Emphasis has been placed on one of the several pathogenetic mechanisms which underpins PH and these include, pulmonary

vasoconstriction, abnormal remodelling, and exaggerated perivascular infiltration of inflammatory cells. However, highlighting the role of vasoconstriction has been the rationale for the development of most currently available treatment modalities which focuses on vasodilation, with little or no attention on the therapeutic significance of the other pathogenetic mechanisms of the disease (Gamen et al., 2016).

1.1.1 Epidemiology

Pulmonary hypertension is a challenging health issue with an estimated global prevalence of one percent in the general population. It is widely distributed across the different age groups but the highest prevalence of about 10 percent is seen in the geriatric age group. Currently, there are limited information on the burden of PH in developing countries, however an estimated 80 percent of the 75 million people who suffer from PH are domicile in the developing countries (Maarman et al., 2020). These are mostly low-middle income countries where the burden of PH related to infectious diseases is high and likewise experiencing the epidemiologic transition in the burden of non-communicable diseases similarly responsible for PH in such regions (Hoeper et al., 2016; Borges, 2017).

The variation in the prevalence of PH has observed by the differences in the study methodologies and geographic distribution of PH. The discrepancy is further expressed in the non-uniformity in the definition, and assessment of PH especially in the utilization of RHC which is the standard for diagnosis. For instance, a Brazilian study reported schistosomiasis as a major of cause of PH due to the high burden of schistosomiasis and likewise in some regions in Africa like Egypt. This has been recognized by the WHO as a neglected and endemic cause of PH in developing countries (Piscoya Roncal et al., 2019; Farrag et al., 2012). On the contrary, the European and North American registries have reported connective tissue diseases as the most common cause of PH which is often due to scleroderma (Prins and Thenappan, 2016).

A subgroup of PH is known as pulmonary arterial hypertension (PAH) is related to autoimmune diseases. The incidence of PH, in the developed world varies from 1·1–7·6 per million adults per year while the prevalence varies from 6·6–26·0 per million adult population respectively in the developed world (Hoeper et al., 2016). While the younger age groups are more likely to be affected by PH, and mostly seen in females, particularly in the heritable PAH affecting twice as many females as males before the age of 50 years. The global registry data on PH also supports this female preponderance (2–4 times) of PH over males (Örem, 2017).

In experimental animal models, female animals are protected from the development of PH by the effects of oestrogen in a phenomenon known as the “oestrogen paradox” or “sexual dimorphism”

(Huber et al., 2015). This finding in animals suggest oestrogen to be strongly protective against the development of PH which is contrary to the high occurrence of PH in human subjects. Researchers have shown oestrogen and oestrogen receptor-based therapies to be necessary to ameliorate established pulmonary hypertension in animals but this role is yet to be defined in humans (Umar et al., 2012).

Mutations in the bone morphogenetic protein receptor 2 gene (BMPR2) are associated with familial PH in about 70% of cases and could be sporadic in 11–40% of cases. While genetic predisposition accounts for only 1–5% of PH cases, autoimmune diseases and infections are also closely linked to PAH as possible triggers for the expression in those genetically predisposed (Batton et al., 2018). BMPR2 is a member of the transforming growth factor-beta (TGF-β) receptor superfamily which consist of mediators that regulate cellular functions, like differentiation, apoptosis, proliferation, migration, extracellular matrix secretion and deposition etc (Thenappan et al., 2018).

The racial distribution of PH was explored in the Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL) study, where the patient distribution was 72.8 %, 12.2 %, 8.9 %, 3.3 % and 2.8 % in the Caucasians, African Americans, Hispanics, Asians or Pacific Islanders, and other or unknown groups respectively (Medrek and Sahay, 2018).Although the racial proportion was similar to the US population registry among the Caucasian group, there is a slight variation in the percentages in the other groups, as there was an overrepresentation of African Americans (12.2% vs. 10.9%) and underrepresentation of Hispanics (8.9% vs. 11.5%), and Asians/pacific islander (3.3% vs. 4.3%) in this study (Prins and Thenappan, 2016; Elliott et al., 2019).

Several of the risk factors for PH are hyperendemic in sub-Saharan Africa, among them are HIV infection, rheumatic heart disease, hereditary hemoglobinopathies, schistosomiasis, tuberculosis and chronic obstructive pulmonary diseases. Despite the recent global awareness on PH, the epidemiology in Africa still remains poorly described and yet to be given the deserved attention (Bigna et al., 2017).

HIV/AIDS is characterized by a 2500-fold increase risk of developing PH when compared to the general population and the survival of HIV associated PH (HIV-PH) patients is fifty percent (Zuber et al., 2004). In human subjects, HIV-PH has an average age at presentation of 33 years with slight female predominance (Bloomfield et al., 2014). This prevalence of HIV-PH has remained stable in the developed countries ranging from the initial 0.5% in the Swiss cohort survey of 1200 intravenous drug users who were HIV patients and 0.46% in a prospective cohort study of 7648 HIV-infected individuals in France (Barnett and Hsue, 2013). However, with the huge burden of HIV/AIDS in sub-Saharan Africa, where it constitutes about 70% of global HIV

burden, a critical review of this challenge in this region is necessary to establish the burden of HIV-PH. There are limited studies conducted in Africa on this subject, however, the prevalence of PH among HIV infected people in the available studies varies from 5 to 13 %, which is higher than the reported values in the developed countries (Cicalini et al., 2011; Janda et al., 2010). The reported higher prevalence of HIV-PH in Africa may be connected to the differences in the available diagnostic tools, as the majority of these prevalence studies were determined on the basis of echocardiographic findings without RHC confirmation (Henriques-Forsythe et al., 2015).

1.1.2 Classification

PH is a heterogenous group of diseases classified based on pathophysiology, clinical presentation, and therapeutic conditions into five categories, by the World Health Organisation, viz-a-viz PAH (group 1), PH due to left-sided heart disease (group 2), PH due to lung disease or hypoxia (group 3), chronic thromboembolic PH (CTEPH) and other pulmonary artery obstructions (group 4), and PH with unclear and/or multifactorial mechanisms (group 5) (Simonneau et al., 2019).

Group 1 PH consists of idiopathic, heritable, drug and toxin-induced, HIV associated and connective tissue disease, portal hypertension, and schistosomiasis. In this group, the hereditary PAH and idiopathic PAH are also described, which are linked to mutation in genes like the bone morphogenetic protein receptor 2 gene (BMPR2). This group is selectively known as the PAH, as all have same characteristics features on histology. Most the available therapeutic options are focused on PAH which remains treatable disease but still has high mortality (Condon et al., 2019)

Group 2 PH is attributed to left-sided heart disease, comprising heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF), as well asmitral or aortic valve disease, or congenital/acquired left inflow or outflow tract obstruction andcongenital cardiomyopathies (Goldberg et al., 2017).

Group 3 PH is caused by chronic lung disease and/or hypoxia. It is most commonly associated with chronic obstructive pulmonary disease (COPD), parenchymal lung disease and sleep-disordered breathing (Heresi et al., 2017).

Group 4 is classified as CTEPH, which often results from abnormal clotting cascade, dysfunctional endothelial cells or platelets with organised fibrotic material resulting in the clot formation within the pulmonary vasculature either as residual pulmonary thromboembolism (PTE) or in situ thrombosis causing mechanical obstruction of pulmonary arteries (Hughes et al., 2006).

Group 5 refers to PH with unclear and/or multifactorial mechanisms. This group has represented less-studied forms of PH, with likely multiple pathophysiological phenomena involved in this process, however, many of the PH forms currently in group 5 represent a significant part of the yet unrecognised worldwide burden of PH (Simonneau et al., 2019).

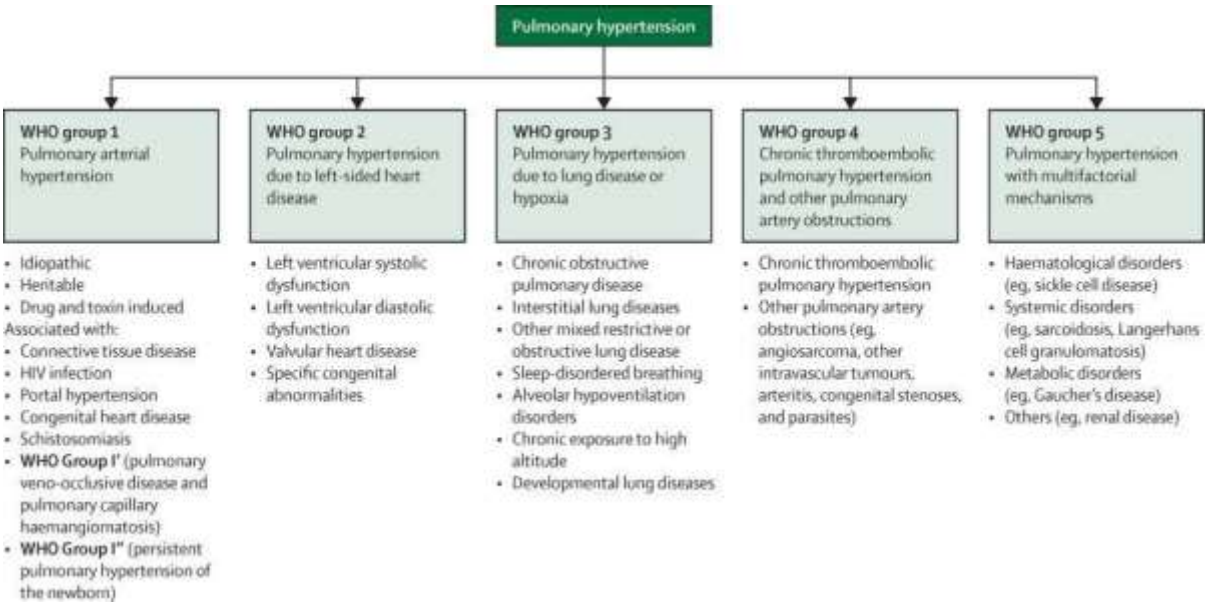

Figure 1: Adapted from a global view of PH. (Hoeper et al., 2016.)

1.1.3 Pathogenesis

The pathogenesis of PH is complex and multifactorial, characterised by the triad of excessive vasoconstriction, microthrombosis and remodelling of pulmonary arteries. Despite the vasoconstrictive process being the most studied while the remodelling is arguably the most important factor, there are complex entities in the pathogenesis that is still poorly understood (Huber et al., 2015). A heterogenous process of endothelial vascular injury results in a chaotic endothelial proliferation and haphazard endothelial cellular migration to all pulmonary arteriolar layers (Humbert et al., 2004). This vascular remodelling of pulmonary arteries which encompasses concentric medial thickening of small arterioles, neomuscularization of previously non-muscular capillary-like vessels, and structural wall thickness in larger pulmonary arteries is characterized by vascular smooth muscle cell (SMC) hyperplasia and hypertrophy (Aggarwal et al., 2013).

The most emphasized of the several proposed mechanisms for the observed pathogenesis of PH is the vasoconstrictive changes which is hinged on three processes that prevent pulmonary vasodilation. The three key signalling pathways outlined affect nitric oxide (NO), prostacyclin (PGI_2) and endothelin-1 (ET-1) (Lan et al., 2018). There is a decrease in the bioavailability of

NO from an enzymatic uncoupling of endothelial nitric oxide synthase (eNOS) resulting in the reduction in NO synthesis. This manifests as pulmonary vasoconstriction and increased smooth muscle cell proliferation, inflammation and thrombosis (Alp and Channon, 2004). Likewise, in the prostacyclin (PGI_2) pathway, PGI_2 produced by the endothelial cells binds to specific receptor known as the I-prostanoid (IP) receptors in the vascular smooth muscle cells which activates smooth muscle relaxation and vasodilation by cyclic AMP generation from ATP. In addition, PGI_2 prevents platelet aggregation and smooth muscle proliferation. This receptor blockage has been demonstrated in IP-knockout mice with subsequent vascular remodelling with exposure to chronic hypoxia resulting in severe PH and also demonstrated in humans (Hoshikawa et al., 2001). In the endothelin-1 (ET-1) pathway, ET-1 which is a potent vasoconstrictor acts on ET_A and ET_B receptors and the resultant PH. An increased plasma and pulmonary vascular endothelial concentrations of ET-1 in cells is responsible for the vasoconstriction (Giaid et al., 1993).

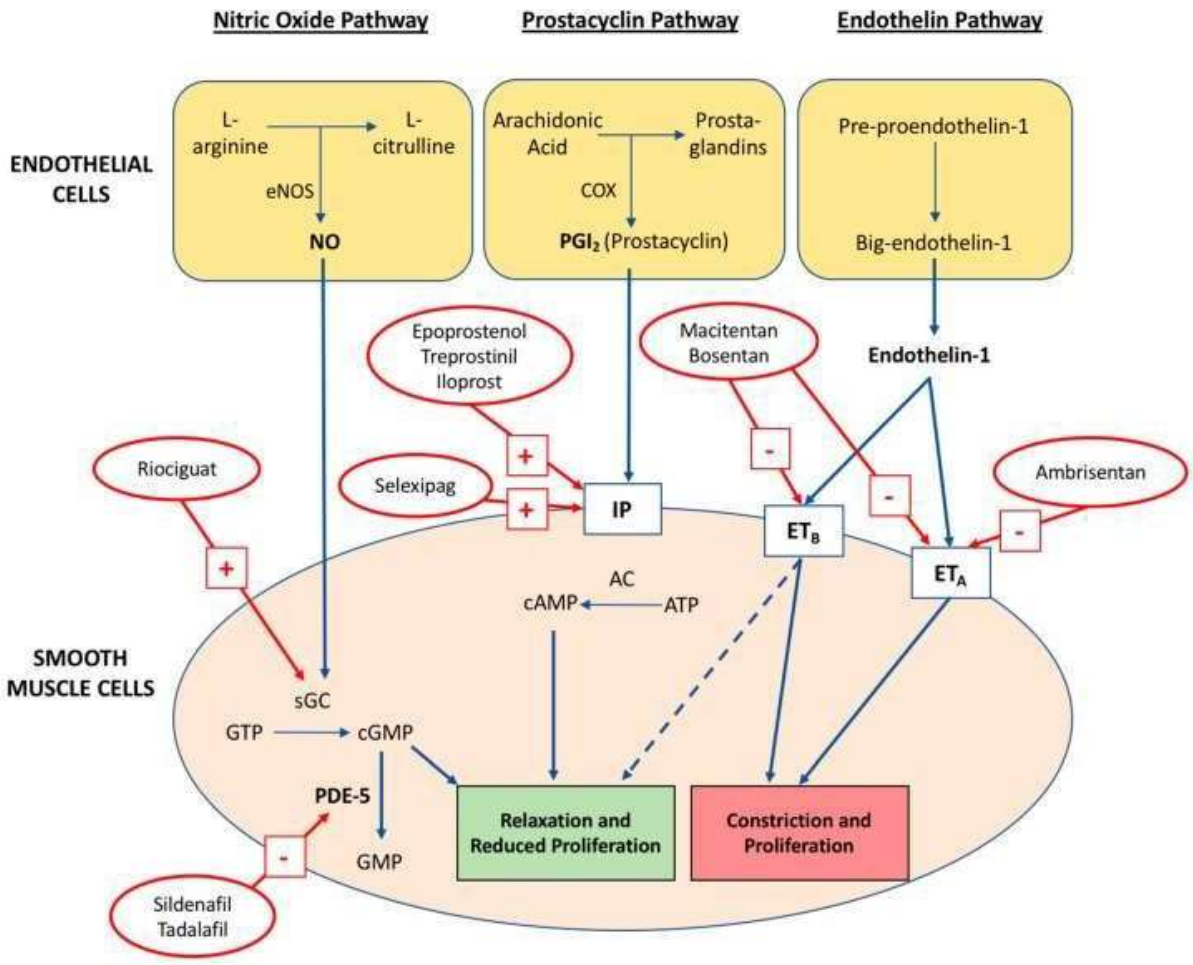

Figure 2: Adapted from the key abnormal pathways targeted in the pharmacological treatment of PH and the mechanism of action for contemporary drugs. (Lan et al., 2018)

With the remodelling, there is an imbalance in the pro-proliferative and apoptotic cellular activities within the vessels, which is triggered by BMPR2. Therefore, the mutation in BMPR2 on chromosome 2q33 alters the cellular processes and determine the developmental fate during embryogenesis. This predisposes the mutant carrier to develop PH in the presence of a favourable exogenous stimuli (Said, 2006).

In addition, inflammation is an emerging concept in the pathogenesis of PH. Several inflammatory cells are shown to infiltrate the pulmonary vasculature in response to infectious agents and injurious endothelial damage (Huber et al., 2015). This has been demonstrated in humans and animal models where inflammatory cells including lymphocytes, macrophages, dendritic cells and mast cells invade the pulmonary vasculature. Similarly, infectious diseases and related diseases like HIV/AIDS, systemic sclerosis and systemic lupus erythematosus have exhibited qualitative and quantitative abnormalities in their T-regulatory cells. Despite this available body of knowledge, researchers are yet to fully explore drugs with anti-inflammatory properties or design medications to specifically target inflammatory processes (Hemnes and Humbert, 2017). This might have played a significant role in the steady prevalence of HIV-PH as antiretroviral medications have demonstrated effects on viral antigens in vascular changes among HIV patients (Szturmowicz et al., 2015). In rat model of PH, increased IL-6 has been associated with the development of PH which was reversed by an early administration of anti-inflammatory drugs (dexamethasone) as well as attenuation of the PH developmental process (Bhargava et al., 1999). In addition, monitoring of the inflammatory process, using an acute phase reactant like C-reactive protein (CRP) has been reported as a predictor of outcome and response to therapy in PH. Further evidence suggests that this inflammatory mechanism could potentially be the target for management of remodelling in PH (Sydykov et al., 2018).

Furthermore, enhanced production and dysregulated ROS/RNS signalling has been implicated in the pathogenesis of PH. This is evident by the elevation in the ROS and direct mop-up of NOby superoxide resulting in a disequilibrium in ROS/RNS. This further encourages pro- proliferation, pro-survival phenotypes and DNA damage in the pulmonary vascular (Jernigan etal., 2004; Ranchoux et al., 2016). Gratton et *al* found that the administration of a protease inhibitor (antiretroviral medications) in a cell line, significantly increased tROS production, andapoptosis (Gratton et al., 2018). Furthermore, ROS causes contraction of pulmonary vessels which is mediated by hypoxia, and the release of RNS from the endothelium of blood vessels (Fulton et al., 2017).

1.2 Animal models of pulmonary hypertension

Animal models were developed to study the functional and structural changes in PH due to the limited human tissue samples. However, no single preclinical model is a flawless replica of the PH presentation in humans. In the bid to unravel this fatal condition, researchers have employed experimental animal models to execute mechanistic studies, and evaluate potential therapeutic options (Huetsch et al., 2016).

The most extensively used animal models of PH have been chronic hypoxia and the MCT-induced PH rodent models (Nogueira-Ferreira et al., 2015). These models have complemented the understanding of the mechanisms of pulmonary vascular remodelling in PH but are limited by the lack of the typical complex vascular neointimal lesions and severe disease phenotype seen in PAH (Dickinson et al., 2013). Although, widely used, it has been characterized predominantly by media hypertrophy of the pulmonary vessels but lack the vaso-occlusive and plexiform lesions that are the hallmark of severe human PAH (Lahm et al., 2014). Few animal studies are available in the literature regarding the other categories of PH aside PAH that is widely studied (Breitling et al., 2015).

1.2.1 Monocrotaline-induced pulmonary hypertension

In the discovery by J. M. Kay and his colleagues, powdered *Crotolaria spectabilis* seeds induced PH in rats due to an alkaloid monocrotaline (MCT) (Scupinari et al., 2020; Kay et al., 1967). Mice do not develop PH from MCT due to the deficiency of CYP3A isoenzyme in the mouse liver, required to metabolize MCT to its active metabolite. MCT is an 11-membered macrocyclic pyrrolizidine alkaloid derivative of the *Crotalaria spectabilis* from the seeds of the plant. It is activated to the reactive pyrrole metabolite dehydromonocrotaline (MCTP) in the liver, a reaction that is highly dependent on cytochrome P-450 (CYP3A4) (Gomez-Arroyo et al., 2012). The application of MCT model of PAH has been in existence in the last five decades. This is induced by a single dose injection of either intraperitoneal or subcutaneous administration of MCT into the rat. Following its administration, the circulating MCTP has high affinity for the pulmonary arterial endothelial lining and leads to irreversible cell injury, vascular inflammation, and remodelling (Feng et al., 2017; Barman et al., 2009). In the first two weeks following MCT administration, usually no overt physical disorder may be noticed, however, after three weeks, the animal begins to deteriorate from progressive thickening of the media and muscularization of non-muscularized arteries, along with adventitial proliferation (Santos-Ribeiro et al., 2016).

The mechanisms underlying this progression remain poorly understood, although there has been established endothelial damage following MCT administration. Several researchers have postulated possible explanations like the development megalocytosis by the pulmonary arterial endothelial cells (PAEC), disruptions of intracellular membrane trafficking that affect several cell membrane proteins and the deregulation of nitric oxide signalling, leading to lung vascular changes (Gomez-Arroyo et al., 2012). In addition, MCT also causes hepatic veno-occlusive disease in rats as well as biventricular myocarditis resulting in right ventricular hypertrophy and failure (Sztuka and Jasinska-Stroschein, 2017).

1.2.2 Global Burden of HIV/AIDS

HIV epidemic is a major global health issue. According the WHO, since the onset of this epidemic, over 70 million people have been infected with HIV and close to 35 million people have died as a result of HIV/AIDS. In 2018, approximately 37.9 million people were living with HIV, 1.7 million newly infected people with HIV, 770 000 people have died from AIDS-related illnesses, with about 23.3 million accessing antiretroviral therapy. To further reduce the prevalence of HIV/AIDS, the Joint United Nations Programme on HIV and AIDS (UNAIDS) set a target known as the 90-90-90 in the year 2014 that 90% of all HIV positive people will be diagnosed, 90% of all those diagnosed will be on treatment and 90% of those on treatment will be virally suppressed (Jones et al., 2019; UNAIDS, 2019). The transmission of HIV infection is through several routes like sexual contact, blood transfusions, mother-to-child transmission, intravenous drug use and needle-stick injuries (Jones et al., 2019). Ever since the first case of HIV associated PH was identified in 1987, it has become a well-recognized complication of HIV infection and increased morbidity (Barnett and Hsue, 2013).

1.2.3 Burden of HIV/AIDS in Africa

HIV/AIDS is a major cause of morbidity and mortality in sub-Saharan Africa. This region represents about 12% of the global population yet it harbours 71% of the global burden of HIV infection. Since HIV was first recognized in the 1980s, notable scientific breakthroughs have changed the once fatal condition into manageable one, with the introduction of anti-retroviral therapy (ART). Despite the discovery of ART since the mid-2000s and the resulting decline in mortality, 34% of people in east and southern Africa and 60% of people in west and central Africa living with HIV are currently not receiving any treatment (Colombe et al., 2018; Bain and Gwain, 2019). Sub-Saharan African countries lag behind in HIV treatment cascade, with significant pressure on the limited health care facilities and the concomitant rise in the burden of

non-communicable disease (NCD) which has great implications for ART programmes (Smit et al., 2018). With the enormous disease burden in Africa, Southern Africa is still the most severely affected region in the continent with an estimate of about 11.3 million people living with HIV and AIDS. In 2013, it was estimated that 10% of the total population of South Africa was HIV positive (Mohapi and Pitsoane, 2017).

1.2.4 Pathogenesis of HIV-induced PH

Following the transmission of the virus, the viral entry into the human cells is facilitated by the CD4 receptors: C-C chemokine receptor-5 (CCR5) and C-X-C chemokine receptor-4 (CXCR4). These viruses may hibernate in cells by downregulating the key host receptors and evading the host immune surveillance or commence active replication and reinfection of other cells. There are nine genes that encode HIV-I proteins with three of these genes (gag, pol, and env) found in all retroviruses. While the gag and pol regions encode for the reverse transcriptase and integrase enzymes, the env gene encodes for gp160 which is the precursor for the envelope proteins gp120 and gp41 necessary for the viral cell entry (Porter and Sutliff, 2012).

Although HIV has not been detected in the pulmonary vasculature lesions of patients but the viral proteins are strongly linked to have a cause-effect relationships by promoting apoptosis, growth, and proliferation in the pulmonary vasculature (Almodovar et al., 2010) Several studies have implicated HIV proteins like trans-activator of transcription (Tat), glycoprotein (gp) 120, negative regulator factor (nef) and envelope protein (env) in the development of HIV-PH. However, the occurrence of HIV-PH has no correlation in several studies with the viral load, CD4 count or co-existing opportunistic infections in HIV patients (Almodovar et al., 2011; Henriques-Forsythe et al., 2015).

The role of chemokines and chemokine receptors in PH progression has been elucidated where significant upregulation of the CCR5 receptor was found in PH patients (Amsellem et al., 2014). HIV-Nef, downregulates the CD4 receptor, which stimulates viral replication in primary T-cells. HIV envelope gp-120, which is essential for viral attachment and fusion to the host cellular membrane, also it induces apoptosis and increases the secretion of endothelin-1 from human lung endothelial cells (Barnett and Hsue, 2013). Similarly, HIV env stimulates monocytes and macrophages to release proinflammatory cytokines, while the HIV Tat protein activates endothelial cells and has angiogenic properties. (Almodovar et al., 2010).

1.2.4.1 Pharmacology of antiretroviral drugs

HIV is a retrovirus which causes AIDS. It was co-discovered by Barre-Sinoussi et al. and Gallo et al. about four decades ago and has two strains which are HIV-1 and HIV-2. The HIV-1 strain is widespread but predominant in America, Europe and Asia while HIV-2 represents a significant minority of HIV infections, mostly in West Africa countries, which is less readily transmitted and less pathogenic than HIV-1 (Almodovar, 2014).

HIV produces more than 10 billion virions per day, which is a rapid viral turnover (new generation every 2.6 days) (Almodovar, 2014). The different classes of antiretroviral drugs act by preventing viral replication and thereby reduce the viral load to undetectable level in the blood. In 1986, the first antiretroviral drug zidovudine (ZDV) was introduced, and otherantiretroviral drugs followed. There are currently 26 drugs divided into 6 classes based on their mechanisms of action. The combination of ART in the different groups was adopted to address the development of drug resistance, hence currently three or more ARVs are recommended worldwide for the treatment of people with HIV infection (Arts and Hazuda, 2012).

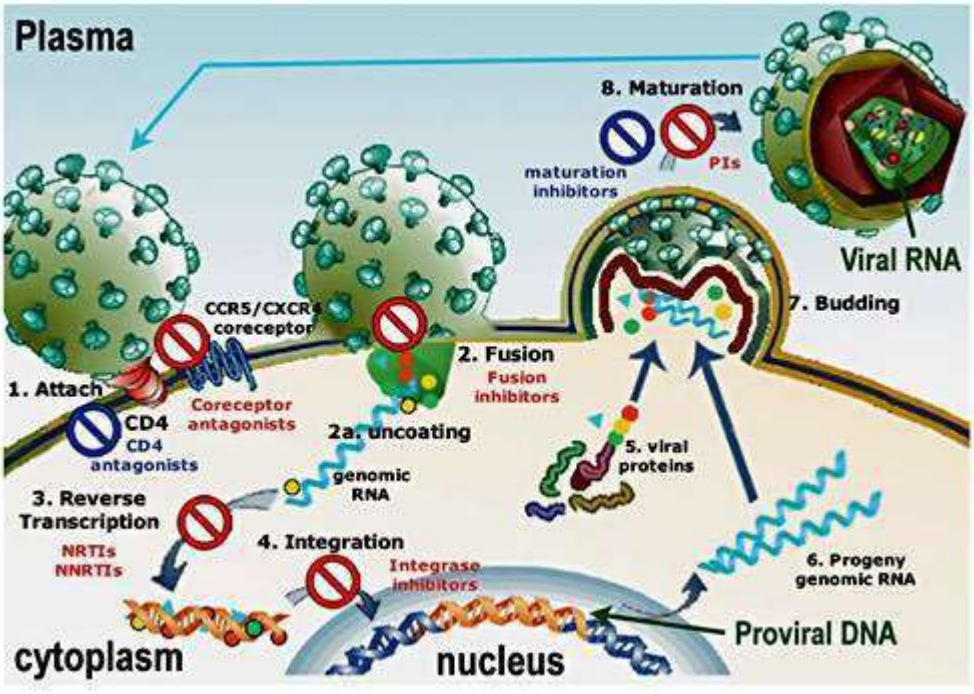

Figure 3: Adapted from HIV-1 life cycle and current targets for antiretroviral therapy (Sierra-Aragon and Walter, 2012).

The first approved class of ARVs was the nucleotide reverse transcriptase inhibitors (NRTIs), which when administered, requires the host cell entry and phosphorylation by cellular kinases

for effective antiviral action. NRTIs lack 30-hydroxyl group at the sugar (20-deoxyribosyl) moiety and requires bonding of the 50-nucleoside triphosphates for the formation of a 30-50-phosphodiester bond for the termination of the growing viral DNA chain. The approved drugs in this category are abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, zidovudine, and Tenofovir disoprovil fumarate (Barroso et al., 2019).

The prototype in the NRTI group is zidovudine. It is a thymidine analogue that inhibits the mitochondrial adenylate kinase and adenosine nucleotide translocator in isolated mitochondria and diminishes oxidative phosphorylation. It causes haematological complications like bone marrow suppression (pancytopenia), malaise, anorexia, nausea, vomiting, lactic acidosis, and loss of limb fat (Rai et al., 2018).

Non-nucleotide reverse transcriptase inhibitors (NNRTIs) are non-competitive inhibitors of reverse transcriptase enzyme. They alter the conformation of the substrate-binding site and reduce polymerase activity after binding with an allosteric non-bonding site of the viral reverse transcriptase. This group is highly specific for the HIV-1 reverse transcriptase (RT), as they do not inhibit the HIV-2. The approved NNRTIs are etravirine, delavirdine, efavirenz, nevirapine, and rilpivirine however delavirdine is no longer used as a component of ART treatments (Famiglini and Silvestri, 2016).

Protease inhibitors (PIs) prevent the cleavage of the viral gag and gag-pol polyprotein precursors during virion maturation. Due to their interaction with GLUT1 and GLUT4 glucose transporters, insulin resistance and hyperglycemia could occur. Approved PIs are amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir (Arts and Hazuda, 2012; Barroso et al., 2019).

Fusion inhibitors bind to gp41 on the viral envelope and prevent peptide fusion inhibitors required for the two homologous domains in the viral gp41 protein interaction. The available drug in this category is enfuvirtide, with local reactions at the injection site being the most common adverse effect. (Barroso et al., 2019).

CCR5 chemokine antagonists bind to hydrophobic pockets within the transmembrane helices of CCR5 and prevent the binding by HIV-1 envelope. CCR5 is a G-protein coupled chemokine receptors determining HIV-1 cellular tropism. The viruses preferentially utilize CCR5 (R5 strains) co-receptor mainly expressed on macrophages for entry into the host cell. Lack of CCR5 receptors in certain individuals make them resistant to HIV. The approved drugs in this category are maraviroc, vicriviroc and aplaviroc (Abrol et al., 2014; Tilton et al., 2010).

Integrase strand transfer inhibitors (InSTIs) prevent HIV replication by inhibiting the integration of HIV DNA to human genome. InSTIs have fewer adverse effects than other classes of antiretroviral agents. They target the strand transfer reaction and interact with two essential

elements of the virus, the integrase enzyme as well as the viral DNA, which is the substrate for integration. Approved drugs are raltegravir, elvitegravir, dolutegravir (Chen et al., 2019).

1.2.4.2 Effects of antiretroviral drugs in PH

There is ambiguity in the literature on the role of ART in HIV-PH, while some reports suggest that ART has no effect on the presence or severity of PH, the French cohort showed that ART improved exercise tolerance and physical performance in PH patients (Humbert et al., 2006) . In animal models, protease inhibitors have been shown to significantly reverse hypoxia-induced PH (Barnett and Hsue, 2013; Degano et al., 2009).

Chronic inflammation is considered as an important component of PH that contributes to the structural pulmonary-vessel remodelling (Amsellem et al., 2014). Hence, corticosteroids and immunosuppressive therapy were found to reduce the manifestations in severe PH which further emphasizes the critical role of inflammation in this condition (Morris et al., 2012).

Some studies have reported the efficacy of ART in decreasing morbidity and mortality in patients with HIV-PH (Jarrett and Barnett, 2017). On the contrary, a retrospective study found a higher risk of PH in patients treated with protease inhibitor compared to patients treated with nucleoside reverse transcriptase inhibitors (Dhadwal et al., 2009). This finding may be due to the fact that protease inhibitors (PI) have been associated with several cardiovascular risk factors like hyperlipidemia, lipodystrophy and insulin resistance which correlation with endothelial dysfunction (Stein et al., 2001). However, in an animal model, first generation PIs partially prevented the development of hypoxia and monocrotaline-induced PH. PIs inhibit aspartyl protease required for HIV viral replication. The growth-inhibiting and insulin-resistance-inducing effects of PIs ameliorate pulmonary artery smooth muscle proliferation which supports the pulmonary vascular remodelling as a possible mechanism for the PH (Gary-Bobo et al., 2010). There is a gap in the literature on the effects of oxidative stress in the development of PH following the administration of ARVs.

1.2.4.3 Novel Biomarkers

PH is a multifactorial and heterogeneous disorder with varying aetiopathogenesis. Therefore, the search for minimally invasive, high specificity and sensitivity, biomarkers for the purpose of diagnosis, prognosis, and treatment response would be ideal in the management of PH. However, currently, there are no known available single marker for accurate diagnosis or monitoring of disease progression in PH (Huetsch et al., 2016).

1.2.4.4 B-Type natriuretic peptide (BNP)

BNP was first isolated from the porcine brain in 1988, but it was later found to originate from the ventricular myocardium. The synthesized and secreted BNP is a prehormone of 108 amino acid which cleaves to the active 32 amino acid BNP and its inactive 76 amino acid N-terminal fragment (Weber and Hamm, 2006).The release of this hormone is usually triggered by myocardial wall stress, ischemia or infarction resulting in heart failure (Omland et al., 2007). BNP is stable at room temperature in whole blood with the addition of EDTA as anticoagulant for at least 24 hours, whereas NT-proBNP is stable for at least 72 hours. However, both BNP and NT-proBNP are stable during freezing and thaw processes (Weber and Hamm, 2006).

The usefulness of BNP has been demonstrated in right ventricular dysfunction, prediction of elevated pulmonary artery pressure and prognostication of outcome of the PH (Goto et al., 2010). Its role as a potent pulmonary vasodilator and anti-hypertrophic agent has been explored in the inhibition of pulmonary vascular remodelling following maladaptive changes in the ventricular myocardium (Casserly and Klinger, 2009). Leuchte et al demonstrated the association between this non-invasive blood test and pulmonary hemodynamics as well as the negative correlation with 6 minutes exercise test in human subjects. BNP remains the most widely used biomarker in clinical practice for prognosticating at diagnosis and monitoring PH progression (Leuchte et al., 2004)..

1.2.4.5 Apoptosis signal-regulating kinase-1 (ASK-1)

ASK-1 is a signal pathway activated in response to oxidative stress that stimulate apoptosis, inflammation, and fibrosis within pulmonary vascular and right ventricular remodelling in PH. Its inhibition prevents pathological vascular remodelling and terminates the progression of PH in a rat model (Budas et al., 2018). Furthermore, the regulation of ASK-1 has been linked to cardiac hypertrophy, cardiac remodelling, atherosclerosis and systemic hypertension (Liu et al., 2017). ASK-1 is also required for the execution of reactive oxygen species (ROS)-induced apoptosis essentially through mitochondria-dependent caspase activation. Hayakawa et al. reported the protective role of ASK-1 in HIV infection through its downregulation following its interaction with the viral nef protein which enables apoptosis of surrounding T-cells (Hayakawa et al., 2012).

1.2.4.6 Reactive Oxygen Species

ROS play a significant role in vascular dysfunction which accounts for PH (Adesina et al., 2015). ROS are naturally formed free radicals which are by-products of oxygen metabolism. They include hydroxyl, superoxide, peroxyl and non-radical species, such as hydrogen peroxide. Due to their unpaired and highly reactive outer shell, they react with the vascular endothelial causing oxidative damage to the cells (Aggarwal et al., 2013). The resultant uncontrolled proliferation and impaired apoptosis of pulmonary artery smooth muscle cells (PASMC) contributes to vascular obstruction and ultimately pulmonary hypertension (Archer et al., 2010).

1.2.4.7 Inflammatory biomarkers

Animal models of PH and humans have varying extent of perivascular inflammatory cells, which include T- and B-lymphocytes, dendritic cells (DCs), mast cells and macrophages (Rabinovitch et al., 2014). Identification of a suitable biomarker can serve as a less invasive tool for diagnosis, monitoring, prognosticating, and assessment of response to treatment. Several inflammatory mediators, including IL-1α, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-12p70, TNF-α, and CRP are upregulated in PH but none is documented to be specific for this purpose (Sydykov et al., 2018).

1.3 Future research

1.3.1 Cancer research

An investigation into the major similarities between PH and cancer cells, could improve the knowledge on PH and further help in oncology, especially in the explanation of the possible mechanisms in the prevention of tissue invasion and metastasis (Guignabert et al., 2013). Specifically, the pro-proliferative and antiapoptotic characteristics of PH which is typical of the manifestations in cancers (Cottrill and Chan, 2013). A common phenomenon in both conditions, Warburg metabolism, is a failure of two fundamental pathways glucose metabolism and mitochondrial oxygen sensing (Archer, 2017). A possible biomarker which could also be explored is survivin, an apoptosis protein inhibitor, often expressed in cancerous tissue and also demonstrated in pulmonary vascular remodelling in rats developing monocrotaline-induced PH (Adnot, 2005). This mediator can serve as a nexus in the pathogenesis of both diseases.

1.4 Hypothesis

This study will test the hypothesis that ARVs administration ameliorates monocrotaline-induced PH in rat model.

1.5 Aims of the study

i. To understand the effect of ARVs on the progression of MCT-induced PH in rats.
ii. To investigate the protective effect of ARVs in the development of MCT-induced PH in a rat model.

1.6 Objectives of the study

i. To investigate the histopathological features in the pulmonary vasculature, right heart thickness and perivascular changes in the lungs.
ii. To investigate the involvement of ASK-1 in pulmonary vascular and right ventricular remodelling.
iii. To investigate the generated ROS and BNP in the rats.

REFERENCES

Abrol R, Trzaskowski B, Goddard WA, 3rd, et al. (2014) Ligand- and mutation-induced conformational selection in the CCR5 chemokine G protein-coupled receptor. *Proc Natl Acad Sci U S A* 111: 13040-13045.

Adesina SE, Kang BY, Bijli KM, et al. (2015) Targeting mitochondrial reactive oxygen species to modulate hypoxia-induced pulmonary hypertension. *Free Radic Biol Med* 87: 36-47.

Adnot S. (2005) Lessons learned from cancer may help in the treatment of pulmonary hypertension. *J Clin Invest* 115: 1461-1463.

Aggarwal S, Gross CM, Sharma S, et al. (2013) Reactive oxygen species in pulmonary vascular remodeling. *Compr Physiol* 3: 1011-1034.

Almodovar S. (2014) The complexity of HIV persistence and pathogenesis in the lung under antiretroviral therapy: challenges beyond AIDS. *Viral Immunol* 27: 186-199.

Almodovar S, Cicalini S, Petrosillo N, et al. (2010) Pulmonary hypertension associated with HIV infection: pulmonary vascular disease: the global perspective. *Chest* 137: 6s-12s.

Almodovar S, Hsue PY, Morelli J, et al. (2011) Pathogenesis of HIV-associated pulmonary hypertension: potential role of HIV-1 Nef. *Proc Am Thorac Soc* 8: 308-312.

Alp NJ and Channon KM. (2004) Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol* 24: 413-420.

Amsellem V, Lipskaia L, Abid S, et al. (2014) CCR5 as a treatment target in pulmonary arterial hypertension. *Circulation* 130: 880-891.

Archer SL. (2017) Pyruvate Kinase and Warburg Metabolism in Pulmonary Arterial Hypertension: Uncoupled Glycolysis and the Cancer-Like Phenotype of Pulmonary Arterial Hypertension. *Circulation* 136: 2486-2490.

Archer SL, Marsboom G, Kim GH, et al. (2010) Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. *Circulation* 121: 2661-2671.

Arts EJ and Hazuda DJ. (2012) HIV-1 antiretroviral drug therapy. *Cold Spring Harb Perspect Med* 2: a007161.

Bain LE and Gwain GC. (2019) Cardiovascular Disease and HIV Infection in Sub-Saharan Africa: Misplaced Priorities in the Public Health and Research Agendas? *Front Cardiovasc Med* 6: 35.
Barman SA, Zhu S and White RE. (2009) RhoA/Rho-kinase signaling: a therapeutic target in pulmonary hypertension. *Vasc Health Risk Manag* 5: 663-671.
Barnett CF and Hsue PY. (2013) Human immunodeficiency virus-associated pulmonary arterial hypertension. *Clin Chest Med* 34: 283-292.
Barroso S, Moren C, Gonzalez-Segura A, et al. (2019) Metabolic, mitochondrial, renal and hepatic safety of enfuvirtide and raltegravir antiretroviral administration: Randomized crossover clinical trial in healthy volunteers. *PLoS One* 14: e0216712.
Batton KA, Austin CO, Bruno KA, et al. (2018) Sex differences in pulmonary arterial hypertension: role of infection and autoimmunity in the pathogenesis of disease. *Biol Sex Differ* 9: 15.
Bhargava A, Kumar A, Yuan N, et al. (1999) Monocrotaline induces interleukin-6 mRNA expression in rat lungs. *Heart Dis* 1: 126-132.
Bigna JJ, Noubiap JJ, Nansseu JR, et al. (2017) Prevalence and etiologies of pulmonary hypertension in Africa: a systematic review and meta-analysis. *BMC Pulm Med* 17: 183.
Bloomfield GS, Khazanie P, Morris A, et al. (2014) HIV and noncommunicable cardiovascular and pulmonary diseases in low- and middle-income countries in the ART era: what we know and best directions for future research. *J Acquir Immune Defic Syndr* 67 Suppl 1: S40-53.
Borges GM. (2017) Health transition in Brazil: regional variations and divergence/convergence in mortality. *Cad Saude Publica* 33: e00080316.
Breitling S, Ravindran K, Goldenberg NM, et al. (2015) The pathophysiology of pulmonary hypertension in left heart disease. *Am J Physiol Lung Cell Mol Physiol* 309: L924-941.
Budas GR, Boehm M, Kojonazarov B, et al. (2018) ASK1 Inhibition Halts Disease Progression in Preclinical Models of Pulmonary Arterial Hypertension. *Am J Respir Crit Care Med* 197: 373-385.
Casserly B and Klinger JR. (2009) Brain natriuretic peptide in pulmonary arterial hypertension: biomarker and potential therapeutic agent. *Drug Des Devel Ther* 3: 269-287.
Chen GJ, Sun HY, Cheng A, et al. (2019) Risk of elevation of serum creatine kinase among HIV-positive individuals receiving dolutegravir-based combination antiretroviral therapy. *Medicine (Baltimore)* 98: e16235.
Cicalini S, Almodovar S, Grilli E, et al. (2011) Pulmonary hypertension and human immunodeficiency virus infection: epidemiology, pathogenesis, and clinical approach. *Clin Microbiol Infect* 17: 25-33.
Colombe S, Machemba R, Mtenga B, et al. (2018) Impact of schistosome infection on long-term HIV/AIDS outcomes. *PLoS Negl Trop Dis* 12: e0006613.
Condon DF, Nickel NP, Anderson R, et al. (2019) The 6th World Symposium on Pulmonary Hypertension: what's old is new. *F1000Res* 8.
Cottrill KA and Chan SY. (2013) Metabolic dysfunction in pulmonary hypertension: the expanding relevance of the Warburg effect. *Eur J Clin Invest* 43: 855-865.
Degano B, Yaici A, Le Pavec J, et al. (2009) Long-term effects of bosentan in patients with HIV-associated pulmonary arterial hypertension. *Eur Respir J* 33: 92-98.
Dhadwal AK, Wang X, Annambhotla S, et al. (2009) Capsaicin blocks HIV protease inhibitor ritonavir-induced vascular dysfunction in porcine pulmonary arteries. *Med Sci Monit* 15: Br1-5.
Dickinson MG, Bartelds B, Borgdorff MA, et al. (2013) The role of disturbed blood flow in the development of pulmonary arterial hypertension: lessons from preclinical animal models. *Am J Physiol Lung Cell Mol Physiol* 305: L1-14.

Elliott CG, Austin ED, Badesch D, et al. (2019) United States Pulmonary Hypertension Scientific Registry (USPHSR): rationale, design, and clinical implications. *Pulm Circ* 9: 2045894019851696.
Famiglini V and Silvestri R. (2016) Focus on Chirality of HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors. *Molecules* 21.
Farrag A, El-Aroussy W, Zaghloul S, et al. (2012) Prevalence and severity of pulmonary hypertension in asymptomatic rural residents with schistosomal infection in the Nile Delta. *Trop Med Int Health* 17: 112-118.
Feng S, Chen S, Yu W, et al. (2017) H2S inhibits pulmonary arterial endothelial cell inflammation in rats with monocrotaline-induced pulmonary hypertension. *Lab Invest* 97: 268-278.
Fulton DJR, Li X, Bordan Z, et al. (2017) Reactive Oxygen and Nitrogen Species in the Development of Pulmonary Hypertension. *Antioxidants (Basel)* 6.
Galie N, Hoeper MM, Humbert M, et al. (2009) Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Respir J* 34: 1219-1263.
Galie N, McLaughlin VV, Rubin LJ, et al. (2019) An overview of the 6th World Symposium on Pulmonary Hypertension. *Eur Respir J* 53.
Gamen E, Seeger W and Pullamsetti SS. (2016) The emerging role of epigenetics in pulmonary hypertension. *Eur Respir J* 48: 903-917.
Gary-Bobo G, Houssaini A, Amsellem V, et al. (2010) Effects of HIV protease inhibitors on progression of monocrotaline- and hypoxia-induced pulmonary hypertension in rats. *Circulation* 122: 1937-1947.
Giaid A, Yanagisawa M, Langleben D, et al. (1993) Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med* 328: 1732-1739.
Goldberg AB, Mazur W and Kalra DK. (2017) Pulmonary hypertension: diagnosis, imaging techniques, and novel therapies. *Cardiovasc Diagn Ther* 7: 405-417.
Gomez-Arroyo JG, Farkas L, Alhussaini AA, et al. (2012) The monocrotaline model of pulmonary hypertension in perspective. *Am J Physiol Lung Cell Mol Physiol* 302: L363-369.
Goto K, Arai M, Watanabe A, et al. (2010) Utility of echocardiography versus BNP level for the prediction of pulmonary arterial pressure in patients with pulmonary arterial hypertension. *Int Heart J* 51: 343-347.
Gratton R, Tricarico PM, Guimaraes RL, et al. (2018) Lopinavir/Ritonavir Treatment Induces Oxidative Stress and Caspaseindependent Apoptosis in Human Glioblastoma U-87 MG Cell Line. *Curr HIV Res* 16: 106-112.
Guignabert C, Tu L, Le Hiress M, et al. (2013) Pathogenesis of pulmonary arterial hypertension: lessons from cancer. *Eur Respir Rev* 22: 543-551.
Hadri L, Kratlian RG, Benard L, et al. (2013) Therapeutic efficacy of AAV1.SERCA2a in monocrotaline-induced pulmonary arterial hypertension. *Circulation* 128: 512-523.
Hayakawa R, Hayakawa T, Takeda K, et al. (2012) Therapeutic targets in the ASK1-dependent stress signaling pathways. *Proc Jpn Acad Ser B Phys Biol Sci* 88: 434-453.
Hemnes AR and Humbert M. (2017) Pathobiology of pulmonary arterial hypertension: understanding the roads less travelled. *Eur Respir Rev* 26.
Henriques-Forsythe M, Annangi S and Farber HW. (2015) Prevalence and hospital discharge status of human immunodeficiency virus-associated pulmonary arterial hypertension in the United States. *Pulm Circ* 5: 506-512.
Heresi GA, Platt DM, Wang W, et al. (2017) Healthcare burden of pulmonary hypertension owing to lung disease and/or hypoxia. *BMC Pulm Med* 17: 58.
Hoeper MM, Bogaard HJ, Condliffe R, et al. (2013) Definitions and diagnosis of pulmonary hypertension. *J Am Coll Cardiol* 62: D42-50.
Hoeper MM, Humbert M, Souza R, et al. (2016) A global view of pulmonary hypertension. *Lancet Respir Med* 4: 306-322.

Hoshikawa Y, Voelkel NF, Gesell TL, et al. (2001) Prostacyclin receptor-dependent modulation of pulmonary vascular remodeling. *Am J Respir Crit Care Med* 164: 314-318.
Huber LC, Bye H and Brock M. (2015) The pathogenesis of pulmonary hypertension--an update. *Swiss Med Wkly* 145: w14202.
Huetsch JC, Suresh K, Bernier M, et al. (2016) Update on novel targets and potential treatment avenues in pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 311: L811-l831.
Hughes RJ, Jais X, Bonderman D, et al. (2006) The efficacy of bosentan in inoperable chronic thromboembolic pulmonary hypertension: a 1-year follow-up study. *Eur Respir J* 28: 138-143.
Janda S, Quon BS and Swiston J. (2010) HIV and pulmonary arterial hypertension: a systematic review. *HIV Med* 11: 620-634.
Jarrett H and Barnett C. (2017) HIV-associated pulmonary hypertension. *Curr Opin HIV AIDS* 12: 566-571.
Jernigan NL, Walker BR and Resta TC. (2004) Endothelium-derived reactive oxygen species and endothelin-1 attenuate NO-dependent pulmonary vasodilation following chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* 287: L801-808.
Jones J, Sullivan PS and Curran JW. (2019) Progress in the HIV epidemic: Identifying goals and measuring success. *PLoS Med* 16: e1002729.
Kay JM, Harris P and Heath D. (1967) Pulmonary hypertension produced in rats by ingestion of Crotalaria spectabilis seeds. *Thorax* 22: 176-179.
Lahm T, Tuder RM and Petrache I. (2014) Progress in solving the sex hormone paradox in pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 307: L7-26.
Lan NSH, Massam BD, Kulkarni SS, et al. (2018) Pulmonary Arterial Hypertension: Pathophysiology and Treatment. *Diseases* 6.
Liu T, Zhou HJ and Min W. (2017) ASK family in cardiovascular biology and medicine. *Adv Biol Regul* 66: 54-62.
Mohapi BJ and Pitsoane EM. (2017) Life skills as a behaviour change strategy in the prevention of HIV and AIDS: Perceptions of students in an open and distance learning institution. *Sahara j* 14: 77-84.
Morris A, Hillenbrand M, Finkelman M, et al. (2012) Serum (1-->3)-beta-D-glucan levels in HIV-infected individuals are associated with immunosuppression, inflammation, and cardiopulmonary function. *J Acquir Immune Defic Syndr* 61: 462-468.
Nogueira-Ferreira R, Vitorino R, Ferreira R, et al. (2015) Exploring the monocrotaline animal model for the study of pulmonary arterial hypertension: A network approach. *Pulm Pharmacol Ther* 35: 8-16.
Omland T, Sabatine MS, Jablonski KA, et al. (2007) Prognostic value of B-Type natriuretic peptides in patients with stable coronary artery disease: the PEACE Trial. *J Am Coll Cardiol* 50: 205-214.
Örem C. (2017) Epidemiology of pulmonary hypertension in the elderly. *J Geriatr Cardiol* 14: 11-16.
Piscoya Roncal CG, Mendes AA, Muniz MTC, et al. (2019) Schistosomiasis-associated pulmonary arterial hypertension: survival in endemic area in Brazil. *Int J Cardiol Heart Vasc* 25: 100373.
Porter KM and Sutliff RL. (2012) HIV-1, reactive oxygen species, and vascular complications. *Free Radic Biol Med* 53: 143-159.
Prins KW and Thenappan T. (2016) World Health Organization Group I Pulmonary Hypertension: Epidemiology and Pathophysiology. *Cardiol Clin* 34: 363-374.
Rabinovitch M, Guignabert C, Humbert M, et al. (2014) Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res* 115: 165-175.

Rai MA, Pannek S and Fichtenbaum CJ. (2018) Emerging reverse transcriptase inhibitors for HIV-1 infection. *Expert Opin Emerg Drugs* 23: 149-157.
Ranchoux B, Meloche J, Paulin R, et al. (2016) DNA Damage and Pulmonary Hypertension. *Int J Mol Sci* 17.
Said SI. (2006) Mediators and modulators of pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol* 291: L547-558.
Santos-Ribeiro D, Mendes-Ferreira P, Maia-Rocha C, et al. (2016) Pulmonary arterial hypertension: Basic knowledge for clinicians. *Arch Cardiovasc Dis* 109: 550-561.
Scupinari T, Mannochio Russo H, Sabino Ferrari AB, et al. (2020) Crotalaria spectabilis as a source of pyrrolizidine alkaloids and phenolic compounds: HPLC-MS/MS dereplication and monocrotaline quantification of seed and leaf extracts. *Phytochem Anal*.
Sierra-Aragon S and Walter H. (2012) Targets for inhibition of HIV replication: entry, enzyme action, release and maturation. *Intervirology* 55: 84-97.
Simonneau G, Montani D, Celermajer DS, et al. (2019) Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J* 53.
Smit M, Olney J, Ford NP, et al. (2018) The growing burden of noncommunicable disease among persons living with HIV in Zimbabwe. *Aids* 32: 773-782.
Stein JH, Klein MA, Bellehumeur JL, et al. (2001) Use of human immunodeficiency virus-1 protease inhibitors is associated with atherogenic lipoprotein changes and endothelial dysfunction. *Circulation* 104: 257-262.
Studer SM and Gilkin RJ, Jr. (2014) Clinical trial designs in PAH: shifting from functional measurements to long-term clinical outcomes. *Am J Manag Care* 20: S115-122.
Sydykov A, Mamazhakypov A, Petrovic A, et al. (2018) Inflammatory Mediators Drive Adverse Right Ventricular Remodeling and Dysfunction and Serve as Potential Biomarkers. *Front Physiol* 9: 609.
Sztuka K and Jasinska-Stroschein M. (2017) Animal models of pulmonary arterial hypertension: A systematic review and meta-analysis of data from 6126 animals. *Pharmacol Res* 125: 201-214.
Szturmowicz M, Kacprzak A, Blasinska-Przerwa K, et al. (2015) Pulmonary hypertension in the course of diffuse parenchymal lung diseases - state of art and future considerations. *Pneumonol Alergol Pol* 83: 312-323.
Tilton JC, Amrine-Madsen H, Miamidian JL, et al. (2010) HIV type 1 from a patient with baseline resistance to CCR5 antagonists uses drug-bound receptor for entry. *AIDS Res Hum Retroviruses* 26: 13-24.
Umar S, Rabinovitch M and Eghbali M. (2012) Estrogen paradox in pulmonary hypertension: current controversies and future perspectives. *Am J Respir Crit Care Med* 186: 125-131.
UNAIDS. (2019) *Global HIV & AIDS statictics-2019 fact sheet*. Available at: https://www.unaids.org/en/resources/fact-sheet.
Weber M and Hamm C. (2006) Role of B-type natriuretic peptide (BNP) and NT-proBNP in clinical routine. *Heart* 92: 843-849.

CHAPTER 2

Manuscript

Oxidative stress and gene expression of antioxidants enzymes in monocrotaline-induced pulmonary hypertension following the administration of antiretroviral medications in rats.

Submitted to RESPIRATORY PHYSIOLOGY AND NEUROBIOLOGY

(Ref No:* RESPNB-D-20-00161*)

Oxidative stress and gene expression of antioxidants enzymes in monocrotaline-induced pulmonary hypertension following the administration of antiretroviral medications in rats.

Adeoti AO[1], Nadar A[1], and Channa ML[1]

[1]Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, South Africa

ABSTRACT

Background: Pulmonary hypertension (PH) is a severe life-threatening pulmonary vasculopathy. Studies in animal models have identified the role of oxidative stress in PH. However, conflicting reports on the effects of antiretroviral medications.
Aim: This study investigated the effects of zidovudine and /or ritonavir in the oxidative process of monocrotaline-induced PH in rats.

Method: Forty male Sprague-Dawley rats weighing between 200 and 250 g were randomized into 5 different groups (n=8 per group). A single dose intraperitoneal injection of monocrotaline (60mg/kg) was administered to all the rats in four of the groups while the control group was excluded. There was daily oral administration of the antiretroviral medications-zidovudine (AZT)-only (100 mg/kg); ritonavir (RTV) only (30 mg/kg); AZT+ RTV (100+30 mg/kg); and the untreated group had equivalent volume of saline for 28 days respectively. The rats were euthanized by decapitation and the organs harvested at the end of the 28 days for analysis. Gene expression using RT-PCR for the antioxidants and laboratory assay for lipid peroxidation were analysed.

Results: A significantly higher mRNA gene expression of catalase, superoxide dismutase, and glutathione peroxidase in the treated rats was observed compared to the untreated. There was an increase in malondialdehyde (MDA) in the heart tissues of untreated rats (37.01±1.16nmol/g, $p<0.0001$) compared to the control group (3.46±0.97 nmol/g) with an associated reduction in MDA by the antiretrovirals. Furthermore, an increase in the total antioxidant capacity (TAC) in AZT (0.85±0.02 nmol/g, $p<0.0001$), RTV (0.63±0.03 nmol/g, $p<0.0001$) and combination of AZT/RTV (0.77±0.06 nmol/g, $p<0.0001$) compared to untreated (0.28±0.03) rats. Oxidative stress with accompanied lipid peroxidation was observed in the experimental group without antiretroviral medications. The converse was observed in the groups treated with the antiretroviral medications, as there was a reduction in oxidative stress and evidence of lipid peroxidation.

Conclusion: Zidovudine and ritonavir ameliorate oxidative stress in experimental rats. Further studies are required to ascertain the role of oxidative stress in the reversal of established pulmonary hypertension by the administration of antiretroviral medications

Keywords: Pulmonary Hypertension, Oxidative stress, Reactive oxygen species, Monocrotaline

INTRODUCTION

Pulmonary hypertension (PH) is a severe and life-threatening disease characterized by progressive vasculopathy and remodelling of pulmonary arteries leading to sustained pressure elevation in the pulmonary circulation (Sydykov et al., 2018). Pulmonary artery remodelling is vital in the development of pulmonary hypertension and right ventricular hypertrophy. This process involves oxidative reactions, mitochondrial injury, inflammation, and apoptosis (Chang et al., 2019).

Oxidative stress is one of the identified factors, of the several complex processes, that culminate in the vascular remodelling towards the development and progression of PH (Sharma et al., 2016). In which case, there is an imbalance in reactive oxygen species (ROS) handling due to either the over-production of ROS and/or decreased capability of antioxidant defenses. ROS causes vasoconstriction through smooth muscle cell contraction by triggering a rise in cytosolic free Ca^{2+} concentration (Aggarwal et al., 2013). Upregulation of ROS in the pathogenesis of PH has been reported in both animal models and humans with PH (Csiszar et al., 2009; Ghasemzadeh et al., 2014; Redout et al., 2010). Furthermore, the continuous elevation of ROS can also uncouple nitric oxide (NO) by an oxidative process resulting in either the production of superoxide ($O_2^{\bullet-}$) or conversion to peroxynitrite ($ONOO^-$). These free radicals can reduce endothelial nitric oxide synthase and prostacyclin synthase leading to endothelial dysfunction, vasoconstriction and increased vascular tone which ultimately result in PH (Smukowska-Gorynia et al., 2019).

HIV-associated PH (HIV-PH) is a recognized complication of HIV/AIDS, with a stable prevalence both before and following the introduction of antiretroviral drugs in the treatment of people living with HIV/AIDS (PLHIV). However, the reported prevalence of HIV-PH is known to be relatively higher in Africa than developed countries (Almodovar et al., 2011; Bigna et al., 2015). The pathogenesis of HIV-PH remains unclear, as neither is there evidence that HIV infects pulmonary artery endothelial cells nor its proteins identified in significant amount in tissues during this process (Zuber et al., 2004). Furthermore, there is ambiguity in the role of the antiretroviral medications in the development and progression of the disease as some studies claim antiretroviral treatment decrease pulmonary arterial pressure (PAP). On the contrary, some

studies reported higher prevalence of HIV-PH in HIV/AIDS patients (Opravil et al., 1997; Zuber et al., 2004). Similarly, it has been observed that the use of zidovudine and indinavir in animal models may lead to PH through endothelin-1 production (Hebert et al., 2004). These antiretroviral medications have been linked with an increase in the production of reactive oxygen species (ROS) and endothelial mitochondrial dysfunction (Jiang et al., 2007). Nonetheless, zidovudine also has potent anti-inflammatory and antioxidant activities while protease inhibitors like ritonavir have demonstrated a protective role in animal model of HIV-PH (Gary-Bobo et al., 2010; Liu et al., 2018). Likewise, several translational studies have shown the beneficial effect of antiretroviral medications on the risk reduction of HIV-PH and the variable outcomes among established early stage of HIV-PH patients (Pal et al., 2013; Quezada et al., 2012). This present study was conducted to unravel the critical role of antiretroviral medications-zidovudine and ritonavir, in the development of HIV-PH as it relates to oxidative stress in MCT-induced rat model of PH. Hence the study investigated the hypothesis that regular administration of antiretroviral medications in monocrotaline-treated rats ameliorates pulmonary hypertension via oxidative stress dependent mechanism.

METHODS AND MATERIALS

Animals

Animal experiment was approved by the Animal Research Committee of the University of KwaZulu-Natal, South Africa with the approval number AREC/066/018M. All experiments were conducted in accordance with the standard protocol and the ARRIVE guideline at the Biomedical Resource Centre in the University of KwaZulu-Natal, South Africa (Nam et al., 2018). Forty adult male Sprague Dawley rats (200–250 g) were housed at 18-22 °C under a 12-h light/12-h dark cycle with free access to food and water.

Experimental design

The forty adult male Sprague-Dawley rats were randomly assigned into five groups. One of the groups (n=8) served as the control while the remaining 32 rats were the PH model group that received a single 60 mg/kg intraperitoneal dose of MCT (Replamed, South Africa). Similarly, the rats in the control group were injected with the same volume of sterile saline. The rats in the treatment group were randomized into four subgroups (n=8 per group) and three of the four subgroups had daily administration of antiretroviral over a period of 4 weeks. The fourth was observed for same period although no other medication was administered as side the initial MCT. Following the MCT, rats in the treatment groups were dosed once daily on the antiretroviral

drugs of zidovudine (100mg/kg), ritonavir (30 mg/kg) or combination of both zidovudine/ritonavir (100mg/kg/30 mg/kg) or saline administration.

Measurements of Oxidative Stress

The heart and lung tissues were homogenized in ice-cold saline (0.9%) in a glass homogenizer. Then the homogenate was centrifuged at 2500 r/min for 10 min at 4 ˚C temperature. Supernatant of homogenate (10%, w/v) was employed for the subsequent tests. Total antioxidant capacity (TAC) activity was determined using the colorimetric assay. TAC determination (ElabScience, Biotechnology, MD, USA) was carried out following the manufacturer's protocol. While malondialdehyde (MDA) assay (Oxis Research, Portland, OR, USA) was used by colorimetric quantification of MDA, as a lipid peroxidation marker. MDA concentration (µM) was measured at 532 nm and calculated using an MDA standard curve. The activities of MDA and TAC in the supernatant was determined according to the instructions of the respective testing kit (TAC assay kit, ElabScience, Biotechnology, MD, USA) and a microplate reader (Spectrostar Nano Bmg Labtech, Germany).

Morphometric analysis

The rats' lungs and heart were harvested, immersed in 10% neutral buffered formalin, and embedded in paraffin. Tissues were sectioned at 4 µm, stained with hematoxylin and eosin (H&E), and analysed. A computerized morphometric system (Leica DM 500, Germany) was connected to a light microscopy for the assessment of the slides. The slides were evaluated by light microscopy at 400 x magnification, and the reviewers (AO, AN) who assessed the extent of vascular remodelling were blinded to the grouping. We randomly selected ten fields containing terminal arterioles (50–150 mm external diameters) and the dimensions of the external elastic lamina and the internal elastic lamina of each vessel were measured. The wall thickness (percent) of the arterioles were calculated using the formula as wall thickness (%) =100 x (1– internal elastic lamina/external elastic lamina). (Han et al., 2016).

Quantitative real-time PCR

Total RNA was isolated from the rat heart using TRIzol (Zymo Research), subsequently transcribed into cDNA and qPCR was performed. Primers for catalase (5'-CCTCAGAAACCCGATGTCCTG-3′ and 5'- GTCAAAGTGTGCCATCTCGTCG -3′), MnSOD (5'-ACCGAGGAGAAGTACCACGA-3′ and 5'- TAGGGCTCAGGTTTTGTCCAG -3′), GPX-1 (5'-TGAGAAGTGCGAGGTGAATG-3′ and 5'- CGGGGACCAAATGATGTACT-3′) and GAPDH (5'-TGATTCTACCCACGGCAAGTT-3' and 5'-

TGATGGGTTTCCCATTGATGA-3') were purchased from Inqaba (South Africa). Primers for gene expression analysis were designed using published sequence information (Ren et al., 2019). PCR was performed in a 0.1 mL tube containing 2 µL cDNA, 1 µL (10 pmol) rat forward primer and reverse primer, 2 µL dNTP (1.25 mM each nucleotide), 5 µL SybrGreen, and 1µL dH_2O. After denaturation at 95 ◦C for 10 min, the mixture underwent PCR for 40 cycles at 95 ◦C for 60 s, annealing temperature for 60 s, and elongation at 72 ◦C for 60 s. The relative expression of protein mRNA level to GAPDH mRNA was calculated from cycle threshold (Cq) value by a ΔΔCq method and presented as relative to control.

Drugs

MCT was purchased from Replamed (South Africa), while zidovudine from Aspen Pharmacare Limited (Aspen, South Africa) and ritonavir from Abbvie Limited (Abbvie, South Africa). The dosage of the medications was based on previous studies involving rats (Adebiyi et al., 2015; Gary-Bobo et al., 2010). Of the major classes of antiretroviral drugs, NRTIs, which includes zidovudine, and protease inhibitors (PIs) like ritonavir have been the most widely implicated drug classes in development of endothelial dysfunction (Hansen et al., 2013).

Statistical Analysis

Statistical differences were analysed by one-way ANOVA with Tukey multiple comparison post-test. Data were recorded as mean ± SEM with $P < 0.05$ considered significant. All analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

RESULTS

Assessment on lipid peroxidation

Figure 1 shows significant increase in the level of MDA in the heart tissues of experimental rats treated with MCT (37.01 ± 1.16 nmol/g, $p < 0.0001$) compared to the rats in the control group (3.46 ± 0.97 nmol/g). The administration of zidovudine (8.80 ± 1.21 nmol/g, $p < 0.0001$), ritonavir (8.47 ± 1.24 nmol/g, $p < 0.0001$) and a combination of both drugs (9.18 ± 0.98 nmol/g,$p < 0.0001$) was associated with mitigation of the effect of MCT by the reduction in the MDA levels in the heart of treated rats.

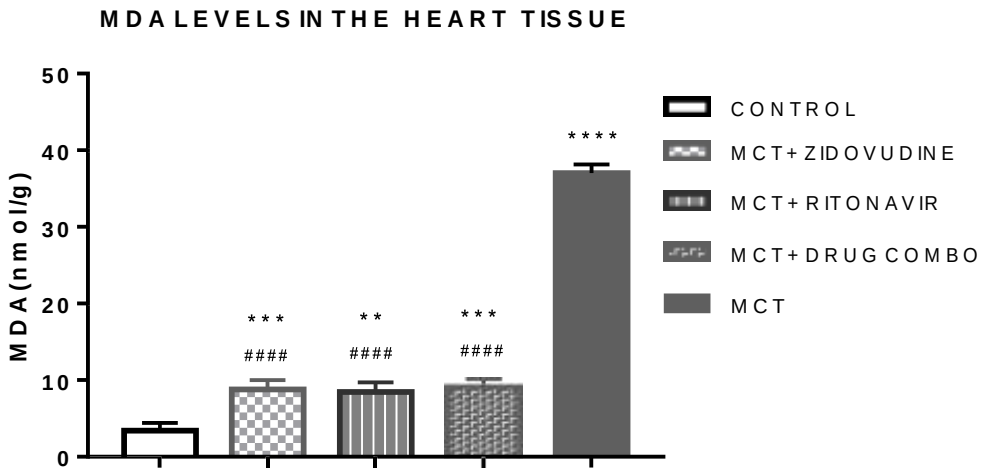

Figure 1: Level of lipid peroxidation in rat heart tissue
Values are expressed as mean ± SEM (n=8/group). In comparison with the control: *P < 0.05 , **P < 0.01, ***P < 0.001 and ****P < 0.0001 while in comparison with MCT $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 and $^{\#\#\#\#}$P < 0.0001. MCT + DRUG COMBO represents MCT plus Drug combination of zidovudine and ritonavir.

Assessment of total antioxidant capacity

As presented in figure 2, there was a significant difference in the total antioxidant capacity (TAC) of the heart tissue of the rats in the control group as compared to the treatment group exposed to only MCT, without any antiretroviral drugs given. A significant increase in TAC levels in the heart tissues of the rats treated with zidovudine (0.85 ± 0.02 nmol/g, P < 0.0001), ritonavir (0.63 ± 0.03 nmol/g, p < 0.0001) or a combination of both drugs (0.77 ± 0.06 nmol/g, p < 0.0001 was observed when compared to the group administered only MCT (0.28 ± 0.03).

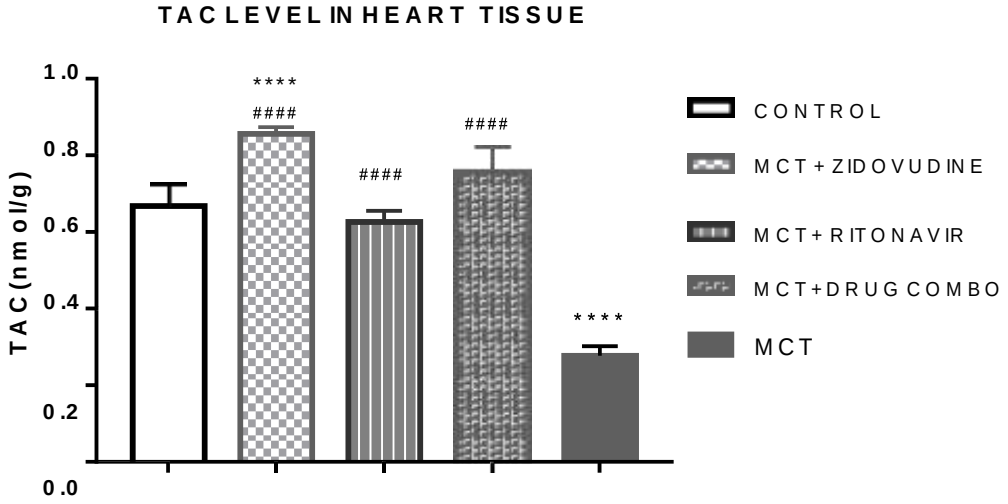

Figure 2: Total antioxidant capacity of the rat heart tissues
Values are expressed as mean ± SEM (n=8/group). In comparison with the control: *P < 0.05 , **P < 0.01, ***P < 0.001 and ****P < 0.0001 while in comparison with MCT $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 and $^{\#\#\#\#}$P < 0.0001. MCT + DRUG COMBO represents MCT plus Drug

combination of zidovudine and ritonavir.

Relative mRNA expression of superoxide dismutase in the rat heart tissue

Figure 3 shows the relative mRNA expression of superoxide dismutase in the heart tissue of rats exposed to only MCT to be significantly lower (1.39 ± 0.14, p <0.0001) than the control group (6.02 ± 0.17). Figure 3 also revealed that MCT exposed animals treated with zidovudine (6.25 ± 0.26, p), ritonavir (5.78 ± 0.25, p <0.0001) and a combination of both drugs (6.45 ± 0.42, p <0.0001) expressed a significantly higher level of superoxide dismutase mRNA in comparison to the animals exposed to MCT without any treatment with antiretroviral drugs.

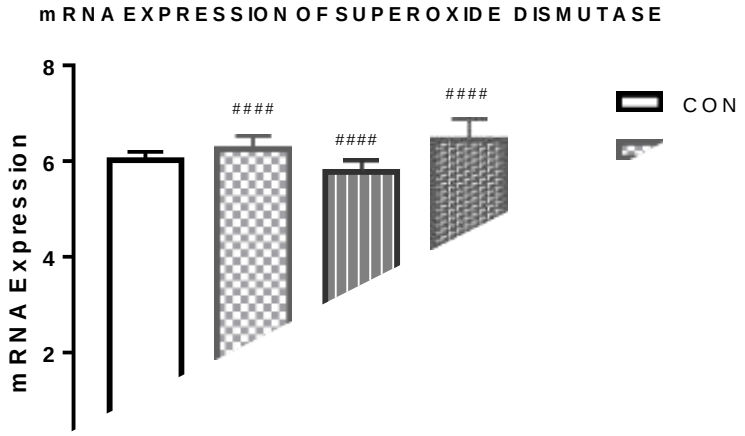

Figure 3: Relative mRNA expression of superoxide dismutase in the rat heart tissue
Values are expressed as mean ± SEM (n=8/group). In comparison with the control: *P < 0.05 , **P < 0.01, ***P < 0.001 and ****P < 0.0001 while in comparison with MCT $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 and $^{\#\#\#\#}$P < 0.0001. MCT + DRUG COMBO represents MCT plus Drug combination of zidovudine and ritonavir.

Relative mRNA expression of catalase in the rat heart tissue

The mRNA expression of catalase in the heart tissue of animals exposed to only MCT was significantly lower (0.91 ± 0.06, P <0.0001) than the control group (1.88 ±0.15, p <0.0001). It was also observed that MCT exposed animals treated with zidovudine (1.99 ± 0.09, p <0.0001), ritonavir (1.90 ± 0.02, p <0.0001) and a combination of both drugs (1.96 ± 0.04, p =0.0001) expressed a significantly higher level of catalase mRNA in comparison to the animals exposed to MCT without treatment with any antiretroviral drugs.

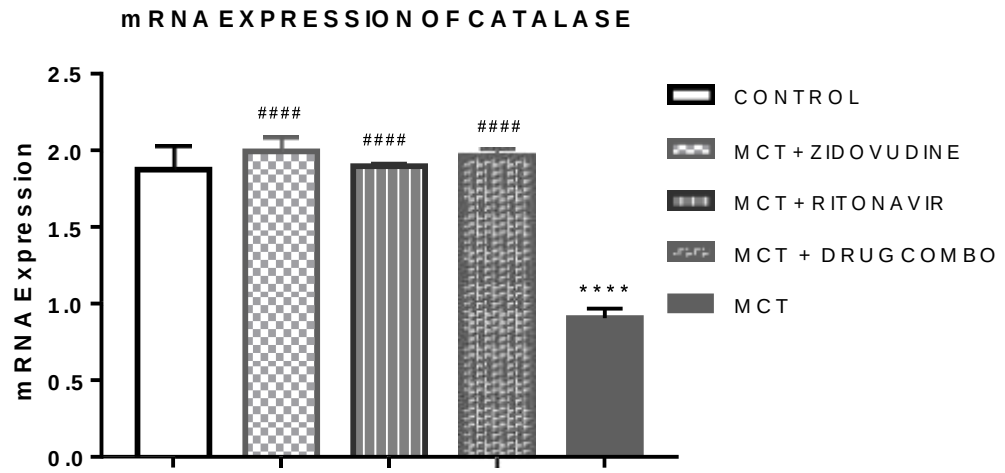

Figure 4: Relative mRNA expression of catalase in the heart tissue of experimental rats
Values are expressed as mean ± SEM (n=8/group). In comparison with the control: *P < 0.05 , **P < 0.01, ***P < 0.001 and ****P < 0.0001 while in comparison with MCT $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 and $^{\#\#\#\#}$P < 0.0001. MCT + DRUG COMBO represents MCT plus Drug combination of zidovudine and ritonavir

Relative mRNA expression of glutathione peroxidase in the rat heart tissue

Glutathione peroxidase mRNA expression in the heart of experimental animals exposed to MCT only (0.51 ± 0.17, p <0.0001) was significantly lower than that of the control group (2.255 ± 0.16). The level of glutathione peroxidase mRNA expressed in MCT animals treated with zidovudine (2.35 ± 0.19, p <0.0001), ritonavir (2.06 ± 0.09, p < 0.0001) and a combination of both drugs (2.28 ± 0.19, p < 0.0001) was similar to that of the control group but it was significantly higher than the glutathione mRNA expressed in the heart of animals exposed to only MCT.

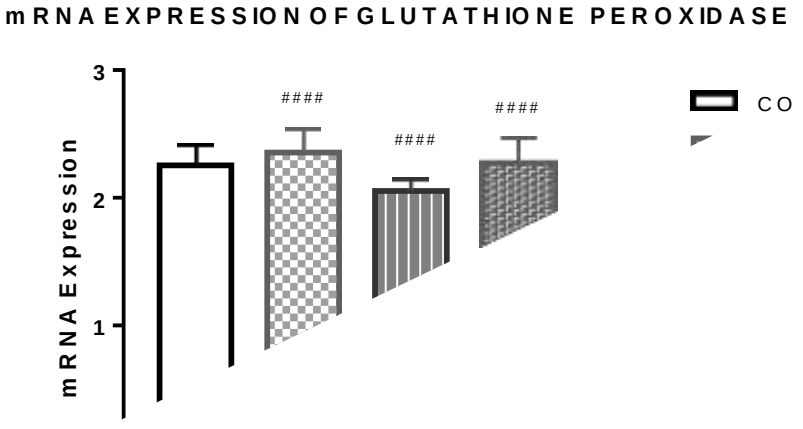

Figure 5: mRNA expression of glutathione peroxidase in the heart tissue of experimental rats
Values are expressed as mean ± SEM (n=8/group). In comparison with the control: *P < 0.05 , **P < 0.01, ***P < 0.001 and ****P < 0.0001 while in comparison with MCT $^{\#}$P < 0.05, $^{\#\#}$P <

0.01, ###P < 0.001 and ####P < 0.0001. MCT + DRUG COMBO represents MCT plus Drug combination of zidovudine and ritonavir.

Histological examination of the lung tissues of rats

Histological examination of the slides under light microscope following H and E staining shows profound thickening in the media wall of the muscular pulmonary arteries in the lungs of rats treated with MCT. The arteries of rats treated with antiretroviral medications were thicker than controls but not as those with only MCT.

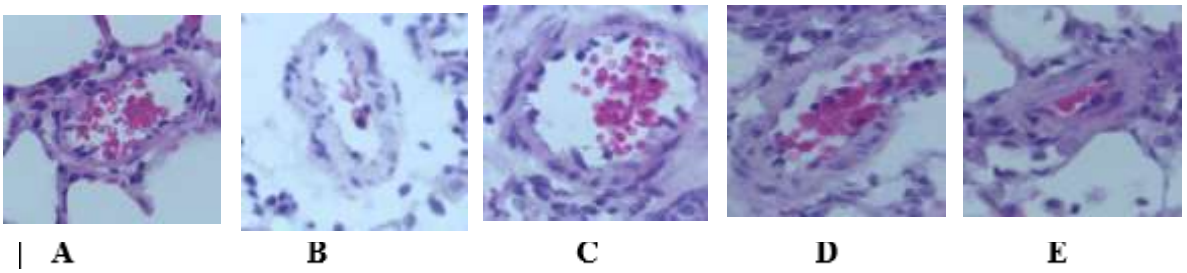

Figure 6a: shows representative images of lung stained with H and E for the different groups (Image A- Control, Image B- MCT+Zidovudine, Image C- MCT+Ritonavir, Image D- MCT+Drugs combination of Zidovudine and Ritonavir and Image E- MCT)

Figure 6b: shows arteriolar wall thickness in the different groups. In the treatment group with MCT shows significantly higher percentage wall thickness than the control (67.47 ± 1.22% versus 31.83 ± 1.09%, p < 0.0001). The anti-retroviral drug-zidovudine (41. 17 ± 0.52% versus 67.47 ± 1.22%, P < 0.0001), drug-ritonavir (39.83 ± 0.32% versus 67.47 ± 1.22%, P < 0.0001) and a combination of drugs zidovudine and ritonavir (39.53 ± 0.84% versus 67.47 ± 1.22, p < 0.0001 when compared to MCT group shows significantly reduction in the percentage wall thickness.

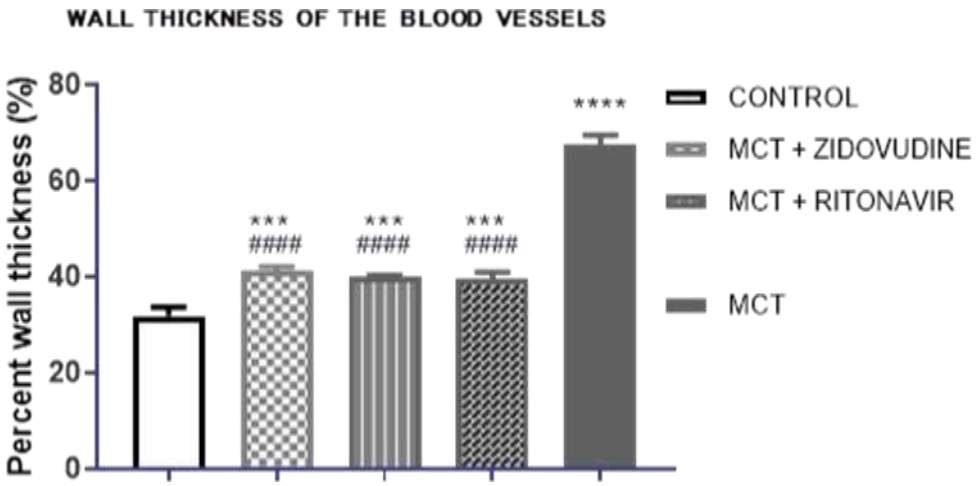

Figure 6b shows wall thickness of pulmonary arterioles in lung tissues of experimental rats. Values are expressed as mean ± SEM (n=8/group). In comparison with the control: *P < 0.05 , **P < 0.01, ***P < 0.001 and ****P < 0.0001 while in comparison with MCT $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 and $^{\#\#\#\#}$P < 0.0001. MCT + DRUG COMBO represents MCT plus Drug combination of zidovudine and ritonavir.

DISCUSSION

This study showed the effects of ARVs against MCT-induced PH. There was a reduction in MDA levels while there were increased levels in TAC, SOD, CAT and GPx. In addition, there was reduced thickness in the pulmonary arterioles of the lungs of MCT rats following the ARVs administration.

In our study, the critical period of 28 days following the intraperitoneal administration of MCT was observed, as revealed in the literature, for the establishment of PH model in rats. However, beyond 28 days, at the single dose of 60 mg/kg of MCT, there is an increased likelihood of mortality of rats (Kishimoto et al., 2015). This finding was also observed in our study where the mortality was recorded in the experimental rats after the first 28 days.

Similar to previous studies, the role of ROS in the development of PH following MCT administration but the effects of antiretroviral medication have not been explored. The mitigating effects of molecular hydrogen and several anti-ROS medications have been demonstrated to improve MCT-induced PH in rats (Ghasemzadeh et al., 2014; Kishimoto et al., 2015). The generation of ROS in monocrotaline-injected rats responsible for the development of PH results from the reduction in nitric oxide levels with the proliferation of pulmonary arterial smooth muscle cells, and ultimately enhanced pulmonary vasoconstriction and remodelling (Feng et al., 2020; Wang et al., 2017).

In the study, marked thickening in the pulmonary arteriolar media walls was observed on the histopathology results of the lungs in the MCT treated rats compared those treated on antiretroviral medications. This observed finding was like those reported in a previous study by

Lee *et al*. where prominent medial hypertrophy of the muscular pulmonary arteries was seen in MCT-induced PH rats (Lee et al., 2005). This structural change in the MCT treated rats when compared to the control and the rats that were administrated zidovudine and ritonavir could be explained by the mitigating effects of the antiretroviral medications on MCT treated rats.

In animal models, increased MDA level has been reported in pulmonary hypertension compared to controls and the degree of elevation of MDA has been correlated with the disease severity (Chang et al., 2019; Irodova et al., 2002; Smukowska-Gorynia et al., 2019). This similar finding was observed in our study, as the heart tissues of rats exposed to MCT showed elevated levels of MDA, which was significantly mitigated by administration of zidovudine, ritonavir or both drugs in combination. This was likewise elucidated in the study by Feng et al., where an increased MDA level in MCT-induced pulmonary hypertension in rats revealed the administration of redox regulatory agent was protective against pulmonary hypertension (Feng et al., 2020).

The overall balance between oxidant and antioxidants is reflected by the level of TAC which is an indicator of available enzymatic and no enzymatic antioxidant in a tissue (Smukowska-Gorynia et al., 2019). Our study found depletion in TAC in the MCT treated rats without antiretroviral therapy while higher TAC in the groups on the anti-retroviral drugs.

The former three are the main enzymatic antioxidant system where the SODs catalysis the conversion of superoxide radical to H_2O_2 and catalase (CAT) further converts it into water. This activity can reverse pulmonary vascular remodelling and PH. Similar to our findings, significantly lower SOD mRNA expression has been reported in human and animal models (Mata et al., 2012; Masri et al., 2008). In animal models, MCT induces oxidative stress by downregulating the expression of genes responsible for mopping up free radicals, hence superoxide dismutase (SOD) preserves NO *in vivo* by scavenging superoxide and preventing the consumptive reactions (Masri et al., 2008). This present study also proves that pulmonary hypertension is associated with decreased expression of SOD mRNA especially in the MCT treated group. However, this finding further substantiated the treatment of MCT- induced pulmonary hypertension in rats with antiretroviral medications (zidovudine and ritonavir) ameliorated the depressive effect of MCT on SOD mRNA expression.

The same was observed for the mRNA expression of CAT and GPX mRNA expression. MCT induced severe depression of GPX and CAT mRNA gene expression which was significantly mitigated by the administration of the anti-retroviral drug namely zidovudine and ritonavir. An imbalance in oxidative-antioxidative process in the blood vessel walls with increased reactive oxygen species produced by these cells result in vasoconstriction, smooth muscle cell proliferation, and vascular remodelling which if limited could help alleviate PH (Zhang et al., 2019).

The findings from this study demonstrated that the antiretroviral drugs namely zidovudine, ritonavir or the combination of the two drugs ameliorated the distortion in the oxidant-antioxidant system in rats exposed to MCT. However, antiretroviral may not be sufficient as a sole therapy for HIV-PH, hence its combination with PH-specific therapy is of paramount importance. Further studies are also required to ascertain the role of oxidative stress following antiretroviral medications in the reversal of PH.

To the best of our knowledge, this is the first study to demonstrate the effects of antiretroviral medications and oxidative stress as a biomarker in the development of PH in experimental animals. A limitation of our study is the non-pressure measurement by right heart catheterization which is an invasive procedure to establish an increased pressure in the right ventricle due to PH. However, the histologic examination of the media wall of pulmonary arteries was reviewed.

Conclusion

This study has demonstrated that MCT-induced PH generates ROS in rats and the protective role of antiretroviral drugs (zidovudine and ritonavir) in the treatment of PH. Therefore, this further support the beneficial role of antiretroviral drugs in the treatment of PH, but future research should be conducted to confirm these beneficial ARV effects in a HIV-PH model.

Competing Interests: The authors declare that they have no competing interests

Acknowledgement: This study was supported by the research grant provided by College of Health Sciences (CHS) of the University of KwaZulu-Natal, South Africa.

REFERENCES

Adebiyi OO, Adebiyi OA and Owira PM. (2015) Naringin Reverses Hepatocyte Apoptosis and Oxidative Stress Associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-Induced Metabolic Complications. *Nutrients* 7: 10352-10368.

Aggarwal S, Gross CM, Sharma S, et al. (2013) Reactive oxygen species in pulmonary vascular remodeling. *Compr Physiol* 3: 1011-1034.

Almodovar S, Hsue PY, Morelli J, et al. (2011) Pathogenesis of HIV-associated pulmonary hypertension: potential role of HIV-1 Nef. *Proc Am Thorac Soc* 8: 308-312.

Bigna JJ, Sime PS and Koulla-Shiro S. (2015) HIV related pulmonary arterial hypertension: epidemiology in Africa, physiopathology, and role of antiretroviral treatment. *AIDS Res Ther* 12: 36.

Chang CY, Shih HJ, Huang IT, et al. (2019) Magnesium Sulfate Mitigates the Progression of Monocrotaline Pulmonary Hypertension in Rats. *Int J Mol Sci* 20.

Csiszar A, Labinskyy N, Olson S, et al. (2009) Resveratrol prevents monocrotaline-induced pulmonary hypertension in rats. *Hypertension* 54: 668-675.

Feng W, Hu Y, An N, et al. (2020) Alginate Oligosaccharide Alleviates Monocrotaline-Induced Pulmonary Hypertension via Anti-Oxidant and Anti-Inflammation Pathways in Rats. *Int Heart J* 61: 160-168.
Gary-Bobo G, Houssaini A, Amsellem V, et al. (2010) Effects of HIV protease inhibitors on progression of monocrotaline- and hypoxia-induced pulmonary hypertension in rats. *Circulation* 122: 1937-1947.
Ghasemzadeh N, Patel RS, Eapen DJ, et al. (2014) Oxidative stress is associated with increased pulmonary artery systolic pressure in humans. *Hypertension* 63: 1270-1275.
Han X, Zhang Y, Zhou Z, et al. (2016) Hydroxysafflor yellow A improves established monocrotaline-induced pulmonary arterial hypertension in rats. *J Int Med Res* 44: 569-584.
Hansen L, Parker I, Roberts LM, et al. (2013) Azidothymidine (AZT) leads to arterial stiffening and intima-media thickening in mice. *J Biomech* 46: 1540-1547.
Hebert VY, Crenshaw BL, Romanoff RL, et al. (2004) Effects of HIV drug combinations on endothelin-1 and vascular cell proliferation. *Cardiovascular toxicology* 4: 117-131.
Irodova NL, Lankin VZ, Konovalova GK, et al. (2002) Oxidative stress in patients with primary pulmonary hypertension. *Bull Exp Biol Med* 133: 580-582.
Jiang B, Hebert VY, Li Y, et al. (2007) HIV antiretroviral drug combination induces endothelial mitochondrial dysfunction and reactive oxygen species production, but not apoptosis. *Toxicol Appl Pharmacol* 224: 60-71.
Kishimoto Y, Kato T, Ito M, et al. (2015) Hydrogen ameliorates pulmonary hypertension in rats by anti-inflammatory and antioxidant effects. *J Thorac Cardiovasc Surg* 150: 645-654.e643.
Lee YS, Byun J, Kim JA, et al. (2005) Monocrotaline-induced pulmonary hypertension correlates with upregulation of connective tissue growth factor expression in the lung. *Exp Mol Med* 37: 27-35.
Liu H, Gambino F, Jr., Algenio C, et al. (2018) Zidovudine protects hyperosmolarity-stressed human corneal epithelial cells via antioxidant pathway. *Biochem Biophys Res Commun* 499: 177-181.
Masri FA, Comhair SA, Dostanic-Larson I, et al. (2008) Deficiency of lung antioxidants in idiopathic pulmonary arterial hypertension. *Clin Transl Sci* 1: 99-106.
Mata M, Sarrion I, Milian L, et al. (2012) PGC-1α induction in pulmonary arterial hypertension. *Oxid Med Cell Longev* 2012: 236572.
Nam MH, Chun MS, Seong JK, et al. (2018) Ensuring reproducibility and ethics in animal experiments reporting in Korea using the ARRIVE guideline. *Lab Anim Res* 34: 11-19.
Opravil M, Pechere M, Speich R, et al. (1997) HIV-associated primary pulmonary hypertension. A case control study. Swiss HIV Cohort Study. *Am J Respir Crit Care Med* 155: 990-995.
Pal J, Sen K, Sarkar G, et al. (2013) Effect of antiretroviral therapy on pulmonary hypertension in HIV patients. *J Indian Med Assoc* 111: 845-846, 849.
Quezada M, Martin-Carbonero L, Soriano V, et al. (2012) Prevalence and risk factors associated with pulmonary hypertension in HIV-infected patients on regular follow-up. *Aids* 26: 1387-1392.
Redout EM, van der Toorn A, Zuidwijk MJ, et al. (2010) Antioxidant treatment attenuates pulmonary arterial hypertension-induced heart failure. *Am J Physiol Heart Circ Physiol* 298: H1038-1047.
Ren SX, Zhan B, Lin Y, et al. (2019) Selenium Nanoparticles Dispersed in Phytochemical Exert Anti-Inflammatory Activity by Modulating Catalase, GPx1, and COX-2 Gene Expression in a Rheumatoid Arthritis Rat Model. *Med Sci Monit* 25: 991-1000.
Sharma S, Ruffenach G, Umar S, et al. (2016) Role of oxidized lipids in pulmonary arterial hypertension. *Pulm Circ* 6: 261-273.

Smukowska-Gorynia A, Rzymski P, Marcinkowska J, et al. (2019) Prognostic Value of Oxidative Stress Markers in Patients with Pulmonary Arterial or Chronic Thromboembolic Pulmonary Hypertension. *Oxid Med Cell Longev* 2019: 3795320.

Sydykov A, Mamazhakypov A, Petrovic A, et al. (2018) Inflammatory Mediators Drive Adverse Right Ventricular Remodeling and Dysfunction and Serve as Potential Biomarkers. *Front Physiol* 9: 609.

Wang X, Shults NV and Suzuki YJ. (2017) Oxidative profiling of the failing right heart in rats with pulmonary hypertension. *PLoS One* 12: e0176887.

Zhang M, Chang Z, Zhao F, et al. (2019) Protective Effects of 18β-Glycyrrhetinic Acid on Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats. *Front Pharmacol* 10: 13.

Zuber J-P, Calmy A, Evison JM, et al. (2004) Pulmonary Arterial Hypertension Related to HIV Infection: Improved Hemodynamics and Survival Associated with Antiretroviral Therapy. *Clinical Infectious Diseases* 38: 1178-1185.

CHAPTER 3

Manuscript

Role of Apoptosis signal regulating kinase-1 (ASK-1) in monocrotaline-induced Pulmonary hypertension following administration of antiretroviral medications in rat model.

Submitted to FREE RADICAL BIOLOGY AND MEDICINE

*(**Ref No**:* FRBM-D-20-00235*)*

Role of Apoptosis signal regulating kinase-1 (ASK-1) in monocrotaline-induced Pulmonary hypertension following administration of antiretroviral medications in rat model.

Adeoti AO[1], Nadar A[1], and Channa ML[1]

[1]Department of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, South Africa

ABSTRACT

Background: Apoptosis resistance is a recognized pathogenetic mechanism in pulmonary hypertension (PH). However, the link between apoptosis signal-regulating kinase-1 (ASK-1) and PH is unclear. The present study was conducted to elucidate ASK-1 as a potential biomarker in PH. The study aimed to identify the role of ASK-1 in the mechanism of monocrotaline-induced PH in rats.

Methods: Forty adult male Sprague-Dawley rats (body weight: 200-250 g) were randomly divided into five groups (n=8 per group). The treatment groups received a single intraperitoneal injection of MCT (60 mg kg^{-1}) while the control group received an equivalent volume of intraperitoneal saline injection. Zidovudine (100 mg kg^{-1}), ritonavir (30mg kg-1), or combination of both drugs (zidovudine 100mg kg-1 and ritonavir 30mg kg-1) were administrated daily for the study period of 28 days to the rats in three of the four groups with MCT for 28 days respectively. On the twenty-eight day of the study, rats were sacrificed, and organ harvested with the heart analyzed using RT-PCR for ASK-1. Antioxidant enzyme activities were determined using the colorimetric method.

Results: Animal survival rate was one hundred percent in the treated and control groups while the untreated group recorded 62% survival rate. There was significantly lower mRNA gene expression of ASK-1 in the heart of the treated rats with zidovudine (2.67 ± 0.09, $p < 0.0001$), ritonavir (2.57 ± 0.11, $p < 0.0001$) and a combination of both (2.75 ± 0.06, $p < 0.0001$) when compared to rats in the untreated group. An overexpressed mRNA gene of ASK-1 in the untreated rats (12.0 ± 0.90, $p < 0.0001$) when compared to the control.

Conclusion: ASK-1 is a veritable biomarker for anti-apoptotic characteristics of PH. Our findings will spur new investigations on the role of ASK-1 in PH and the potential therapeutic benefits of antiretroviral medications in the prevention of PH.
Keywords: Pulmonary hypertension, apoptosis signal-regulating kinase 1 (ASK-1), zidovudine, ritonavir, HIV/AIDS.

INTRODUCTION

Pulmonary hypertension (PH) is a fatal progressive disease involving an increase in pulmonary vascular resistance and pulmonary arterial pressure (PAP) which leads to right ventricular (RV) dysfunction, right heart failure and ultimately death (Zhang et al., 2017; Varshney et al., 2016). It involves vascular endothelial dysfunction, chronic inflammation, smooth muscle cell proliferation, pulmonary arteriolar occlusion, apoptosis resistance, and the hallmark feature of pulmonary vascular remodelling (Zhang et al., 2019; Cai et al., 2018). During this process, the pulmonary vascular pathology evolves from an inert state to pro-proliferative, apoptotic-resistant vascular cells (Gamen et al., 2016).

Apoptosis signal-regulating kinase-1 (ASK-1) is an upstream kinase in the p38 mitogen-activated protein kinases (MAPKs) signalling pathway that respond to stimuli by activating cellular proliferation, differentiation, survival, apoptosis, and inflammation (Qin et al., 2016; Tesch et al., 2015). Its inhibition has been associated with decline in vascular remodelling in MCT-induced PH experimental rats however the contrary is observed when activated (Qin et al., 2016; Budas et al., 2018). The activation of apoptotic pathway by the continuous induction of ASK-1 induces mitochondria-dependent caspases, triggering the intrinsic apoptosis pathway which regulates programmed cell death (Tesch et al., 2015). ASK-1 is activated in response to several stimuli most especially to oxidative stress and it is widely expressed in diverse tissues like heart and lungs (Courboulin et al., 2011; Tesch et al., 2015).

Human immunodeficiency virus infection is a known risk for the development of severe PH, however, the prevalence of HIV associated PH (HIV-PH) has remained stable from the pre-anti-retroviral therapy era (Opravil and Sereni, 2008). The impact of antiretroviral medications on the development of PH is controversial, however, nucleoside reverse transcriptase inhibitors (NRTI) and protease inhibitors like zidovudine and ritonavir respectively have been reported to improve outcome and survival of HIV-PH (Pugliese et al., 2000; Gary-Bobo et al., 2010). Animal models of PH have been developed, following the administration of monocrotaline and other techniques, which gives a good representation of the expected findings in humans (Nogueira-Ferreira et al., 2015). Using this modality to explore the effects of anti-retroviral therapy on the development

of PH with the aim of identifying potential biomarker would give clarity to the pathogenesis of PH.

Despite the current therapeutic advances such as endothelin-1 receptor blockers, type 5 phosphodiesterase inhibitors or platelet derived growth factors (PDGF) receptor blockers in the management of PH, no available literature has explored the role of antiretroviral medications in the development of PH using ASK-1 as a biomarker (Courboulin et al., 2011). The study aimed at investigating the effect of antiretroviral medication on ASK-1 in monocrotaline-induced PH in rats.

MATERIALS AND METHODS

Animals

Forty adult male Sprague Dawley rats (weighing 200-250 g) were supplied by the Biomedical Resource Unit of the University of KwaZulu-Natal, South Africa, and allowed to acclimatize for one week before commencement of the experiments. All the rats were housed in standard cages under standard laboratory conditions of 18-22 ^{o}C room temperature, 50-70% humidity and 12 hours light/dark cycle with *ad libitum* access to dry feed and water. Animal experiment was approved by the Animal Research Ethics Committee of the institution (AREC/066/018M) and the use of rats complied with the 3R guidelines (3R: Replace, reduce, and refine) for the use of experimental animals (Nam et al., 2018).

Experimental design

The 40 rats were randomly assigned into five groups (n=8), while the treatment groups received a single intraperitoneal injection of monocrotaline (MCT) at the dose of 60 mg. kg^{-1}), the control group received equivalent volume of intraperitoneal saline injection. MCT (Replamed, South Africa) was dissolved in 1N HCl and buffered to pH 7.0 with 1N NaOH before injection. Subsequently, a daily dose of zidovudine (100mg. kg-1), ritonavir (30 mg. kg-1) and combination of each zidovudine/ritonavir was administered to rats in three of the four treatment groups respectively for 28 days.

RNA extraction and measurement by RT-quantitative PCR.

Total RNA was extracted from lung tissues using Trizol (Zymo research) agent. Quantitative real-time RT-PCR (qPCR) was performed to assess mRNA expression of the following genes. The primer for the genes were as follows: ASK-1 (FW, 5'-TGAATCTGAGCCAACACTACAG-3' and RV, 5'-CATCAGCAAGCACGTGCCAAA-3'), GAPDH (FW, 5'-TGATTCTACCCACGGCAAGTT-3' and RV, 5'-TGATGGGTTTCCCATTGATGA-3') which were purchased from Inqaba (South Africa). This primer for gene expression was designed

using published sequence information from previous related study (Kherrouche et al., 2006). PCR Detection System (BioRad Co., CA, USA) was employed to execute Real-Time Polymerase Chain Reaction (PCR) with 0.1 mL tube containing 2 µL cDNA, 2 µL dNTP (1.25 mM each nucleotide), 1 µL reverse and forward primer, 1 µL dH20 and 5 µL SYBRGreen . The PCR conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 60 s at 95 °C, 30 s at annealing temperature, and 60 s at 72 °C. We verified the specificity of PCR by measuring the melting curve of the PCR products at the end of the reaction. Fluorescence data were specified for collection during primer extension. The relative expression of mRNA to GAPDH mRNA was calculated from the cycle threshold (Cq) value by a delta delta quantification cycle (ΔΔCq) method and presented as a relative to control (Coates et al., 2017).

Histomorphometric analysis

The harvested lung and heart tissues were placed with 10% formalin, embedded at 4℃ in paraffin, and sliced to a thickness of 4 µm. Subsequently, the sections were stained with the hematoxylin staining method for 5 min, washed under running water for 1 min, dissimilated 30 s in hydrochloric acid ethanol, and immersed in tap water for 15 min or warm water (about 50℃) for 5 min. Sections were stained for 2 min this time with eosin, each section was mounted with DPX and a coverslip. The histological changes were observed under an optical microscope (Leica DM 500, Germany). Ten fields containing terminal arterioles (50–150 mm external diameters) were randomly selected and the dimensions of the external elastic lamina and the internal elastic lamina of each vessel were measured. The wall thickness (percent) of the arterioles were categorized depending on the degree of vascular muscularization as 1–3, of which: 1=no muscularization, not occluded; 2=partial muscularization, not fully occluded; 3=muscularization, fully occluded(Han et al., 2016).

Drugs

MCT (Sigma-Aldrich, St. Louis, MO, USA) was purchased from Replamed, SA. Zidovudine was obtained from Aspen Pharmacare Limited (Aspen, South Africa) while ritonavir was obtained from Abbvie Limited (South Africa). Previous animal studies involving rats were used as template for the dosage of the medications (Gary-Bobo et al., 2010; Adebiyi et al., 2015).

Data Analysis

Data analysis was performed using one-way ANOVA for statistical differences with Tukey post-hoc tests. Differences for all tests were considered significant when the P value was <0.05 and data recorded as mean ± standard error of mean (SEM). All the analyses were performed using GraphPad Prism, version 6 (GraphPad, San Diego, CA, USA)

RESULTS

Animal survival rate

All rats survived the 1-, 2-, and 3-week and rats in the control and treated groups remained active even till the end of the study. In contrast, three rats in the group treated with MCT only, died in the 4th week of the study on days on days 23 and 25 with 38% death rate at the end of the experiment ($p = 0.0097$). During the study, none of the rats in the control group (unexposed) died as well as those on antiretroviral medication viz a viz zidovudine and ritonavir or a combination of both drugs.

Figure 1: Survival rate of the experimental animals within 28 days. Values are expressed as percent survival.

Relative organ weight

There was no significant difference ($p = 0.3165$) in the relative heart weights (RHW) of rats across all groups: control (0.38 ± 0.02%), MCT (0.31 ± 0.01%), MCT and zidovudine (0.48 ± 0.03%), MCT and ritonavir (0.41 ± 0.02%) and MCT and a combination of zidovudine and ritonavir (0.43 ± 0.01%).

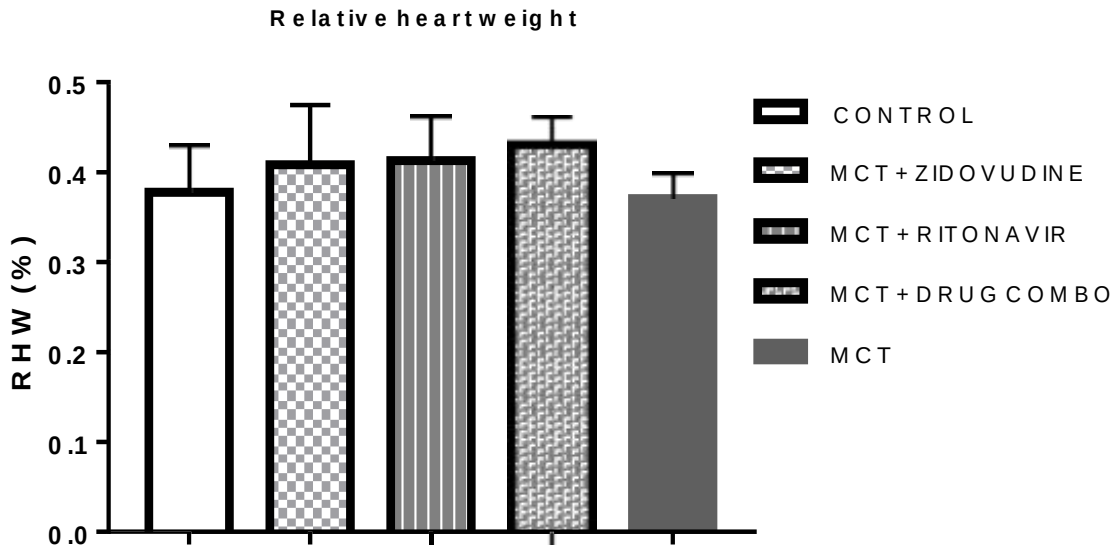

Figure 2: Relative heart weight (RWH) of the experimental animals

Values are expressed as mean ± standard error of mean (SEM).

Assessment on the relative mRNA expression of ASK-1 in rat heart

Figure 3 shows an overexpression of ASK-1 in the heart tissue of only MCT treated rats (12.0 ± 0.90, $p < 0.0001$) when compared to the rats in the control group. The mRNA expressions in the hearts of rats with zidovudine (2.67 ± 0.09, $p < 0.0001$), ritonavir (2.57 ±0.11, $p < 0.0001$) and a combination of both (2.75 ± 0.06, $p < 0.0001$) were lower and statistically significant when compared to rats in the untreated group. Likewise, the mRNA expression of ASK-1 was similar to the control rats (2.22 ± 0.08) but not statistically significant.

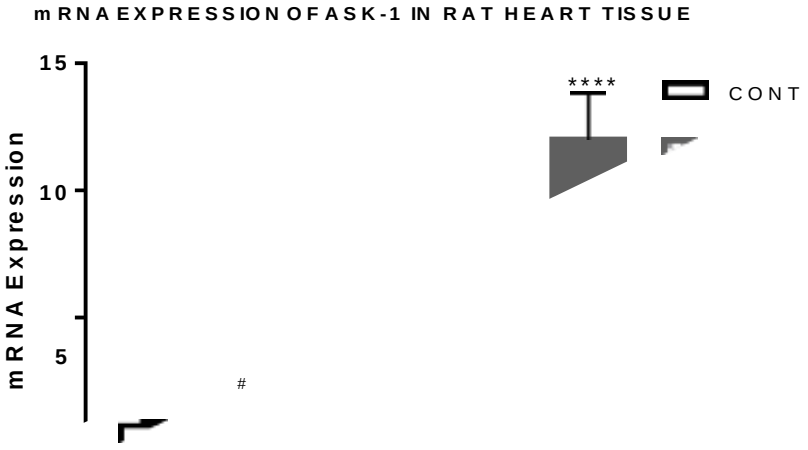

Figure 3: Relative mRNA expression of ASK-1 in the heart tissue of experimental rats

Values are expressed as mean ± standard error of mean (SEM). In comparison with the control, $^{*}P < 0.05$, $^{**}P < 0.01$, ***$P < 0.0001$ while in comparison with MCT $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$. MCT + DRUG COMBO represents MCT plus a combination of zidovudine and ritonavir.

Histopathological indices

The light microscope examination of the slides with representative pictorial features shows marked thickening in the media walls of the muscular pulmonary arteries in the lungs of the untreated rats following MCT administration. The rats with anti-retroviral drugs (zidovudine, ritonavir and both drugs combined) show significant improvement in their media wall thickness when compared to the untreated MCT group.

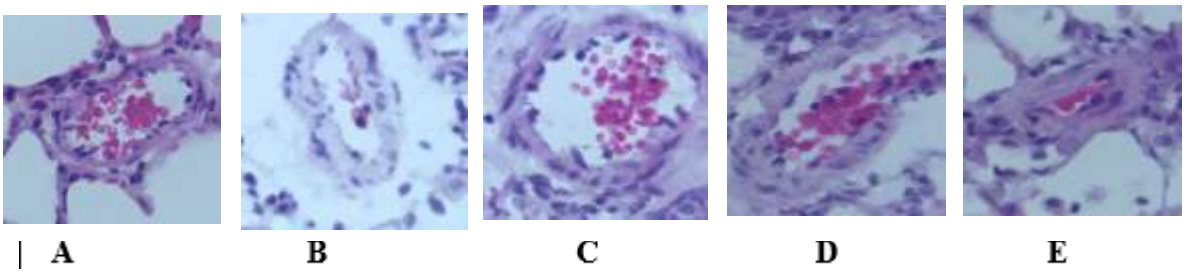

Figure 4a-Representative images of lung stained with H and E for the different groups (Image A- Control, Image B- MCT+Drug A, Image C- MCT+Drug B, Image D- MCT+Drugs combo and Image E- MCT)

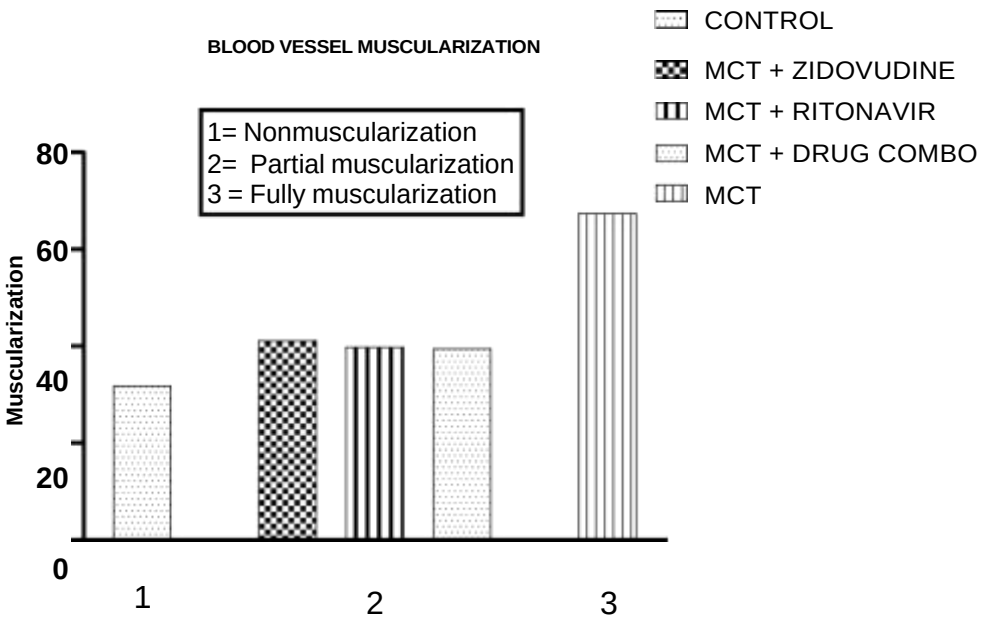

Figure 4b shows degree of pulmonary arterioles muscularization in lung tissues of experimental rats.

Discussion

In this study, there was a reduction in the animal survival rate in the only MCT treated group but no significant difference in the relative heart weight in the experimental groups. Our study showed a significantly lower mRNA gene expression of ASK-1 in the heart tissues of the treated rats when compared to rats in the untreated group as well as an overexpressed mRNA gene of ASK-1 in the untreated rats relative to the control.

The survival rate of the untreated rats dropped to 87.0% on day 23 which further declined to 62.0% by day 25. This reduction in the animal survival rate of untreated rats after the three weeks was similar to previous studies where survival rates of 70% to 90% was observed by the 21st days following MCT injection (Itoh et al., 2004; Nagaya et al., 2003). As observed in our study and previous studies, rats were reported to have died after the third week of MCT administration in the untreated rats. This findings could be due to the manifestations of PH in the rats due to the MCT and this has been reported to be maximum after this period (Ruiter et al., 2013). Therefore, the therapeutic effects of the antiretroviral in the prevention of mortality among the rats in the treatment would suggest its beneficial role in mitigating the development of PH in these groups.

In our study, the ASK-1 mRNA gene expression in the treated rats was higher compared to the control rats but lower than in the untreated rats. This agreed with previous studies where ASK-1 stimulation of cellular apoptotic response plays a major role in the signalling pathways and treatment modalities (Kolliputi and Waxman, 2009; Kherrouche et al., 2006). ASK-1 is fundamental to programmed cell death, of which its failure affects apoptosis, and its inhibition reduced pathological remodelling of the pulmonary vasculature and the right ventricle and this progresses to pulmonary hypertension in rodent models(Xu et al., 2016).

In a similar study involving monocrotaline-induced pulmonary hypertension in rats, the overexpression of ASK-1 mRNA gene in the untreated rats in relation to the control group could be evidence of the ASK-1 resistance which is anti-apoptotic (Budas et al., 2018). Such presentations of apoptosis-resistant could manifest as increased proliferative vascular changes of the endothelial cells (EC) thereby obliterating the vessels due to the anti-apoptotic effects (Suzuki et al., 2016).

The histopathology results of the lungs revealed marked thickening in the media walls of the muscular pulmonary arteries in the untreated rats. However, improvement in this media wall thickness was observed in the rats treated with zidovudine and or ritonavir following the MCT-induced PH. This histologic finding is comparable to those reported prominent medial hypertrophy of the muscular pulmonary arteries in MCT-induced PH rats (Lee et al., 2005). This is an indication that zidovudine, ritonavir, and a combination of both drugs were able to mitigate the deteriorative effects of MCT.

In conclusion, this research indicates that zidovudine and ritonavir attenuate MCT-induced PH in rats by down-regulation of ASK-1 which plays a major role in the anti-apoptotic characteristics in the development of PH.

Abbreviations CTRL: Control; Combo: Combination; HIV: Human immunodeficiency virus; MCT: Monocrotaline; PH: Pulmonary hypertension; RV: Right ventricle; S: Septum; SEM: Standard error of the mean.

Acknowledgments**:** This study was supported by the research grant provided by CHS of the University of KwaZulu-Natal, South Africa.

Competing interests: The authors declare that they have no competing interests.

REFERENCES

Adebiyi OO, Adebiyi OA and Owira PM. (2015) Naringin Reverses Hepatocyte Apoptosis and Oxidative Stress Associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-Induced Metabolic Complications. *Nutrients* 7: 10352-10368.

Budas GR, Boehm M, Kojonazarov B, et al. (2018) ASK1 Inhibition Halts Disease Progression in Preclinical Models of Pulmonary Arterial Hypertension. *Am J Respir Crit Care Med* 197: 373-385.

Cai Z, Li J, Zhuang Q, et al. (2018) MiR-125a-5p ameliorates monocrotaline-induced pulmonary arterial hypertension by targeting the TGF-beta1 and IL-6/STAT3 signaling pathways. *Exp Mol Med* 50: 45.

Coates DE, Zafar S and Milne TJ. (2017) Quantitative Real-Time Gene Profiling of Human Alveolar Osteoblasts. *Methods Mol Biol* 1537: 447-459.

Courboulin A, Tremblay VL, Barrier M, et al. (2011) Kruppel-like factor 5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial hypertension. *Respir Res* 12: 128.

Gamen E, Seeger W and Pullamsetti SS. (2016) The emerging role of epigenetics in pulmonary hypertension. *Eur Respir J* 48: 903-917.

Gary-Bobo G, Houssaini A, Amsellem V, et al. (2010) Effects of HIV protease inhibitors on progression of monocrotaline- and hypoxia-induced pulmonary hypertension in rats. *Circulation* 122: 1937-1947.

Han X, Zhang Y, Zhou Z, et al. (2016) Hydroxysafflor yellow A improves established monocrotaline-induced pulmonary arterial hypertension in rats. *J Int Med Res* 44: 569-584.

Itoh T, Nagaya N, Fujii T, et al. (2004) A combination of oral sildenafil and beraprost ameliorates pulmonary hypertension in rats. *Am J Respir Crit Care Med* 169: 34-38.

Kherrouche Z, Blais A, Ferreira E, et al. (2006) ASK-1 (apoptosis signal-regulating kinase 1) is a direct E2F target gene. *Biochem J* 396: 547-556.

Kolliputi N and Waxman AB. (2009) IL-6 cytoprotection in hyperoxic acute lung injury occurs via suppressor of cytokine signaling-1-induced apoptosis signal-regulating kinase-1 degradation. *Am J Respir Cell Mol Biol* 40: 314-324.

Lee YS, Byun J, Kim JA, et al. (2005) Monocrotaline-induced pulmonary hypertension correlates with upregulation of connective tissue growth factor expression in the lung. *Exp Mol Med* 37: 27-35.

Nagaya N, Okumura H, Uematsu M, et al. (2003) Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *Am J Physiol Heart Circ Physiol* 285: H2125-2131.
Nam MH, Chun MS, Seong JK, et al. (2018) Ensuring reproducibility and ethics in animal experiments reporting in Korea using the ARRIVE guideline. *Lab Anim Res* 34: 11-19.
Nogueira-Ferreira R, Vitorino R, Ferreira R, et al. (2015) Exploring the monocrotaline animal model for the study of pulmonary arterial hypertension: A network approach. *Pulm Pharmacol Ther* 35: 8-16.
Opravil M and Sereni D. (2008) Natural history of HIV-associated pulmonary arterial hypertension: trends in the HAART era. *Aids* 22: S35-S40.
Pugliese A, Isnardi D, Saini A, et al. (2000) Impact of highly active antiretroviral therapy in HIV-positive patients with cardiac involvement. *J Infect* 40: 282-284.
Qin N, Yang W, Feng D, et al. (2016) Total ginsenosides suppress monocrotaline-induced pulmonary hypertension in rats: involvement of nitric oxide and mitogen-activated protein kinase pathways. *J Ginseng Res* 40: 285-291.
Ruiter G, de Man FS, Schalij I, et al. (2013) Reversibility of the monocrotaline pulmonary hypertension rat model. *Eur Respir J* 42: 553-556.
Suzuki YJ, Ibrahim YF and Shults NV. (2016) Apoptosis-based therapy to treat pulmonary arterial hypertension. *J Rare Dis Res Treat* 1: 17-24.
Tesch GH, Ma FY, Han Y, et al. (2015) ASK1 Inhibitor Halts Progression of Diabetic Nephropathy in Nos3-Deficient Mice. *Diabetes* 64: 3903-3913.
Varshney R, Ali Q, Wu C, et al. (2016) Monocrotaline-Induced Pulmonary Hypertension Involves Downregulation of Antiaging Protein Klotho and eNOS Activity. *Hypertension* 68: 1255-1263.
Xu D, Li Y, Zhang B, et al. (2016) Resveratrol alleviate hypoxic pulmonary hypertension via anti-inflammation and anti-oxidant pathways in rats. *Int J Med Sci* 13: 942-954.
Zhang M, Chang Z, Zhao F, et al. (2019) Protective Effects of 18beta-Glycyrrhetinic Acid on Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats. *Front Pharmacol* 10: 13.
Zhang Z, Zhang L, Sun C, et al. (2017) Baicalin attenuates monocrotaline-induced pulmonary hypertension through bone morphogenetic protein signaling pathway. *Oncotarget* 8: 63430-63441.

CHAPTER 4: SYNTHESIS, CONCLUSION AND RECOMMENDATION

4.1 Synthesis

ROS are reactive chemical species containing oxygen which include molecules such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$) (Pan et al., 2010). They play a vital role in the regulation of biological processes such as cell proliferation, differentiation, apoptosis, and cellular senescence which are important in aging, cancer and pulmonary vascular diseases (Finkel, 2003; Rana, 2008).

Apoptosis (programmed cell death) is a strictly controlled process in which cells undergo auto-destruction due to DNA damage, senescence, inflammation or oxidative stress (Kerr et al., 1972). The regulation of apoptosis is crucial for the maintenance of normal cellular homeostasis; however, the deregulation of apoptosis has been linked to several pathologies (Fulda et al., 2010). Oxidative stress which results from an imbalance in the concentration of pro-oxidants which are ROS and antioxidant within cells, is majorly stimulated by apoptosis. Reactive oxygen containing molecules such as H_2O_2 can cause the release of cytochrome *c* from mitochondria into the cytosol and also activate nuclear transcription factors, like NF-κB, AP-1, and p53 and other proteins or genes which are also involved in the apoptotic pathway. Similarly, nitric oxide has been reported to induce apoptosis by increasing ceramide generation through caspase-3 activation, induction of mitochondrial permeability transition, and activation of the Fas system (Denning et al., 2002).

ASK-1 is one of the ROS-regulated inducers of apoptosis. It is a member of the MAP3K family of protein kinases that could regulate a wide range of physiological processes ranging from cell proliferation, differentiation and apoptosis (Ichijo et al., 1997; Takeda et al., 2011; Tobiume et al., 2001). Oxidative stress induces ASK-1-dependent production of inflammatory cytokines such as tumour necrotic factor-α, interleukin-6, and interleukin-1β which are necessary for apoptosis (Nakagawa et al., 2011). Although ASK-1 is abundant in the cells, but it is usually repressed by thiol-containing antioxidant proteins in the cytosol and mitochondria. Furthermore, the liberal production of ROS could overwhelm the anti-oxidant defence system resulting in oxidative stress and the activation of ASK-1(Budas et al., 2018; Saitoh et al., 1998).

There is increasing evidence within the literature that ROS contribute significantly to apoptosis and ultimately play a vital role in the pathophysiology of PH (Xu et al., 2011; Matsuzawa et al., 2005). Our study demonstrated an increased oxidative stress with accompanied lipid peroxidation in the untreated group (without antiretroviral medications). On the contrary, the treated group showed a reduction in oxidative stress and evidence of lipid peroxidation. Lipid peroxidation has

been implicated in studies as a mediator in the development of PH as therapy against lipid peroxidation has been found effective in the treatment of PH (Mohammadi et al., 2012). Similar to previous studies, the higher MDA levels were found in PH compared to the control and the treated groups (Wen et al., 2019; Jin et al., 2008). On the contrary, Awodele et al. found a non-significant decrease in the MDA level of zidovudine treated rats when compared with other groups which could be due to the lower dose of zidovudine and the combination of the antiretroviral with antituberculosis drugs in their study (Awodele et al., 2011).

The finding of decreased lipid peroxidation marker MDA in rats and an accompanying elevated TAC in the antiretroviral treated MCT-induced pulmonary hypertension rats supports theunlikely development of PH. Hence the treatment with zidovudine and/or ritonavir ameliorated these oxidative stress indices. The level of TAC was lowest in the untreated rats compared to thecontrol and treated groups. This decrease in TAC in our study is similar to findings in other related studies where low TAC is reported in untreated animal groups (Liu et al., 2019). ROS enhances endothelin-1 production and down-regulation of eNOS expression leading to PH (Fulton et al., 2017).

An upregulation of ASK-1 gene would result in uncontrolled activity in the ASK-1-p38 pathway and ultimately results in apoptosis. Our study also revealed that pulmonary hypertension induced by MCT in rats heart tissues of rats significantly increased the expression of ASK-1 when compared with the control group. The administration of antiretroviral drugs (zidovudine and/or ritonavir) significantly reduced oxidative stress induced by exposure to MCT and resulted in the downregulation of ASK-1 mRNA expression. The reversal of MCT-induced oxidative stress and ASK-1 overexpression by the anti-retroviral drugs as observed could be due to an unexplained interplay of actions of the MCT and antiretroviral although other studies has associated most anti-retroviral drugs to oxidative stress (Manda et al., 2011; Sharma, 2014; Williams et al., 2017). This is not surprising as it has been elucidated that reduced levels of antioxidants results in the continued activation on ASK-1 (Budas et al., 2018; Saitoh et al., 1998)

4.2 Conclusion

Our study demonstrated that the antiretroviral drugs namely zidovudine, ritonavir or the combination of the two drugs ameliorated the distortion in the oxidant-antioxidant system in rats exposed to MCT. However, antiretroviral may not be sufficient as a sole therapy for HIV-PH, hence its combination with PH-specific therapy is of paramount importance. Further

studies are also required to ascertain the role of oxidative stress following antiretroviral medications in the reversal of PH.

4.3 Limitations

A limitation of our study is the non-pressure measurement by right heart catheterization which is an invasive procedure to establish an increased pressure in the right ventricle due to PH.

4.4 Recommendation

The data from this research further highlights the need to explore the use of ASK-1 as a biomarker for the anti-apoptotic characteristics of PH. Although the antiretroviral medications ameliorated the distortion in the oxidant-antioxidant system in rats exposed to MCT. It may not be sufficient as a sole therapy for HIV-PH, hence its combination with PH-specific therapy could be paramount to the understanding of this disease entity. Further studies are also required to ascertain the role of inflammatory cytokines such as tumour necrotic factor -α, interleukin-6, and interleukin-1β in ROS-ASK-1 linkage.

REFERENCES

Awodele O, Agbaje EO, Adesina EA, et al. (2011) Hepatoprotective role of neutrosecR on hepatic damage induced by combination of zidovudine and combined anti-tuberculous agents in rats. *Tokai J Exp Clin Med* 36: 31-36.

Budas GR, Boehm M, Kojonazarov B, et al. (2018) ASK1 Inhibition Halts Disease Progression in Preclinical Models of Pulmonary Arterial Hypertension. *Am J Respir Crit Care Med* 197: 373–385.

Denning TL, Takaishi H, Crowe SE, et al. (2002) Oxidative stress induces the expression of Fas and Fas ligand and apoptosis in murine intestinal epithelial cells. *Free Radical Biology & Medicine* 33: 1641–1650.

Finkel T. (2003) Oxidant signals and oxidative stress. *Curr Opin Cell Biol* 15: 247-254.

Fulda S, Gorman AM, Hori O, et al. (2010) Cellular stress responses: cell survival and cell death. *Int J Cell Biol* 2010: 214074.

Fulton DJR, Li X, Bordan Z, et al. (2017) Reactive Oxygen and Nitrogen Species in the Development of Pulmonary Hypertension. *Antioxidants (Basel)* 6.

Ichijo H, Nishida E, Irie K, et al. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275: 90-94.

Jin HF, Du SX, Zhao X, et al. (2008) Effects of endogenous sulfur dioxide on monocrotaline-induced pulmonary hypertension in rats. *Acta Pharmacol Sin* 29: 1157-1166.

Kerr JF, Wyllie AH and Currie AR. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26: 239-257.

Liu Z, Duan YL, Ge SL, et al. (2019) Effect of estrogen on right ventricular remodeling of monocrotaline-induced pulmonary arterial hypertension in rats and its mechanism. *Eur Rev Med Pharmacol Sci* 23: 1742-1750.

Manda KR, Banerjee A, Banks WA, et al. (2011) Highly active antiretroviral therapy drug combination induces oxidative stress and mitochondrial dysfunction in immortalized human blood-brain barrier endothelial cells. *Free Radic Biol Med* 50: 801-810.

Matsuzawa A, Saegusa K, Noguchi T, et al. (2005) ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. *Nat Immunol* 6: 587-592.

Mohammadi S, Najafi M, Hamzeiy H, et al. (2012) Protective effects of methylsulfonylmethane on hemodynamics and oxidative stress in monocrotaline-induced pulmonary hypertensive rats. *Adv Pharmacol Sci* 2012: 507278.

Nakagawa H, Hirata Y, Takeda K, et al. (2011) Apoptosis signal-regulating kinase 1 inhibits hepatocarcinogenesis by controlling the tumor-suppressing function of stress-activated mitogen-activated protein kinase. *Hepatology* 54: 185-195.

Pan J, Chang Q, Wang X, et al. (2010) Reactive oxygen species-activated Akt/ASK1/p38 signaling pathway in nickel compound-induced apoptosis in BEAS 2B cells. *Chem Res Toxicol* 23: 568-577.

Rana SV. (2008) Metals and apoptosis: recent developments. *J Trace Elem Med Biol* 22: 262-284.

Saitoh M, Nishitoh H, Fujii M, et al. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17: 2596–2606.

Sharma B. (2014) Oxidative stress in HIV patients receiving antiretroviral therapy. *Curr HIV Res* 12: 13-21.

Takeda K, Naguro I, Nishitoh H, et al. (2011) Apoptosis signaling kinases: from stress response to health outcomes. *Antioxid Redox Signal* 15: 719-761.

Tobiume K, Matsuzawa A, Takahashi T, et al. (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2: 222-228.

Wen J, Wang J, Tang X, et al. (2019) DHT deteriorates the progression of monocrotaline-induced pulmonary arterial hypertension: effects of endogenous and exogenous androgen. *Am J Transl Res* 11: 5752-5763.

Williams AA, Sitole LJ and Meyer D. (2017) HIV/HAART-associated oxidative stress is detectable by metabonomics. *Mol Biosyst* 13: 2202-2217.

Xu D, Guo H, Xu X, et al. (2011) Exacerbated pulmonary arterial hypertension and right ventricular hypertrophy in animals with loss of function of extracellular superoxide dismutase. *Hypertension* 58: 303-309.

APPENDIX – AREC APPROVAL LETTER

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

31 May 2019

Dr Adekunle Olatayo Adeoti (218058908)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Dr Adeoti,

Protocol reference number: AREC/066/018M
Project title: Biomarkers and Histopathologic changes in rats with Monocrotaline-induced Pulmonary Hypertension following administration of Antiretroviral medications

Full Approval – Research Application

With regards to your revised application received on 26 March 2019. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 31 May 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, PhD
Acting Chair: Animal Research Ethics Committee

/ms

cc Supervisor: Dr Anand Nadar
Cc Academic Leader Research: Dr Brenda de Gama
Cc Registrar: Mr Simon Mokoena
Cc BRU – Dr Linda Bester

ANIMAL RESEARCH ETHICS COMMITTEE
Postal Address: Private Bag X54001, Durban, 4000 South Africa
Telephone: +27 (0)31 260 8350 **Facsimile:** +27 (0)31 260 3093 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville