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The effects of chronic administration of Cannabidiol and Cannabidiol-Selexipag combination on haematological parameters, oxidative stress, BNP, and TNF-α in a rat model of Pulmonary Arterial Hypertension

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Abstract

Background: Pulmonary arterial hypertension (PAH) is a severe condition characterized by elevated pulmonary artery pressure, leading to progressive cardiovascular impairment and high mortality rates. Current therapies often fall short of providing adequate symptom relief and disease management. This study evaluates the combined therapeutic potential of Cannabidiol (CBD) and Selexipag, assessing their effects on key biomarkers and haematological parameters associated with PAH.

Methods: In a preclinical model, PAH was induced in rats using monocrotaline (MCT), followed by treatment with CBD, Selexipag, or their combination. Biomarkers including B-type natriuretic peptide (BNP), total antioxidant capacity (T-AOC), and tumour necrosis factor-alpha (TNF-α) were measured to evaluate cardiovascular and inflammatory responses. Additionally, haematological parameters such as platelet count, and haemoglobin levels were assessed. Statistical analysis was conducted using Welch's ANOVA and unpaired Welch's T-Tests to determine the significance oftreatment effects.

Results: The combination therapy of CBD and Selexipag was found to be significantly more effective in normalizing BNP and T-AOC levels compared to the individual treatments. This suggests that adjunctive therapy might enhance cardiovascular function and reduce oxidative stress.Despite CBD's established anti-inflammatory properties, elevated TNF-α levels in the MCT-CBDgroup indicated a potential exacerbation of inflammation. This finding is at odds with previous literature on CBD's effects, which may be influenced by specific experimental conditions or dosages. Haematological analysis revealed elevated platelet counts and haemoglobin levels, with increased platelet activation noted, which correlates with findings linking these parameters to PAH progression.

Discussion: The study underscores the potential of combining CBD with Selexipag as a promising approach for PAH management, showing improved biomarker profiles indicative of better cardiovascular function and oxidative stress mitigation. However, the unexpected rise in TNF-α levels with CBD treatment highlights the complexity of its anti-inflammatory effects and suggests that its therapeutic benefits may vary based on the disease context. Furthermore, changes in haematological parameters support existing literature linking elevated haemoglobin and platelet activation to PAH, emphasizing the need for careful monitoring of these parameters in clinical settings.

Conclusion: The combination of CBD and Selexipag demonstrates enhanced therapeutic potentialfor PAH compared to monotherapy, with promising improvements in key biomarkers. Nevertheless, the paradoxical increase in TNF-α and associated haematological changes necessitatefurther research to elucidate the mechanisms behind CBD's effects and optimize therapeutic strategies for PAH. Future studies should focus on understanding these complex interactions and exploring the full therapeutic potential of CBD and Selexipag in cardiovascular disease management.

Keywords: Pulmonary Arterial Hypertension, Cannabidiol, Selexipag, Brain Natriuretic Peptide, Total antioxidant capacity, Tumour Necrosis Factor Alpha, Monocrotaline, Platelet count, Haemoglobin levels

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A1 (Adenosine Receptor 1)
A2A (Adenosine Receptor 2A)
Akt (Protein Kinase B)
ANOVA (Analysis of Variance)
AREC (Animal Research Ethics Committee)
BNP (Brain Natriuretic Peptide)
BP (Blood Pressure)
BRU (Biomedical Resource Unit)
Ca^{2+} (Calcium Ions)
CBD (Cannabidiol)
cGMP (Cyclic Guanosine Monophosphate)
COX (Cyclooxygenase)
COPD (Chronic Obstructive Pulmonary Disease)
D-PBS (Dulbecco's Phosphate-Buffered Saline)
EDP (End Diastolic Pressure)
ELISA (Enzyme Linked Immunosorbent Assay)
ESP (End Systolic Pressure)
ENT (Equilibrative Nucleoside Transporters)
EP (Eppendorf)
ET-1 (Bone Morphogenic Protein Receptor Type 2 (BMPR2))
FasL (Fas Ligand)
FRAP (Ferric Reducing Ability of Protein)
GPR55 (G Protein-Coupled Receptor 55)
GSH (Glutathione)
GSSG (Glutathione Disulfide)
HCl (Hydrochloric Acid)
HCT (Haematocrit)
HGB (Haemoglobin)
HO-1 (Heme Oxygenase-1)
ICAM-1 (Intercellular Adhesion Molecule-1)
IFN-γ (Interferon Gamma)
IL-1β (Interleukin-1β)
IL-6 (Interleukin-6)
IP (Prostacyclin Receptor)
IP3 (Inositol 1,4,5-Trisphosphate)
IP3R (Inositol 1,4,5-Trisphosphate Receptor)
JNK (Jun N-Terminal Kinase)
KATP [Adenosine Triphosphate (ATP)-Sensitive Potassium]

MAPK (Mitogen-Activated Protein Kinase)
MCH (Mean Corpuscular Haemoglobin)
MCHC (Mean Corpuscular Haemoglobin Concentration)
MCT (Monocrotaline)
MCV (Mean Corpuscular Volume)
MMPs (Matrix metalloproteinases)
MPV (Mean Platelet Volume)
MRI (Magnetic Resonance Imaging)
NADPH (Nicotinamide Adenine Dinucleotide Phosphate)
NaOH (Sodium Hydroxide)
NCX (Sodium-Calcium Exchanger)
NF-κB (Nuclear factor kappa-B)
NO (Nitrogen Oxide)
NPPB (Natriuretic Peptide B)
Nrf2 (Nuclear Erythroid 2-Related Factor 2)
NT (N-Terminal)
OS (Oxidative Stress)
PARP [Poly(ADP-Ribose) Polymerase]
PBS (Phosphate Buffered Solution)
PCT (Procalcitonin)
PLT (Platelets)
PVR (Pulmonary Vascular Resistance)
RAA (Right Atrial Appendage)
RBC (Red Blood Cell)
RDW (Red Cell Distribution Width)
RhoA (Ras Homolog Family Member A)
RNS (Reactive Nitrogen Species)
ROCK (Rho-Associated Protein Kinase)
ROS (Reactive Oxygen Species)
RVH (Right Ventricular Hypertrophy)
SD (Sprague-Dawley)
SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase)
SOD (Superoxide Dismutase)
T-AOC (Total Antioxidant Capacity)
TGF-β (Transforming Growth Factor-Beta)
THC (Delta-9-Tetrahydrocannabinol)
TNF-α (Tumour Necrosis Factor Alpha)
TRPV1 (Transient Receptor Potential Vanilloid 1)
VCAM-1 (Vascular Cell Adhesion Molecule-1)
VSMC (Vascular Smooth Muscle Cell)
WBC (White Blood Cell)

Chapter 1:

Introduction

1.1 Background and Context

Pulmonary arterial hypertension (PAH) is a rare but increasingly recognized condition characterized by abnormally high blood pressure within the pulmonary arteries. This elevated pressure results from a multifaceted pathology involving vascular remodelling, vasoconstriction, and persistent inflammation (Humbert et al., 2014). PAH leads to severe morbidity and mortality, manifesting as symptoms such as exertional dyspnoea, chronic fatigue, and right heart failure (Simonneau et al., 2019). The pathophysiological mechanisms contributing to PAH include endothelial dysfunction, excessive proliferation of vascular smooth muscle cells (VSMCs), and inflammatory responses, all of which exacerbate vascular constriction and remodelling (Kurakula, 2021).

Endothelial dysfunction, a hallmark of PAH, involves impaired nitric oxide (NO) production and increased endothelin-1 (ET-1) levels, leading to abnormal vasoconstriction and remodelling of thepulmonary vasculature (Galiè et al., 2019). The excessive proliferation of VSMCs further contributes to the narrowing of the pulmonary arteries, which impairs blood flow and increases pressure (Böhm et al., 2017). Additionally, inflammation plays a crucial role, with elevated levels of inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) found in PAH patients (Kovacs et al., 2019). These factors collectively contribute to the progressive nature of the disease and its associated complications.

The prevalence of PAH has been rising, which is attributed to improved diagnostic techniques and greater awareness among healthcare providers (McLaughlin et al., 2019). Despite advancements inunderstanding the disease's pathophysiology, the management of PAH remains challenging. Conventional treatments, including endothelin receptor antagonists (e.g., bosentan), phosphodiesterase-5 inhibitors (e.g., sildenafil), and prostacyclin analogues (e.g., epoprostenol), have shown efficacy in improving symptoms and survival (Benza et al., 2019; McLaughlin et al., 2021). However, these therapies are often associated with significant side effects, such as headaches, gastrointestinal disturbances, and hypotension, which can limit their tolerability and effectiveness for some patients (Galiè et al., 2016).

Given these limitations, there is an ongoing need for innovative therapeutic approaches. Exploring alternative or adjunctive treatments could offer new avenues for addressing the unmet needs of PAH patients. Recent research has focused on novel pharmacological agents and non-traditional therapies, such as cannabinoids, which have shown potential in modulating vascular function and inflammation (Rajesh et al., 2011) (Kogan et al., 2021). These emerging therapies may offer improved efficacy and safety profiles compared to conventional treatments, thus providing valuableoptions for managing PAH.

1.2 Problem Statement

Current therapeutic approaches for PAH have seen advancements, with Selexipag- a selective prostacyclin receptor agonist- demonstrating effectiveness in alleviating symptoms and enhancingexercise capacity in PAH patients. Despite its efficacy, Selexipag and other conventional treatmentsface limitations in terms of long-term management and effectiveness for all patients. These treatments are often associated with adverse effects, including headaches, gastrointestinal disturbances, and hypotension, which can negatively impact patient adherence and overall quality of life.

Considering these challenges, there is growing interest in exploring alternative and adjunctive therapies. Cannabidiol (CBD), a non-psychoactive phytocannabinoid derived from cannabis, has garnered attention for its broad spectrum of therapeutic properties. Notably, CBD is recognised for its anti-inflammatory, immunomodulatory, and analgesic effects, which have been well-documented across various inflammatory and neurodegenerative conditions.

While preclinical and clinical research has highlighted the potential of CBD in managing conditions such as arthritis, multiple sclerosis, and neurodegenerative diseases, its specific application in PAH remains underexplored. The therapeutic potential of CBD in PAH is particularly intriguing given its ability to modulate inflammatory responses and oxidative stress- key contributors to PAH pathogenesis.

The hypothesis driving this study is that combining CBD with established PAH treatments like Selexipag could result in a synergistic effect, enhancing therapeutic outcomes through complementary mechanisms. This approach could address the limitations of existing treatments and improve patient outcomes by optimizing inflammation control and vascular function. Thus, this study aims to investigate the combined effects of CBD and Selexipag on key biomarkers of PAH, including oxidative stress and inflammatory markers, in a preclinical model. This research seeks to provide insights into the potential benefits of this novel combination therapy and its impact on managing PAH.

1.3 Research Objectives & Questions

The primary objective of this study is to explore the therapeutic potential of CBD, both as a standalone treatment and in combination with Selexipag, in a rat model of PAH induced by monocrotaline (MCT). Specific objectives include:

1. **Biomarker Assessment**: To determine the impact of CBD and Selexipag on the expressionlevels of inflammatory and hypoxic biomarkers, specifically Tumour Necrosis Factor (TNF-α) and Brain Natriuretic Peptide (BNP), in the context of PAH.
2. **Oxidative Stress Evaluation**: To assess how CBD and Selexipag influence oxidative stressby measuring Total Antioxidant Capacity (T-AOC) in the heart tissue and plasma of the PAHmodel.
3. **Comparative Efficacy**: To compare the effects of CBD and Selexipag individually and in combination, to understand their potential synergistic effects on PAH management.
4. **Haematological Assessmen**t: To assess any significance with regards to platelets and haemoglobin.

The research questions guiding this study are:

1. How does CBD, alone or in combination with Selexipag, affect the levels of TNF-α, BNP, haematology, specifically platelets, in the PAH model?
2. What is the effect of these treatments on oxidative stress, as measured by T-AOC, in the heartand blood?

1.4 Significance of the Study

This study holds significant potential to advance the treatment landscape for PAH by evaluating the therapeutic efficacy of CBD as an adjunctive or alternative treatment. The integration of CBD with established therapies like Selexipag could represent a pivotal shift in PAH management, given CBD's known anti-inflammatory and antioxidant properties. If the combined therapy proves effective, it could lead to the formulation of new, more comprehensive treatment regimens that leverage the synergistic effects of natural compounds and conventional drugs.

The findings from this research could contribute to several key areas:

1. **Enhanced Therapeutic Outcomes**: By combining CBD with Selexipag, this study may reveal an improved approach to managing PAH, potentially offering better symptom relief and enhanced quality of life for patients. The dual action of CBD on inflammatory and oxidative stress pathways could complement the effects of Selexipag, addressing current treatment limitations and providing a more robust therapeutic option.

2. **Reduction of Adverse Effects**: Current PAH treatments, while effective, are often accompanied by significant side effects, such as gastrointestinal issues, headaches, and hypotension. Incorporating CBD could mitigate some of these adverse effects, as its profile suggests potential benefits in reducing inflammation and oxidative stress without the same level of side effects associated with traditional medications.

3. **New Mechanistic Insights**: Understanding how CBD interacts with Selexipag could shed light on novel mechanisms of action in PAH treatment. This could enhance our understanding of how combined therapies can modulate disease pathways more effectively than single-agent treatments. Insights gained from this research may lead to the identificationof new therapeutic targets and strategies for managing PAH.

4. **Broader Implications for Related Conditions**: The successful application of CBD in PAH management may also have implications for other inflammatory and vascular diseases. By demonstrating the efficacy of CBD in a well-characterized PAH model, this research could pave the way for exploring its role in other conditions where inflammation and oxidative stress play a critical role.

5. **Advancement of Personalized Medicine**: The study may contribute to the growing field ofpersonalized medicine by identifying specific patient profiles that could benefit most from the combined therapy. Tailoring treatments based on individual responses to CBD and Selexipag could optimize outcomes and enhance the overall efficacy of PAH management strategies.

Overall, this research aims to provide a foundation for integrating natural compounds like CBD into conventional PAH treatments, potentially leading to more effective and patient-friendly therapeutic options. The study's results could prompt further research and clinical trials to validatethese findings and explore the broader applicability of CBD in various clinical settings.

1.5 Overview of Methodology

The experimental design of this study involved using a monocrotaline (MCT)-induced PAH rat model to simulate the pathological features of the disease. Forty male Sprague-Dawley rats were allocated into five distinct groups: Control, MCT-Control, MCT-Selexipag, MCT-CBD, and MCT-CBD-Selexipag. PAH was induced by administering MCT (60 mg/kg) intraperitoneally over a period of four days. Subsequently, from day twenty-one onwards, the treatment groups received oral gavage of CBD (10 mg/kg), Selexipag (3 mg/kg), or a combination of both. The study durationwas 31 days, during which various parameters were monitored, including gene expression of TNF-α and BNP, plasma BNP levels, and T-AOC in the heart and plasma. Haematological profiles werealso assessed to evaluate broader physiological impacts.

1.6 Outline of Thesis

This thesis is organised into the following chapters:

- **Chapter 2**: Literature Review - A comprehensive review of PAH pathophysiology, currenttreatment options, and the potential role of cannabinoids in therapeutic applications.
- **Chapter 3**: Research Methodology - Detailed description of the experimental design, procedures, and analytical techniques used in this study.
- **Chapter 4**: Research Findings - Presentation and analysis of the data collected, includingstatistical evaluations and comparisons between treatment groups.

- **Chapter 5**: Discussion and Analysis of Findings - Interpretation of the results in the contextof of existing research, implications for PAH treatment, and potential mechanisms of action.
- **Chapter 6**: Synthesis - Summary of key findings, limitations of the study, and recommendations for future research.

Chapter 2:

Literature Review

2.1 Pulmonary Arterial Hypertension

Pulmonary Arterial Hypertension (PAH) is a rare, progressive disorder characterized by elevated blood pressure in the pulmonary arteries, leading to significant morbidity and mortality. As a subclass of pulmonary hypertension, PAH is marked by a progressive increase in pulmonary vascular resistance and arterial pressure, which eventually results in right heart failure and premature death (Galiè et al., 2016). The global prevalence of PAH is estimated at approximately 1%, with a notable rise in older populations, particularly those over 65 years, where prevalence canreach up to 10% (Hoeper et al., 2016). The incidence is approximately 2 to 5 cases per million adults, with a higher prevalence in women, who are affected 3 to 5 times more frequently than menin the 30-60 age range (Simonneau et al., 2013). Recent studies have shown that PAH may be underdiagnosed, particularly in developing regions where healthcare access is limited, suggesting a potential increase in global prevalence as awareness and diagnostic capabilities improve (Galiè et al., 2019).

2.1.1 Pathogenesis and Risk Factors

The pathogenesis of PAH involves complex interactions between genetic, environmental, and physiological factors. Genetic predispositions are crucial, particularly for heritable PAH, where mutations in the bone morphogenetic protein receptor type 2 (BMPR2) gene are identified in approximately 80% of familial cases. Other mutations within the Transforming Growth Factor-beta (TGF-β) superfamily genes are found in about 5% of patients with heritable PAH (Simonneau et al., 2013). Emerging research is expanding the genetic landscape of PAH, identifying additional susceptibility genes and epigenetic modifications that contribute to disease development (Deng et al., 2018) (Runo & Loyd, 2003).

Environmental factors, including exposure to toxins, drugs, and infections, also play a significant role in PAH development. Substances such as appetite suppressants and certain chemotherapy agents have been linked to PAH, and recent studies suggest a connection between COVID-19 and PAH, possibly due to endothelial damage and subsequent vascular remodelling (Varga et al., 2020).

Chronic conditions such as connective tissue diseases (e.g., scleroderma) and chronic lung diseases(e.g., COPD) are also known to contribute to PAH (Humbert et al., 2014).

2.1.2 Clinical Presentation and Diagnosis

PAH is characterized by a sustained elevation in pulmonary arterial pressure, with mean pressuresexceeding 25 mm Hg at rest or 30 mm Hg during exercise. This condition results in progressive symptoms, including severe dyspnoea on exertion, fatigue, weakness, chest pain, and syncope (Farber & Loscalzo, 2004). Diagnosis is primarily based on right heart catheterization, which provides direct measurement of pulmonary arterial pressures and evaluates cardiac output and pulmonary vascular resistance (Anwar et al., 2016). Additional diagnostic tools, such as transthoracic echocardiography and advanced imaging techniques like cardiac MRI, offer valuableinformation on disease severity and progression (Yao et al., 2021). Non-invasive tests, including biomarkers like BNP, also play a role in assessing disease status and therapeutic response (Kovacs et al., 2019).

2.1.3 Current Treatment and Limitations

Current therapeutic strategies for PAH include endothelin receptor antagonists (e.g., bosentan, ambrisentan), phosphodiesterase-5 inhibitors (e.g., sildenafil, tadalafil), and prostacyclin analogues(e.g., epoprostenol, treprostinil) (Galiè et al., 2016). These treatments aim to improve exercise capacity, symptoms, and overall quality of life by targeting different pathways involved in PAH pathogenesis. However, they are often limited by side effects such as headaches, gastrointestinal disturbances, and interactions with other medications (Galie et al., 2019). Additionally, these therapies may not provide adequate relief for all patients, highlighting the need for novel and adjunctive therapeutic approaches.

2.1.4 Emerging Research

Recent research has explored new therapeutic avenues, including the use of cannabinoids like Cannabidiol (CBD). Preclinical studies have demonstrated that CBD has potential benefits due to its anti-inflammatory, antioxidative, and vasodilatory properties (Rajesh et al., 2011). CBD's effectson oxidative stress and inflammation may be particularly relevant for PAH, given the role of theseprocesses in disease progression. The combination of CBD with established treatments such as Selexipag- a selective prostacyclin receptor agonist- offers a promising approach to enhance therapeutic outcomes by targeting multiple pathways involved in PAH pathogenesis (Galiè et al., 2019). Ongoing research aims to determine the efficacy and safety of such novel therapies, potentially providing new options for patients with PAH and addressing unmet needs in the current treatment landscape.

2.2 Cannabinoids and the Endocannabinoid System

The endocannabinoid system is a comprehensive neuromodulatory network composed of cannabinoid receptors (CB_1, CB_2, and the ionotropic receptor transient receptor potential vanilloid 1 [$TRPV_1$]), endogenous cannabinoids (such as anandamide and 2-arachidonoylglycerol), and their associated regulatory, catabolic, and metabolic enzymes (including fatty acid amide hydrolase andmonoacylglycerol lipase). This system plays a crucial role in synthesizing and degrading endocannabinoids, which are widely distributed in both the central nervous system and peripheraltissues. The endocannabinoid system is integral to maintaining homeostatic balance across variousphysiological processes (Lu & Mackie, 2016) (Russo, 2016). Currently, there are two distinct cannabinoid receptors: CB_1 and CB_2. The CB_1 receptor is primarily located in the brain and various other neural tissues, where it plays a key role in modulating neurotransmission. In contrast, the CB_2 receptor is predominantly associated with immune cells, particularly lymphocytes, and isinvolved in regulating immune responses (Bosier et al., 2010). CB_2 receptors are expressed at muchlower levels in the central nervous system compared with CB_1 receptors and are primarily present in microglia and vascular tissues (Lu & Mackie, 2018). Under pathophysiological conditions suchas inflammation or tissue injury, there is an increase in CB_2 receptor expression levels in the cardiopulmonary and cardiovascular system which reflects a protective response to limit any tissueor cell injury (Steffens & Pacher, 2012). According to Bosier et al. (2010), both cannabinoid receptors regulate a variety of central and peripheral physiological functions that include neuronal development, energy metabolism as well as cardiovascular, respiratory, and even reproductive functions. The CB_1 and CB_2 cannabinoid receptors are G protein-coupled receptors that are recognised by a variety of endogenous ligands that activate multiple signalling pathways (Bosier et al., 2010).

Cannabis, more commonly known as ‘marijuana’ or ‘weed,’ is one of the most consumed psychoactive substances in the world. The primary cannabinoids that have been studied include delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) which is responsible for cannabis' physical and psychotropic effects (Millan & Millan-Guerrero, 2020). Exogenous cannabinoids (exocannabinoids) such as THC and CBD produce their biological effects through their interactionswith cannabinoid receptors, although it is not yet fully understood how the exact mechanism of their action works, but further studies are being conducted to help us better understand this. CBD is a nonintoxicating phytocannabinoid that rarely binds to CB_1 and CB_2 but acts as antagonists to these receptors, including $TRPV_1$ (Russo, 2016). Under in vitro conditions, it activates $TRPV_1$ andinhibits 5-HT3 receptors. Several studies suggest CBD has immense potential for therapeutic use as an agent with antiepileptic, antipsychotic, anxiolytic, analgesic, anti-inflammatory and neuroprotective properties, and has promising therapeutic implications in vascular diseases especially in the treatment of pulmonary hypertension (Kozlowska et al., 2007) (Lu & Mackie, 2016).

A well-documented and consistent acute physiological effect of cannabis is tachycardia, making ita reliable biomarker for cannabinoid activity. Administration of CBD, regardless of the delivery method (oral, intravenous, or inhalation), typically results in a reduction in both resting blood pressure (BP) and stress-induced BP elevations. Conversely, while acute CBD use primarily leadsto immediate reductions in BP, chronic use may induce bradycardia, which can contribute to a sustained decrease in BP over time (Malinowska et al., 2019). According to a study by Vandrey etal. (2011) an abrupt cessation following prolonged daily cannabis use can result in elevated blood pressure (BP). Emerging evidence indicates that cannabidiol (CBD) offers cardiovascular benefits,demonstrating direct effects on isolated arteries that induce both acute and chronic vasorelaxation. Additionally, CBD provides protection against vascular damage associated with inflammation (Stanley et al., 2013).

A study by Randall (2007) investigated the effects that CBD has on the haematological system, and found evidence that CBD promotes platelet activation which could lead to thrombocytopaenia. There is a distinct connection between PAH patients, platelets, and thrombocytopaenia, as investigated by Zanjani (2012). In PAH, platelets play a crucial role in clot formation due to the vascular and pulmonary damage associated with the condition. This often leads to pathological conditions such as pulmonary intravascular thrombosis and thrombotic arteriopathy in PAH patients. Thrombocytopenia in these patients may result from the consumption of platelets within the pulmonary vasculature or from platelet shearing caused by pulmonary microangiopathy.

A common theme throughout literature published is that CBD has both anti-inflammatory and antioxidant effects and has a positive role for treatment in the heart, peripheral and cerebral vasculature. It is important to note the addictive potential of cannabis as we see it becoming legalised around the world, either for medicinal or recreational purposes, and although the endocannabinoid system is implicated in cardiovascular functions, there is general concern that cannabis smoking could increase risk of cardiovascular disease particularly in middle-aged adults.

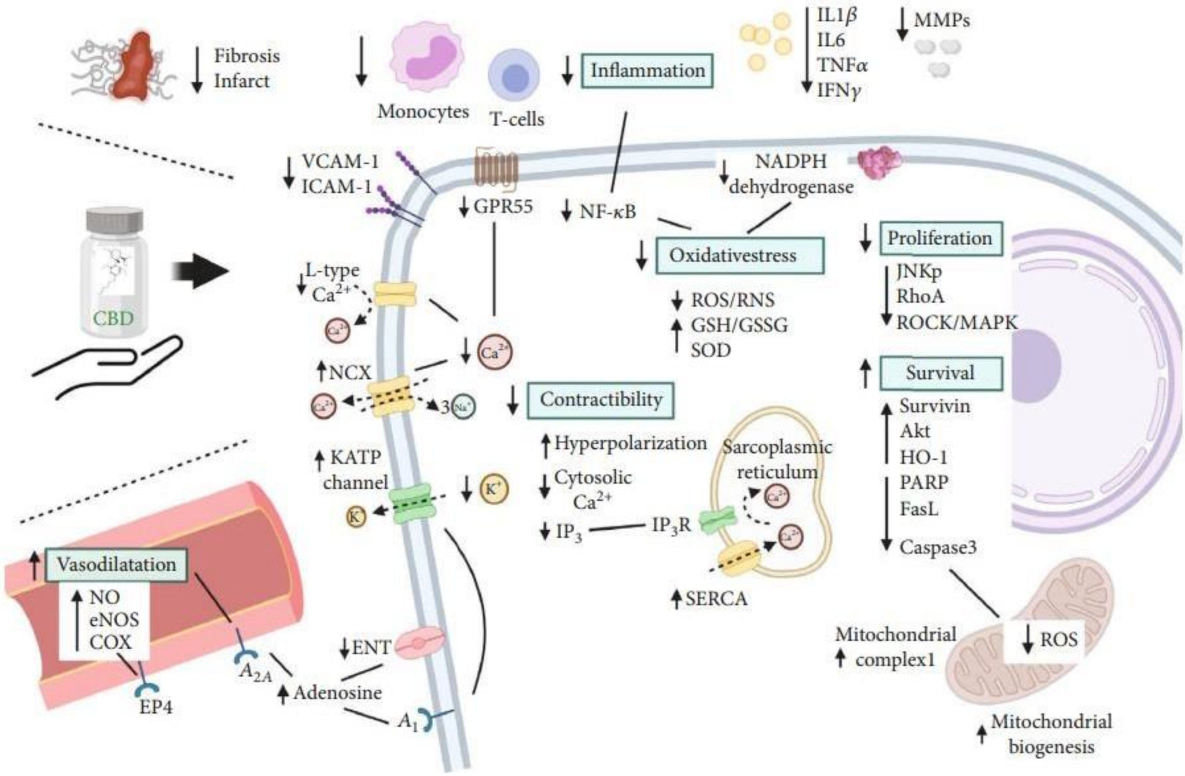

Figure 2. 1 Shows the molecular pathways and mechanisms of CBD administration within the cardiovascular system. CBD is involved in regulating several pathways, as well as regulating the Ca^{2+}/K^{+} intake, protecting cardiomyocytes from inflammation and oxidative stress, promoting cellular survival, and decreasing immune proliferation. CBD also helps to protect mitochondria and regulates their biogenesis, which improves their cellular energy supply. In vascular vessels, CBD causes vasodilatation, lowering blood pressure, and in turn protects the heart. Arrows indicate changes in activity for each inflammatory cell/molecule/mechanism/inflammatory cell (Adapted from Garza-Cervantes et al., 2020) (Generated using Microsoft Word 16 software)

2.3 PAH Biomarkers

Efforts are ongoing to identify a novel biomarker- whether a naturally occurring characteristic or gene- that could enable early detection of PAH through a simple test. Currently, the only biomarkers recommended for risk stratification according to existing guidelines are B-type natriuretic peptide (BNP) and amino-terminal pro-B-type natriuretic peptide (NT-proBNP) (Anwaret al., 2016). Tumour Necrosis Factor-alpha (TNF-α) and Hypoxia-Inducible Factor-1 alpha (HIF- 1α) are key cytokines implicated in the pathogenesis of pulmonary arterial hypertension (PAH). TNF-α is a proinflammatory cytokine found in neurons and glial cells, and it plays a crucial role in various signalling pathways that regulate apoptosis (Silva et al., 2019). Hurst et al. (2017) describes how TNF-α contributes to the development of PAH by suppressing the BNP type-2 receptor, elucidating the mechanisms through which BNP and TNF-α facilitate the disease. The inflammatory response associated with PAH leads to the excessive production of inflammatory cytokines, which may serve as potential biomarkers for the condition. Tuder et al. (1994) describedhow there is a significant influx of inflammatory cells, which includes macrophages and lymphocytes into the plexiform lesions of hypertensive pulmonary vessels. Overexpression of HIF-1α either by hypoxia, or genetic alterations, have been heavily implicated in areas of vascularisation and angiogenesis, which is seen in patients that have PAH with characteristics of remodelled vasculature that include stiffened and thickened pulmonary arteries, and vaso-occlusive lesions (Shimoda & Laurie, 2013). Patients with PAH exhibit pathological changes that include increasedpulmonary arteriole vasoconstriction, remodelling and proliferation of both endothelial and smooth muscle cells and endothelial dysfunction (Lan et al., 2018).

In a joint study by Lu et al. (2021), they found that CBD significantly suppressed mRNA levels of TNF-α in mouse lungs that were hypoxic. The same could be applied to the heart as existing clinicaldata has shown that circulating levels of TNF-a are elevated after heart failure and subsequently higher levels were associated with increased mortality (Irwin et al., 1999). A complete understanding of TNF-α signalling in the failing heart remains elusive, as existing data reveal bothpathogenic and cardioprotective roles of TNF-α in cardiac disease. Targeting the TNF-α pathway could offer therapeutic benefits, particularly through anti-TNF-α therapies, which may mitigate thedetrimental effects associated with heart failure (Rolski & Błyszczuk, 2020).

2.4 Oxidative Stress, CBD, and Hypertension:

Oxidative stress (OS) is associated with both hyperoxia and hypoxia and is characterized by an increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS). This condition typically arises from an underlying disease process or from an imbalance between the production and accumulation of ROS and RNS in cells and tissues, and the biological system's capacity to detoxify these reactive products (Rawat et al., 2022). Excessive accumulation of ROS can cause significant cellular and tissue damage. Although antioxidants such as Vitamin E, flavonoids, and polyphenols have been explored for their potential to counteract oxidative stress, recent research into CBD has highlighted its promising role as a therapeutic agent. CBD possesses both anti-inflammatory and antioxidant properties, making it a potential candidate for addressing OS. Extensive research has been conducted on the chemistry and pharmacology of CBD, including its interactions with cannabinoid receptors and other components of the endocannabinoid system. Clinical studies have further enhanced our understanding of CBD's therapeutic potential across various diseases, including those related to oxidative stress (Atalay et al., 2019).

CBD influences redox balance by modulating the levels and activity of both oxidants and antioxidants. Unlike conventional antioxidants, CBD interrupts free radical chain reactions by capturing free radicals or converting them into less reactive forms. It mitigates oxidative stress byinhibiting the formation of superoxide radicals, which are generated by xanthine oxidase and NADPH oxidase. Additionally, CBD reduces ROS production by chelating transition metal ions that participate in the Fenton reaction, thereby preventing the formation of highly reactive hydroxylradicals. CBD's antioxidant activity also involves the activation of the redox-sensitive transcriptionfactor nuclear erythroid 2-related factor 2 (Nrf2), which regulates the expression of cytoprotectiveand antioxidant genes (Atalay et al., 2019).

Inflammation and OS play a vital role in the development and progression of pulmonary hypertension. Inflammation and OS are essential in PAH with increased lipid peroxidation and reduced antioxidant defences (Reis et al., 2013). Reis et al. (2013) also confirms this fact by the increased levels of circulating cytokines, such as TNF-a in his study investigating the relation of CBD and OS. A potential reason for elevated plasma cytokine levels in PAH patients may be attributed to oxidative stress and the body's attempt to restore vascular homeostasis. This process involves the activation of the inflammatory cascade, which leads to excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and/or a reduction in antioxidant defences.

2.5 Brain Natriuretic Peptide (BNP) in PAH

Natriuretic peptides are a group of hormones secreted mainly by the heart, kidneys, and brain, and they function to induce vasodilation and promote natriuresis. This family includes Brain Natriuretic Peptide (BNP), C-type Natriuretic Peptide, and Urodilatin. BNP is derived from the early-response gene natriuretic peptide B (NPPB) and is predominantly synthesized and secreted by the ventricular myocardium in response to hormonal, mechanical, or sympathetic stimuli. In pulmonary arterial hypertension (PAH), factors such as volume overload, transmural pressure, hypoxia, and pro-inflammatory stimuli trigger the transcription of NPPB, leading to the production of 134-amino acid preproBNP.This precursor undergoes cleavage in the sarcoplasmic reticulum to form 108-amino acid proBNP,which is then further cleaved into two active forms: 32-amino acid BNP and 76-amino acid NT (N-Terminal)- proBNP. BNP binds to Natriuretic Peptide Receptor-A, which is primarily located in the kidneys, aorta, lungs, and adipose tissue (Lewis et al., 2020). Activation of the receptor leads to an increasein the intracellular secondary messenger cyclic guanosine monophosphate (cGMP). This elevation in cGMP triggers vasodilation, promotes natriuresis, inhibits aldosterone secretion, and stimulateslipolysis (Ping et al., 2018). It is well established in current research that BNP and NT-proBNP correlate with several pulmonary haemodynamic metrics that are associated with survival.

Leuchte et al. (2004) investigated the use of plasma BNP as a simple, non-invasive, and observer-independent measure for assessing disease severity in patients with primary pulmonary hypertension. Their study found significant correlations between BNP levels and haemodynamic parameters obtained through right heart catheterization. These findings supported the hypothesis that natriuretic peptides are integral to a physiological counterregulatory system in patients with progressive right heart failure. Specifically, the activation of the natriuretic peptide system, including BNP, can alleviate pulmonary hypertension due to its direct vasodilatory effects.

There is a notable gap in the literature regarding the effects of CBD on BNP levels in pulmonary models, and its implications have yet to be thoroughly explored. Based on current understanding, it is plausible that CBD may elevate BNP levels due to its antioxidant and anti-inflammatory properties.

2.6 Drugs and Therapeutic Strategies for PAH Treatment

Three critical signalling pathways are implicated in the progressive defects observed in pulmonary vascular disease: the Nitric Oxide pathway, the Prostacyclin pathway, and the Endothelin-1 pathway (Lan et al., 2018). Understanding these pathways is crucial for developing targeted pharmacological therapies for PAH. Among these, targeting the prostacyclin pathway has proven to be the most effective treatment approach for PAH. Prostacyclin, produced by endothelial cells, acts via the prostacyclin receptor (IP) to induce vasodilation, inhibit smooth muscle cell proliferation, and prevent platelet aggregation. However, prostacyclin production is diminished in PAH (Del Pozo et al., 2017). Selexipag, a selective IP receptor agonist, is a drug developed to targetthis pathway. It is administered orally, and its pharmacokinetic profile indicates that it should be taken twice daily with food (Kaufmann et al., 2015). Despite its effectiveness, Selexipag is expensive and only addresses the pathway after PAH has progressed. Current research is exploringvarious therapeutic options to treat PAH earlier in its progression, including the potential of cannabinoids as adjunctive treatments.

2.7 The Rat Model of PAH

In a study by Batkal et al. (2004) dedicated to the role of the endocannabinoid system in several hypertensive rat models concluded that ‘targeting the endocannabinoid system offers novel therapeutic treatment strategies for hypertension’. In another study conducted by Wheal et al. (2009), they evaluated the cardiovascular effects of cannabinoids in conscious spontaneously hypertensive rats and found evidence for up-regulation of CB_1 receptors and that antagonism of these receptors caused a rise in blood pressure and administration of the endocannabinoid, anandamide, subsequently caused hypotension. A recent study by Baranowska-Kuczko et al. (2020) aimed to investigate the vasodilatory effects of CBD on isolated human pulmonary arteriesand rat small mesenteric arteries. The study demonstrated that CBD induced significant, concentration-dependent vasorelaxation in both human and rat arteries. Notably, the vasorelaxant effects observed in rat arteries were sensitive to CB_1 receptor modulation and dependent on the specific hypertension model used in the study.

In conclusion, the findings from similar studies underscore the importance of considering the CBD-mediated response when utilizing cannabidiol for therapeutic purposes. These results reinforce thenotion that cannabinoids and the endocannabinoid system may serve as viable therapeutic targets for hypertension. Utilizing this rat model provides an opportunity to further investigate and elucidate the mechanisms underlying pulmonary arterial hypertension and assess the efficacy of cannabinoids as a treatment approach. The ultimate objective is to translate these positive outcomes to human applications, ensuring effective and beneficial therapeutic interventions with cannabinoids.

A particular model that is going to be used is the monocrotaline-induced rat model. Monocrotaline is widely used to establish an animal model of pulmonary hypertension. Xiao et al. (2017) discusses how monocrotaline aggregates on and activates the extracellular calcium-sensing receptor of pulmonary artery endothelial cells to trigger endothelial damage and cause the development of pulmonary hypertension. This model proves useful in helping understand pulmonary hypertensionand find various therapeutic interventions, but according to Hill et al. (2017), limitations of the monocrotaline model must be kept in mind, including its hypoxic component and pulmonary parenchymal involvement. As it stands, this model will likely to continue to be used and serve as acrucial tool for understanding mechanisms and preclinical testing for the near future.

By using this model and all the supporting literature, this research will examine how cannabinoid administration affects hypoxia and inflammatory cytokines in the lungs, as well as the haematological impacts of CBD in a rat model of PAH. It aims to determine if CBD can mitigate cytokine expression, either on its own or in combination with Selexipag, and influence platelet function as a potential therapeutic intervention. Given the growing global legalization of cannabis, this study will explore cannabinoids' effects on pulmonary circulation and the haematological system, focusing on whether CBD offers vasodilatory and antiproliferative benefits and whether it can prevent or reverse PAH-related cardiovascular damage, including right ventricular hypertrophyand vascular remodelling.

Chapter 3:

Research Methodology

Ethical clearance was granted from the Animal Research Ethics Committee (AREC) (**AREC/014/019M**). Animal work was conducted within the validity period of the ethical clearance (2022).

Animal Handling and Competency Training was done at the Biomedical Resource Unit (BRU) housed on the University of KwaZulu-Natal (Westville) campus.

3.1 Animal groupings and Housing

Forty healthy adult male Sprague-Dawley (SD) rats, each with a body weight ranging from 200 to 220 grams, were acquired from the Biomedical Research Unit (BRU) at the University of Kwa-Zulu Natal, Westville Campus. This cohort size was determined to provide sufficient statistical power while accounting for possible sample loss due to unforeseen issues such as health complications orexperimental errors.

The rats were randomly assigned to one of five experimental groups, each containing eight animals: Control, MCT control (to establish baseline, MCT+Selexipag, MCT+CBD, and MCT+CBD+Selexipag (to test for potential synergistic effects, providing insights into combination therapy). Randomizationwas employed to ensure that each group was statistically equivalent at the start of the study, reducing the potential for bias and ensuring that observed effects could be attributed to the experimental treatments rather than pre-existing differences among the groups.

Each group was housed in individual cages, which were designed to offer adequate space for movement and to minimize social stress. Cages were equipped with environmental enrichment, such as nesting materials, chew toys, and shelters, to encourage natural behaviours and improve animal welfare. Environmental conditions within the animal facility were rigorously controlled: the temperature was maintained at 18-22°C, humidity was kept between 50-70%, and a 12-hour light/dark cycle was followed to simulate natural light conditions and support the animals' circadianrhythms. Rats had unrestricted access to a standard laboratory rodent diet and filtered water, provided ad libitum. The diet was formulated to meet the nutritional needs of the rats throughout the study, and fresh food and water were supplied daily to ensure consistent intake. Body weights were recordedtwice a week to monitor health and growth, and any significant deviations from normal growth patterns were noted. Additionally, regular health checks and behavioural observations were conducted to assess the overall well-being of the animals and to detect any potential adverse effects of the treatments. All procedures were conducted in accordance with institutional guidelines and national regulations for the ethical care and use of laboratory animals, ensuring that the research adhered to high standards of animal welfare and scientific integrity.

3.2 Rat model of Monocrotaline-induced PAH

Monocrotaline (MCT), a pyrrolizidine alkaloid, is employed in experimental models to induce pulmonary vascular disease and study related pathophysiological processes. MCT selectively targets and damages the pulmonary vascular endothelium, leading to significant pathological changes such as endothelial cell injury and subsequent vascular remodelling. This process includeshypertrophy of the pulmonary arteries, which results in increased vascular resistance and ultimatelyelevates pulmonary arterial pressure.

In the context of this study, MCT was used to induce a model of pulmonary hypertension, allowing for the evaluation of various therapeutic interventions. The pulmonary vascular effects of MCT typically become observable after 2 to 3 weeks of administration (Buyukakilli et al., 2014), a timeframe selected to ensure that sufficient pathological changes have developed to assess the efficacy of the treatments under investigation. This period is crucial for evaluating the progression of pulmonary hypertension and the impact of the treatments on reversing or mitigating the vascular damage induced by MCT.

The study's methodology involved monitoring the rats for signs of pulmonary vascular pathology during this period, with measurements taken to assess the extent of vascular remodelling and increases in pulmonary arterial pressure. This approach provides a comprehensive evaluation of thetherapeutic effects of Selexipag, CBD, and their combination on MCT-induced pulmonary hypertension, thereby informing the effectiveness of these treatments in altering disease progression and improving pulmonary vascular health. The careful timing of assessments

And interventions reflect the study’s focus on understanding the dynamics of pulmonary vascular changes and the potential for therapeutic modulation.

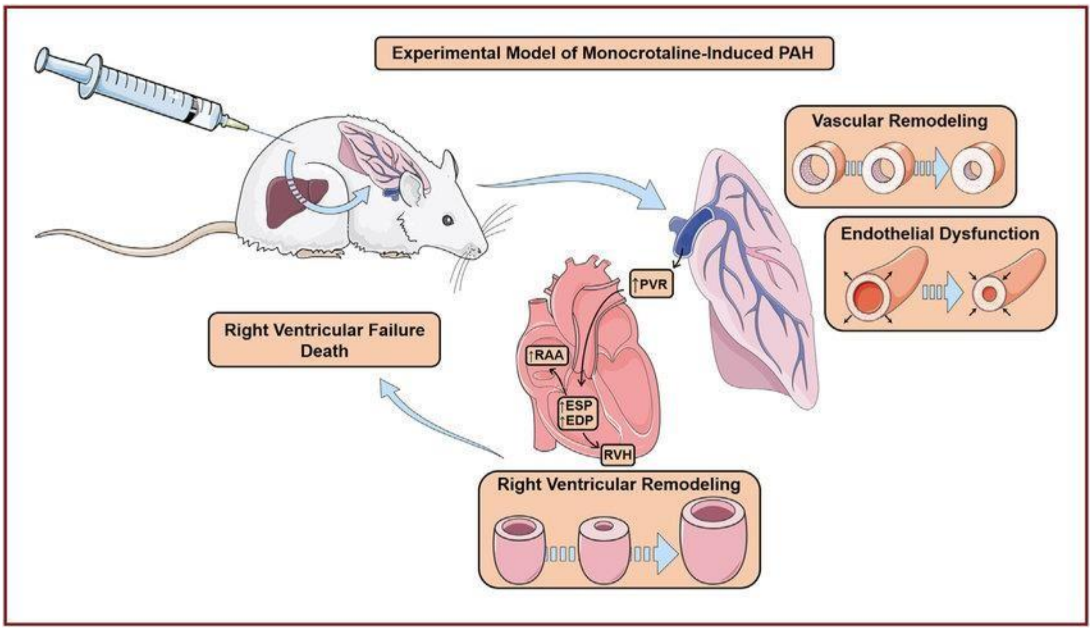

Figure 3. 1 Physiological responses of MCT-induced PAH model (Adapted from Ribeiro et al, 2016) (Generated using Microsoft Word 16 software)

3.3 Drug preparation

Dosages were obtained from a similar study done by Mwewa (2020), a previous Physiology master’s student at the University of KwaZulu-Natal, dealing with PAH using the same MCT rat model and drugs being administered.

3.3.1 Monocrotaline (MCT)

MCT (obtained from Axim South Africa) was prepared for administration at a dose of 50 mg/kg to induce pulmonary hypertension in the rat model. The preparation involved dissolving 500 mg of MCT in 6 ml of Dulbecco’s phosphate-buffered saline (D-PBS), which serves as a carrier solution to maintain the compound in a suitable form for injection. To facilitate the dissolution of MCT, 2.45 ml of 0.1N hydrochloric acid (HCl) was added. This acidic environment helps to dissolve the MCT efficiently. Following dissolution, the solution’s pH was neutralized by adding 14.5 ml of 0.1N sodium hydroxide (NaOH), adjusting the pH to a physiological range to ensure compatibility with the rats' intraperitoneal environment and to minimise potential irritation. The final solution was prepared to a concentration of 5 mg of MCT per 100 µl, with the dosages administered calculated based on the rat's body weight. Each rat received 0.1 ml of the MCT solution per 100 g of body weight, which corresponds to the 50 mg/kg dosage. The MCT solution was administered intraperitoneally using a 27-gauge needle, chosen for its appropriate size to deliver the solution effectively while minimizing tissue damage.

Intraperitoneal injection was selected for its efficiency in delivering the compound directly into theabdominal cavity, allowing for rapid absorption into the systemic circulation. This method ensuresconsistent dosing across the study population and facilitates the induction of pulmonary hypertension, enabling the evaluation of therapeutic effects. Throughout the study, careful monitoring of the rats' health and response to MCT was conducted to ensure the accuracy of the model and the safety of the experimental procedures.

3.3.2 Selexipag

Selexipag (obtained from Axim South Africa) was prepared for administration at a standard dosage of 3 mg/kg. To achieve this dosage,75 mg of Selexipag was thoroughly dissolved in 100 ml of deionized water. This preparation resulted in a concentration of 0.75 mg of Selexipag per 250 g of body weight. The solution was mixed to ensure complete dissolution and uniform distribution of the drug.

For oral administration, the Selexipag solution was delivered to the rats using a syringe. The volume of the solution administered was adjusted according to each rat's body weight to ensure precise dosing. This approach allowed for accurate dosing based on the individual weights of the rats, with each rat receiving a dose corresponding to 3 mg/kg of body weight. Oral administration was chosen to closely replicate clinical administration routes and to evaluate the drug's efficacy in a physiological context similar to potential human treatments.

The process involved careful calculation and measurement to ensure that the concentration and volume of the solution were appropriate for each rat. The solution was administered directly into the stomach of the rats, which was facilitated by a syringe designed for oral gavage. This method ensured consistent and accurate delivery of Selexipag, minimising variability, and optimising the assessment of its therapeutic effects.

Throughout the study, the administration procedure was monitored to ensure all rats received the correct dosage and to assess any potential adverse effects. This rigorous approach was essential forevaluating the effectiveness of Selexipag in mitigating MCT-induced pulmonary hypertension andensuring reliable and reproducible results.

3.3.3 Cannabinoid (CBD)

ADCO CBD Pain 200 (obtained from a local Clicks pharmacy), a purified CBD oil with a concentration of 20 mg per 1.5 ml, was procuredfrom a local pharmacy for use in this study. The preparation involved calculating a dosage of 10 mg/kg body weight for the rats. To achieve this, the required dosage was determined to be 0.2 ml of CBD oil per 250 g of body weight. This volume was calculated to ensure that each rat receiveda precise dose of 10 mg/kg, consistent with the therapeutic goals of the study.

The CBD oil was administered orally using a syringe, specifically designed for gavage. This method allowed for the accurate delivery of the oil into the rat's stomach. The volume administered was carefully adjusted according to each rat’s individual body weight, ensuring that the correct amount of CBD oil was delivered. The oral gavage technique involved gently inserting the syringe into the rat’s mouth and delivering the solution directly into the stomach to ensure full ingestion of the dose.

This route of administration was chosen to mirror clinical settings where oral CBD is used for its potential therapeutic benefits. The oral gavage approach was critical for ensuring consistent dosing and accurate evaluation of CBD's effects on MCT-induced PAH. Throughout the administration process, each rat was monitored closely to observe any immediate adverse reactions or signs of distress, and body weights were recorded to confirm dosage accuracy.

The preparation and administration protocols were designed to minimise variability and maintain the integrity of the study. By using a standardised dosing method and closely monitoring the animals, the study aimed to provide reliable data on the effects of CBD oil in mitigating the vascular damage induced by MCT, while adhering to ethical standards for animal care and use.

3.4 Protocol

Day 0: The study began with the establishment of experimental groups and the initial treatment administration. Eight rats were injected intraperitoneally with a saline solution to serve as the control group, providing baseline data for comparison. To induce PAH, thirty-two rats were administered MCT at a dose of 50 mg/kg via intraperitoneal injection using a 27-gauge needle. Theuse of this dose and administration route ensures that the model reliably mimics human PAH facilitating the evaluation of therapeutic interventions.

Day 18: The therapeutic phase of the study commenced with the administration of CBD and Selexipag. Commercially available CBD oil (ADCO, South Africa), with a concentration of 20 mgper 1.5 ml, was prepared and administered orally. To control for variability in drug absorption dueto the presence of stomach contents, rats were fasted for 8 hours before administration. CBD was administered at a dose of 10 mg/kg, and Selexipag was given at 3 mg/kg. Both drugs were administered every other day for a period of 2 weeks. This dosing regimen was informed by prior research, including Hložek et al. (2017), which demonstrated that oral administration of cannabinoids provides effective drug absorption, prolonged therapeutic effects, and reduced psychoactive side effects compared to other routes such as inhalation or subcutaneous injection. The choice of oral gavage as the method of administration ensures consistent delivery and absorption of the drugs.

Day 31: On the final day of the experimental protocol, all rats were fasted for 12 hours prior to euthanasia to standardize metabolic conditions and ensure accurate baseline measurements. The rats were then humanely euthanised following ethical guidelines to minimize stress and suffering. Tissue and blood samples were collected immediately post-euthanasia. The heart and blood were harvested to assess the impact of treatments on pulmonary and cardiovascular systems. The right lung and kidney were also collected for further analysis, if needed. These samples were promptly stored at -70°C in a bio freezer to prevent degradation and maintain their suitability for subsequentbiochemical assays, haematological analysis, and/or histological examinations.

This detailed protocol timeline provides a comprehensive overview of the study design, from the induction of PAH to the administration of therapeutic agents and final sample collection. The approach emphasises careful planning and execution to ensure accurate and reproducible results while adhering to ethical standards for animal research. The timeline reflects the systematic approach taken to evaluate the efficacy of CBD and Selexipag in managing MCT-induced PAH and provides a clear framework for the assessment of treatment outcomes.

3.4.1 Shortcomings during experimental protocol

During the course of the trial, severe cardiopulmonary complications resulting from MCT administration led to the loss of eleven rats due to pulmonary oedema, a critical outcome of MCT-induced PAH. The autopsy findings (**see appendices**) confirmed the presence of PAH through observations of pulmonary oedema and frothing in the lungs. The lungs appeared engorged and fluid-filled, indicating significant fluid accumulation in the lung tissue. Additionally, frothing or foaming was noted within the lungs, a hallmark of severe pulmonary distress. These findings highlight the acute cardiopulmonary damage caused by MCT, which was used to induce PAH in the experimental model. The presence of excess fluid and frothy secretions underscores the severe vascular leakage and respiratory compromise associated with PAH. In response to these losses and to prevent further animal suffering, the experimental protocol was shortened by one week. This adjustment was made to balance the need for maintaining a statistically valid sample size while minimising additional fatalities.

The distribution of losses across the experimental groups was as follows: three rats from the MCT Control group, one rat from the MCT-Selexipag group, four rats from the MCT-CBD group, and three rats from the MCT-CBD-Selexipag group. The higher mortality observed in the MCT-CBD and MCT-CBD-Selexipag groups suggests potential exacerbation of the PAH condition or an interaction effect that may have increased susceptibility to adverse outcomes. This information is critical for evaluating the safety and efficacy of the combined treatments.

To address these issues in future studies, several considerations could be made. Firstly, reducing the dose of MCT could decrease the severity of the induced PAH and lower the risk of severe complications while still allowing for a meaningful assessment of the treatments. Secondly, dose optimisation for Selexipag and CBD could be explored through incremental dosing studies to finda balance between therapeutic efficacy and safety. Additionally, implementing enhanced supportive care measures, such as monitoring and managing fluid balance and respiratory support,could potentially reduce mortality and improve the overall well-being of the animals during the trial.

Furthermore, future studies might benefit from incorporating intermediate endpoints to assess the progression of PAH and the effects of treatments at various stages, allowing for adjustments in treatment strategies as needed. This approach would contribute to a more comprehensive understanding of the therapeutic potential and safety profile of Selexipag and CBD, ultimately enhancing the robustness of the study findings and the validity of therapeutic claims.

3.5 Haematological Analysis

Two blood samples were collected from each rat immediately following euthanasia. Blood was drawn into EDTA tubes to prevent coagulation. To ensure sample integrity, the blood samples wereplaced on ice and promptly transported to the laboratory for processing.

In the laboratory, a complete haematological profile was conducted using a Beckman-Coulter Haemolyser, which provided detailed information on various blood parameters, including red andwhite blood cell counts, haemoglobin levels, and platelet count. These results were recorded for subsequent analysis and interpretation.

For biochemical analyses, one blood sample from each rat was subjected to centrifugation at 10,000g for 10 minutes. This step was crucial for separating the plasma from the cellular components of the blood. The resulting plasma was then carefully extracted and transferred to polypropylene tubes. To preserve the stability of the plasma components until further analysis, the samples were stored at -70°C. This freezing step was essential for maintaining the integrity of biomarkers BNP and T- AOC, which were to be analysed later.

The detailed processing and storage procedures were implemented to ensure accurate and reliable measurement of the biomarkers and to support the overall validity of the study’s findings related tocardiovascular and oxidative stress parameters.

3.5.1 Plasma Brain Natriuretic Peptide (BNP)

Optical densities for BNP analysis were measured using a microplate reader following the protocolprovided with Elabscience’s Rat BNP Enzyme Linked Immunosorbent Assay (ELISA) Kit (E-EL-R0126). The assay was conducted using a standard competitive ELISA format, adhering meticulously to the manufacturer’s instructions toensure accuracy and reproducibility of the results.

Samples and standards were prepared in duplicate to account for potential variability and to enhancethe reliability of the measurements. This approach allowed for the assessment of BNP concentrations with a higher degree of precision. The use of duplicate measurements also facilitatedthe calculation of average values, reducing the impact of any outliers or inconsistencies and improving the overall robustness of the data.

The microplate reader was used to measure the optical densities, which were then compared against the standard curve generated during the assay to determine the BNP concentrations in the plasma samples. Adhering to the manufacturer’s guidelines and performing assays in duplicate ensured that the results were both accurate and reproducible, critical for the validity of the study's findings regarding BNP levels.

3.5.2 Plasma Total Antioxidant Capacity (T-AOC)

Optical densities were determined with a microplate reader after using Elabscience’s Total Antioxidant Capacity Colorimetric Assay (Ferric Reducing Ability of Protein, FRAP, method) (E-BC-K225-M). The assay was strictly prepared according to the manufacture’s guidelines. All samples and standards were prepared in duplicate.

3.6 Heart tissue preparation

Whole heart samples were meticulously washed with Phosphate Buffered Solution (PBS) to remove any residual blood or debris. After washing, the hearts were thoroughly dried, and their weights were recorded to ensure accurate processing.

The hearts were then homogenised using a PBS medium at a ratio of 9 ml of PBS per gram of heart tissue, with the homogenisation performed at a temperature range of 2-6°C to prevent thermal degradation of sensitive components. This ratio was carefully selected to achieve a uniform homogenate for subsequent analysis.

Following homogenisation, the samples were centrifuged at 10,000g for 10 minutes at 4°C. This centrifugation step was critical for separating cellular debris and insoluble components from the soluble proteins and other analytes in the supernatant. The resulting supernatant was carefully transferred to Eppendorf (EP) tubes and preserved on ice to maintain sample stability until further analysis.

The use of ice for temporary storage of the supernatant was essential to prevent any enzymatic or chemical changes that could affect the integrity of the samples and the accuracy of subsequent assays. This preparation process ensures that the heart homogenate remains in an optimal conditionfor detecting biomarkers and performing biochemical analyses.

3.6.1 Heart Tumour Necrosis Factor-α (TNF-α)

Optical densities were determined with a microplate reader after using Elabscience’s Rat TNF-α ELISA Kit (E-EL-R2856). Standard Sandwich-ELISA preparation was strictly followed accordingto the manufacture’s guidelines. All samples and standards were prepared in duplicate.

3.6.2 Heart Total Antioxidant Capacity (T-AOC)

Optical densities were determined using Elabscience’s Total Antioxidant Capacity Colorimetric Assay (Ferric Reducing Ability of Protein, FRAP, method) (E-BC-K225-M). The assay was strictlyprepared according to the manufacture’s guidelines. All samples and standards were prepared in duplicate.

Before determining the heart T-AOC, total protein concentration of each heart sample had to be determined first to calculate the resulting T-AOC. This was done using Elabscience’s Bradford Protein Colorimetric Assay (E-BC-K168-M). Samples were diluted for fifteen times, and the assaywas conducted strictly according to the manufacturers guidelines with standards and samples prepared in duplicate.

Chapter 4

Research Findings

4.1 Statistical Analysis

All data analyses, including graphical representation, standard curve generation, and statistical evaluation, were conducted using GraphPad Prism 10 statistical software. For the quantification of BNP and TNF-α, concentrations were extrapolated from their respective standard curves (figures 4.1 & 4.8), which were constructed from a series of known concentrations to ensure precise calibration. Protein concentrate ions and heart T-AOC levels were calculated respectively using linear standard curves (figures 4.5 & 4.3) tailored to each specific assay, allowing for accurate measurement of antioxidant capacity relative to protein content. Plasma T-AOC levels were determined from a separate, calibrated standard curve to ensure accurate quantification of antioxidant status in plasma samples.

Given the variability in sample sizes and the potential for unequal variances across the experimental groups, Welch's corrections were utilized instead of traditional T-Tests and analysis of variance (ANOVA). Welch's correction is specifically designed to address situations where the assumption of equal variances—a fundamental assumption of standard parametric tests—is not met. This correction adjusts for differences in variances and sample sizes by recalculating degrees of freedom, which improves the accuracy of significance testing. Welch's ANOVA was employed to assess overall differences among groups, providing a robust method for identifying significant effects despite unequal variances. Welch's T-Tests were used for pairwise comparisons to determine specific differences between groups, accounting for unequal sample sizes and variances.

These statistical adjustments are crucial for reducing the risk of Type I errors (false positives) andType II errors (false negatives), ensuring that observed differences are genuinely reflective of the underlying data and not artifacts of statistical assumptions. By implementing Welch's corrections,the analysis enhances the reliability and validity of the study's findings, providing a more accurate interpretation of the experimental results and ensuring that conclusions drawn are based on rigorous statistical evaluation.

4.2 Haematological Profile

Table 4. 1 Full haematological profile showing the averages of various haematological factors between all groups (with standard deviation)

	Control (8 rats)	**MCT Control (5 rats)**	**MCT+Sel (7 rats)**	**MCT+CBD (5 rats)**	**MCT+Sel +CBD (4 rats)**
WBC (10^3/µL)	6,6 (1.3)	6,6 (2.1)	7,5 (2.1)	7,0 (0.4)	6,0 (2.0)
RBC (10^6/µL)	8,4 (0.7)	8,4 (0.6)	8,5 (1.3)	8,16 (0.9)	8,7 (0.3)
HGB (g/dL)	13,8 (1.0) *	13,8 (0.9)	14,8 (2.5) *	14,4 (2.0)	15,8 (0.6) *
HCT (%)	45,1 (3.3)	45,1 (3.8)	45,5 (8.1)	44,2 (4.0)	47,4 (2.0)
MCV (fL)	54 (0.7)	54 (1.3)	53 2.8)	54 (2.1)	55 (0.8)
MCH (pg)	17,4 (0.3)	17,4 (0.3)	17,3 (0.9)	17,6 (0.7)	18,1 (0.3)
MCHC(g/dL)	32,4 (0.4)	32,4 (0.2)	32,5 (0.3)	32,5 (0.2)	32,7 (0.3)
RDW (%)	9,6 (0.3)	9,6 (0.6)	9,6 (0.9)	9,7 (0.8)	9,6 (0.2)
PLT (10^3/µL)	647 (60.9) **	647 (113)	781 (110) **	678 (30)	836 (97) **
MPV (fL)	5,8 (0.2)	5,8 (0.3)	5,6 (0.2)	5,9 (0.4)	5,9 (0.2)
PCT (%)#	0,368 (0.03)	0,368 (0.1)	0,441 (0.2)	0,393 (0.04)	0,472 (0.05)

WBC (White Blood Cell), RBC (Red Blood Cell), HGB (Haemoglobin), HCT (Haematocrit), MCV (Mean Corpuscular Volume), MCH (Mean corpuscular Haemoglobin), MCHC (Mean Corpuscular Haemoglobin Concentration), RDW (Red Cell Distribution Width), PLT (Platelets), MPV (Mean Platelet Volume), PCT (Procalcitonin) *$p<0.05$ when comparing control to MCT+Sel and MCT+Sel+CBD **$p<0.05$ when comparing control to MCT+Sel and MCT+Sel+CBD
#The inclusion of PCT levels alongside haematological parameters may seem unconventional since PCT is a biochemical marker rather than a direct haematological parameter. Its inclusion in the haematological table is to suggest a possible interest in its relationship with inflammatory and vascular changes in PAH rather than a strict classification under haematology.

The haematological analysis revealed significant changes in both haemoglobin and platelet counts across different treatment groups. The MCT+Selexipag group and the combination group (MCT+Selexipag+CBD) showed significant increases in platelet counts compared to the control group ($p<0.05$). This indicates that Selexipag, both alone and in combination with CBD, has a notable effect on platelet levels under conditions of MCT-induced pulmonary hypertension. Similarly, haemoglobin levels were elevated in the MCT+Selexipag and MCT+Selexipag+CBD groups compared to the control group. The combination group exhibited the highest haemoglobin levels among all groups, reflecting the most substantial increase. For platelet counts, significant differences were also observed between the MCT+Selexipag group and the control group, as well as between the MCT+Selexipag+CBD group and the control group ($p<0.05$). Both treatment groups demonstrated increases in platelet counts, with the highest rise in the combination group.

These findings indicate that Selexipag and CBD treatments positively impact haemoglobin and platelet counts in the context of MCT-induced pulmonary hypertension, with the combination therapy showing the most pronounced effects. This underscores the potential efficacy of these treatments in managing haematological alterations associated with MCT administration.

4.1 BNP

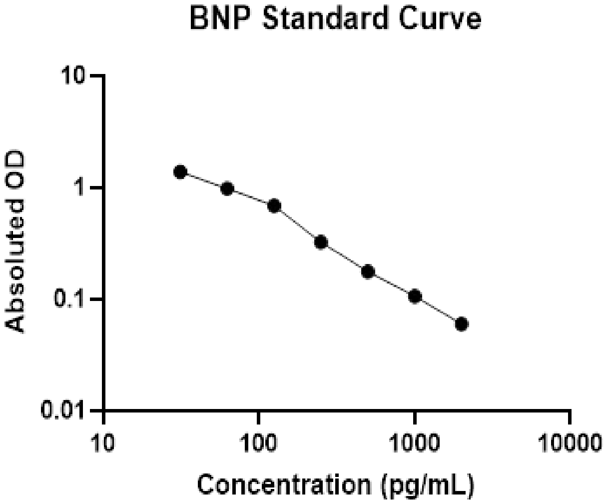

Figure 4. 1 Sigmoidal graph showing the BNP standardcurve.

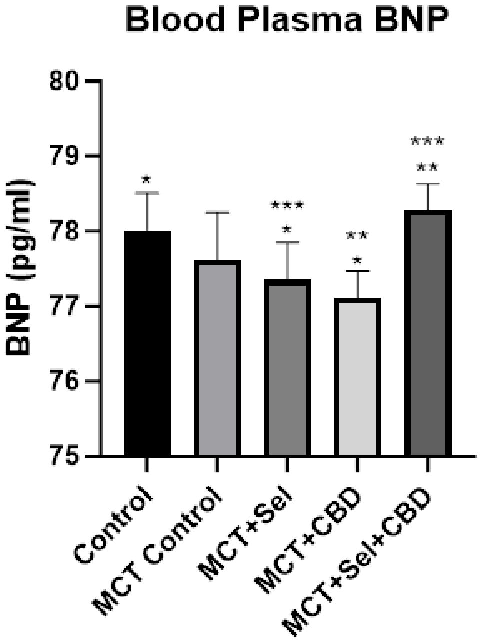

Figure 4. 2 The effect of CBD, Selexipag and Selexipag+CBD administration on BNP expression in plasma of MCT-induced PAH (matching * amount represents significance between two groups where p<0.05)

The data indicates that BNP levels decreased in the MCT-Selexipag and MCT-CBD groups relativeto the MCT Control group. Importantly, the MCT-CBD group demonstrated the most substantial reduction in BNP levels. This decrease was statistically significant ($p<0.05$), suggesting that CBDalone has a notable effect in lowering BNP levels. This finding is significant as it points to the potential of CBD in managing conditions associated with elevated BNP levels, such as heart failure.

Conversely, the combination of CBD and Selexipag (MCT-CBD-Selexipag group) resulted in a significant increase in BNP levels, which were just above the levels observed in the normal control group. This increase was also statistically significant ($p<0.05$). This result implies that the combination treatment could potentially counteract the BNP-lowering effects observed with singletreatments. The increase in BNP levels in this group highlights the complexity of the interaction between CBD and Selexipag and their combined effect on cardiac biomarkers.

Welch’s ANOVA was utilized to assess the differences in BNP levels among all treatment groups. The analysis revealed significant differences across the groups ($p<0.05$), confirming that the observed effects on BNP levels are statistically significant and not due to random variation.

4.2 Blood T-AOC

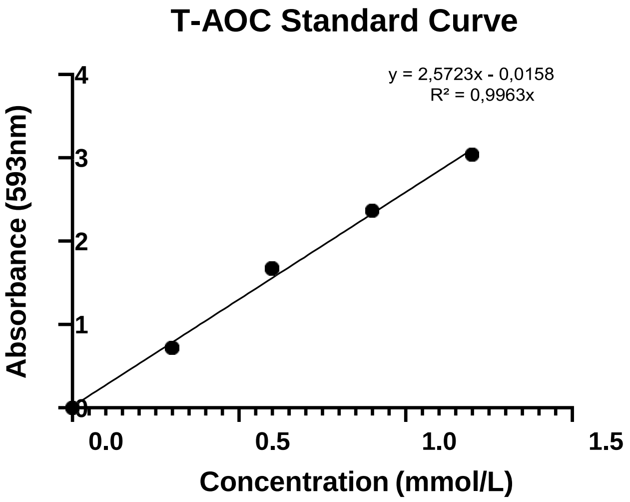

Figure 4. 3 Linear graph showing the T-AOC standard curve.

The T-AOC standard curve generated for this study included only five data points, rather than the planned eight, due to pipetting errors that affected the last three dilutions and resulted in inaccurateoptical densities. Consequently, these three erroneous data points were excluded from the final analysis to produce the linear graph displayed. Despite this, the accuracy of the calculated results remained unaffected, as all measured optical densities were within the range of the five valid data points. The standard curve was consistently applied for both the blood and heart T-AOC calculations, ensuring uniformity in the assessment of antioxidant capacity across the different sample types. This approach maintained the integrity of the results and allowed for reliable comparisons between the experimental conditions.

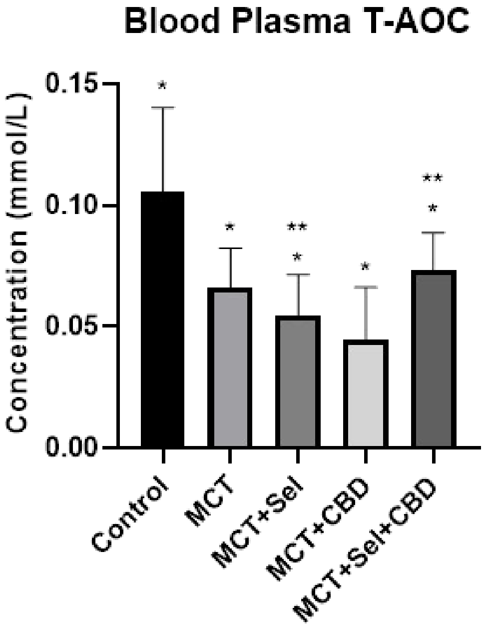

Figure 4. 4 The effect of CBD, Selexipag and Selexipag+CBD administration on T-AOC expression in plasma of MCT-induced PAH (matching * amount represents significance between two groups where $p<0.05$

T-AOC levels were significantly reduced across all treatment groups compared to the MCT Control group, indicating a decrease in overall antioxidant capacity due to the treatments administered. The MCT-CBD group exhibited the most pronounced reduction in T-AOC levels, which was statistically significant with a p-value less than 0.05. This suggests that CBD alone has a substantial impact on reducing antioxidant capacity.

Conversely, the combination treatment of Selexipag and CBD resulted in T-AOC levels that were notably closer to those of the control group, and higher than those observed in the MCT control group. This indicates that the combination therapy may partially mitigate the reduction in antioxidant capacity compared to single treatments. The difference between the combination treatment and the MCT-Selexipag group was statistically significant ($p<0.05$), but there was no significant difference between the combination treatment and the MCT-CBD group.

Welch's ANOVA was employed to assess the differences in T-AOC levels across all experimental groups, revealing statistically significant differences ($p<0.05$). This analysis confirms that the observed variations in antioxidant capacity among the groups are statistically significant and not due to random variation. The findings underscore the differential effects of the treatments on T- AOC levels, with the combination of Selexipag and CBD demonstrating a potentially more favourable outcome compared to single treatments and approaching baseline antioxidant levels seen in the control group.

4.3 Heart T-AOC

Before calculation of the heart T-AOC, total protein concentration had to first be determined. This was done using Bradford's protein colorimetric assay. Before calculating the heart T-AOC, it was essential to first determine the total protein concentration of the heart tissue samples. This was accomplished using Bradford's protein colorimetric assay, a standard method for quantifying protein concentration. The Bradford assay utilizes Coomassie Brilliant BlueG-250 dye, which binds to proteins, causing a shift in the dye's absorption maximum. This shift, which results in a measurable colour change, is directly proportional to the amount of protein present in the sample. The intensity of the blue colour is quantified using a spectrophotometer, and the protein concentration is determined by comparing the absorbance readings to a standard curvegenerated from known protein concentrations. Accurate determination of protein concentration is crucial for normalizing the T-AOC measurements, as it allows for the assessment of antioxidant capacity relative to the total protein content of the heart tissue samples. This normalization ensures that differences in T-AOC are not due to variations in protein concentration but rather reflect true differences in antioxidant capacity.

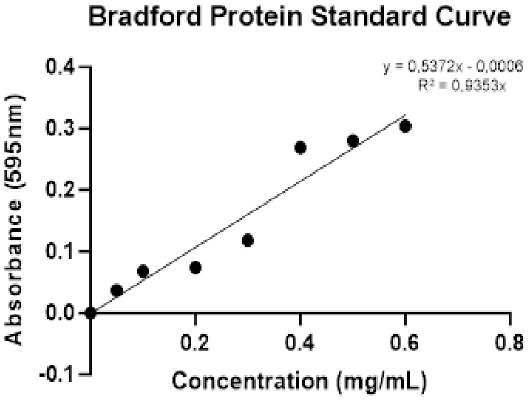

Figure 4. 5 Linear graph showing Bradford protein standard curve.

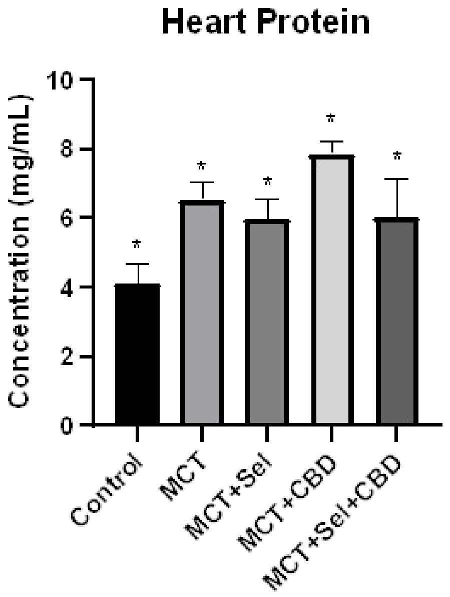

Figure 4. 6 The effect of CBD, Selexipag and Selexipag+CBD administration on protein concentration in heart tissueof MCT-induced PAH (matching * amount represents significance between two groups where p<0.05)

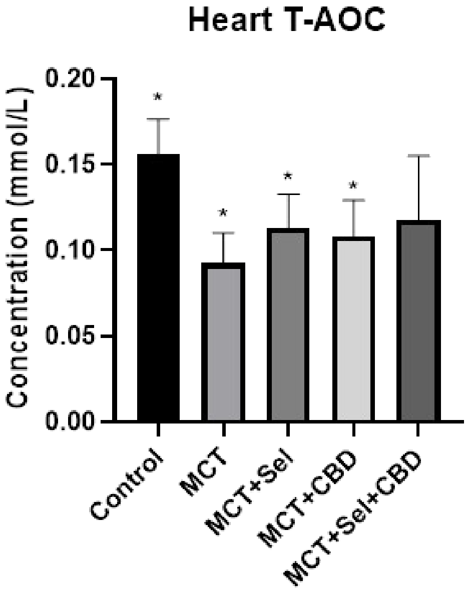

Figure 4. 7 The effect of CBD administration on T-AOC in heart tissue of MCT-induced PAH (*represents significance between groups)

Welch's ANOVA revealed significant differences in T-AOC levels among all experimental groups ($p<0.05$), indicating that the treatments significantly affected antioxidant capacity compared to baseline. To delineate these differences, unpaired Welch's T-Tests were performed, which highlighted significant disparities between the control group and the MCT Control group, as well as between the control group and two specific treatment groups: the MCT+Selexipag group and the MCT+CBD group ($p<0.05$). These findings confirm that both Selexipag and CBD individually led to significant alterations in T-AOC levels relative to the control.

The combination treatment group (MCT+Selexipag+CBD) exhibited the highest T-AOC concentration among all groups and was the only group whose T-AOC levels did not differ significantly from those of the control group. This result suggests that the combination therapy might effectively counteract the decrease in antioxidant capacity observed with the other treatments, potentially restoring T-AOC levels to near baseline values. The absence of a significant difference between the combination group and the control indicates that this therapy might maintain or enhance antioxidant defences more effectively than individual treatments.

4.4 Heart TNF-α

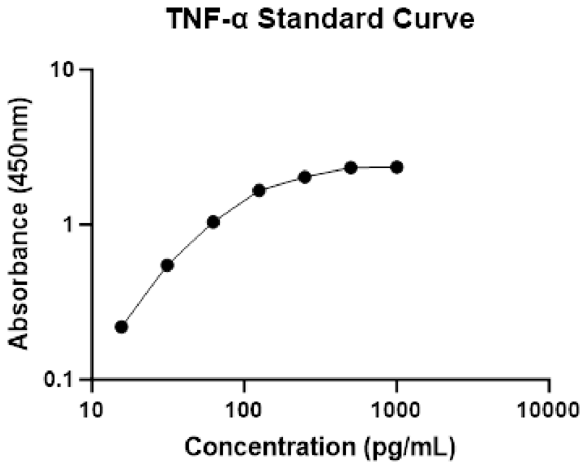

Figure 4. 8 Sigmoidal graph showing the TNF-α standard curve.

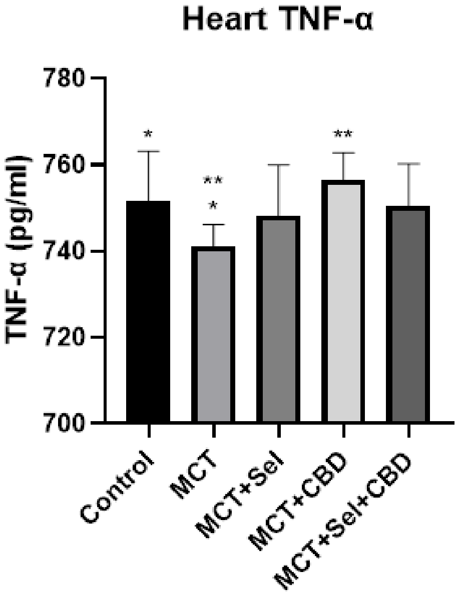

Figure 4. 9 The effect of CBD, Selexipag and Selexipag+CBD administration on TNF-α expression in heart tissue of MCT-induced PAH (matching * amount represents significance between two groups where p<0.05)

The analysis of TNF-α levels revealed distinct variations across the experimental groups. The MCT control group had the lowest TNF-α levels among all groups, suggesting a reduced inflammatory response compared to other treatments. In contrast, the MCT-CBD group showed the highest TNF-α levels, indicating an increased inflammatory response associated with CBD treatment. Statistical analysis using Welch's ANOVA demonstrated significant differences in TNF-α levels among all groups ($p<0.05$), confirming that the treatment effects on TNF-α were statistically significant.

Further, unpaired Welch's T-Tests highlighted significant differences between the control group and the MCT control group, as well as between the MCT control group and the MCT-CBD group ($p<0.05$). This indicates that the MCT treatment significantly altered TNF-α levels compared to the control and that CBD treatment further exacerbated this increase. However, no significant differences were observed between the control group and the MCT+Selexipag group, or between the MCT control group and the MCT+Selexipag group. This suggests that the addition of Selexipag, either alone or in combination with CBD, did not lead to statistically significant changes in TNF-α levels compared to the control or MCT control groups.

These findings indicate that while CBD treatment significantly increased TNF-α levels, the MCT+Selexipag treatment did not produce a notable difference in TNF-α levels compared to other treatments. The data underscore the impact of CBD on inflammatory markers and suggest that its effects may differ from those of Selexipag, both individually and in combination. Further investigation is warranted to explore the underlying mechanisms of these differences and to assess the implications for managing inflammatory responses in similar experimental models.

4.4 Absorbance data between experiments

Absorbance is a fundamental quantitative technique used to determine the concentration of a molecule in solution. According to Beer-Lambert’s law, the absorbance at a specific wavelength is directly proportional to the concentration of the absorbing substance, provided that the path length and the molar absorptivity remain constant. This principle enables accurate concentration measurements by analysing how much light is absorbed by the sample.

In this study, the reliability of absorbance measurements was paramount. The spectrometer used exhibited high absorbance linearity, which means it maintained a consistent linear relationship between absorbance and concentration across a broad range of concentrations. This capability is essential for accurate quantification, especially when working with samples of varying concentrations or when sample volumes are limited. High absorbance linearity also reduces the necessity for multiple dilutions, which can introduce potential errors and variability.

The accuracy and precision of the spectrometer are critical for ensuring reliable results, particularly when comparing measurements across different experiments or conditions. In this study, maintaining high absorbance linearity was crucial for minimizing systematic errors and for validating the consistency of the results. Furthermore, reliable absorbance measurements reflect the robustness of the assay methods employed, demonstrating that the analytical techniques used were both precise and reproducible. Ensuring that the spectrometer's performance adhered to high standards of absorbance linearity underscores the integrity of the experimental data and supports the validity of the conclusions drawn from the study.

Chapter 5

Discussion and Analysis of Findings

5.1 Considerations

It is crucial to consider that the large standard deviations observed across the groups may stem from several factors that impact the reliability and reproducibility of the data. One notable factor is the extended storage time of the samples. Prolonged storage can significantly affect biomarker stability and integrity. For instance, proteins and other analytes may undergo degradation, oxidation, or other modifications over time, especially if storage conditions are suboptimal. Freeze-thaw cycles, temperature fluctuations, and exposure to light or air can exacerbate these issues, potentially leading to inconsistent measurements and increased variability in the data. Implementing stringent storage protocols and using stabilizing agents can help mitigate these effects.

Another critical factor is the precision of pipetting techniques used during sample preparation and assay execution. Variations in pipetting volumes, whether due to technical inconsistencies or equipment calibration issues, can introduce significant error into the assay results. Pipetting precision is essential for accurate reagent addition and sample volume measurement, both of which are critical for reliable ELISA outcomes. Regular calibration of pipettes and adherence to standardized pipetting protocols can minimize these sources of variability.

Additionally, the performance and handling of ELISA kits and their components play a crucial role in the assay’s accuracy. ELISA relies on various components, including antibodies, antigens, and substrates, which must be handled and stored according to the manufacturer's instructions. Deviations from recommended conditions, such as improper storage temperatures or expiration of reagents, can compromise the sensitivity and specificity of the assay. Moreover, issues such as lot-to-lot variability of antibodies or inconsistencies in the preparation of standards can contribute to assay variability. Routine validation of assay performance and adherence to quality control measures, including the use of internal controls and calibration standards, are essential for ensuring consistent and accurate results.

In summary, addressing these factors, storage conditions, pipetting precision, and assay component handling, is critical for reducing variability and improving the reliability of the experimental data. Implementing rigorous quality control practices, including thorough documentation and regular equipment maintenance, will help enhance the reproducibility and robustness of the findings.

5.2 Haematological Analysis

The haematological analysis (table 4.1) focusing on platelet counts and haemoglobin levels provided critical insights into the effects of Selexipag and CBD on MCT-induced pulmonary hypertension. The MCT+Selexipag and MCT+Selexipag+CBD groups exhibited significantly elevated platelet counts compared to the control group, with the combination group showing the highest platelet levels ($p<0.05$). This increase in platelet count could be a response to the thrombotic potential of TNF-α, which stimulates platelet activation through the arachidonic acid pathway (Pignatelli et al., 2005). Furthermore, CBD's potential role in enhancing platelet activation (Randall, 2007) might contribute to the observed increase in platelet levels in these groups.

Haemoglobin levels also increased significantly in the MCT+Selexipag and MCT+Selexipag+CBD groups, with the combination group demonstrating the highest levels. This elevation in haemoglobin is likely a compensatory mechanism to address the hypoxic conditions induced by MCT, which causes pulmonary hypertension and reduces oxygen availability. Enhanced erythropoiesis in response to hypoxia is consistent with the findings of Atsma et al. (2012), who linked elevated haemoglobin levels with hypertension and increased oxygen-carrying capacity of the blood.

Despite the beneficial effects observed, the study highlights potential risks associated with excessive platelet activation. Elevated platelet counts can lead to thrombocythemia, which, although not observed during the trial or postmortem, remains a significant concern. The risk of thrombocythemia and its potential to exacerbate PAH, as noted by Zanjani (2012), underscores the need for careful consideration of the treatment's overall impact. The combination of Selexipag and CBD, while showing the most pronounced improvements in platelet and haemoglobin levels, may also introduce complexities that warrant further investigation.

Additionally, while the combination therapy offered the most pronounced improvements, the long-term safety and efficacy of Selexipag and CBD, both individually and in combination, need further exploration. Understanding the underlying mechanisms through which these treatments affect haematological parameters will be crucial for optimizing therapeutic strategies. Future research should focus on longitudinal studies to assess the sustained impact of these treatments on platelet counts, haemoglobin levels, and overall pulmonary and cardiovascular health. Moreover, investigating alternative dosing strategies and potential side effects will be important for refining treatment protocols and ensuring the safety and efficacy of these therapies in clinical settings.

5.3. Brain Natriuretic Peptide (BNP) and Total Antioxidant Capacity (T-AOC)

5.3.1 BNP Levels

Our results demonstrate that the combination of CBD and Selexipag significantly enhances BNP levels in the blood compared to either CBD or Selexipag alone (figure 4.2). BNP is a key biomarkerfor heart failure, produced by cardiomyocytes in response to increased ventricular pressure. Itslevels are elevated in conditions of chronic pressure overload, such as PAH, where it acts as avasodilator, promoting natriuresis and diuresis. The significant increase in BNP levels with the combined therapy suggests a synergistic effect of CBD and Selexipag in restoring or enhancing the natriuretic peptide system. This combination may improve the overall efficacyof the treatment by amplifying the vasodilatory and pressure-reducing effects of BNP. The observed synergy could be attributed to CBD’s potential anti-inflammatory and antioxidant properties combined with Selexipag’s role in prostacyclin receptor activation, which may together lead to a more substantial improvement in BNP levels than either treatment alone.

5.3.2 T-AOC in Blood

The increase in blood T-AOC levels following the combination therapy aligns closely with the elevation in BNP levels (figure 4.4), suggesting an interrelationship between antioxidant capacity and cardiac function. T-AOC is a comprehensive measure of the antioxidant defence system, encompassing various antioxidants that neutralize reactive oxygen species (ROS) and mitigate oxidative stress. The significant rise in T-AOC with the combined treatment implies that CBDand Selexipag may work synergistically to enhance the body's overall antioxidant capacity. This improvement could reduce oxidative stress, which has been linked to exacerbation of cardiac dysfunction and PAH. Leong et al. (2016) highlighted that increased oxidative stress correlates with ventricular dysfunction and that combining oxidative stress markers with BNPcould enhance early detection of heart failure. Our data support this by showing that increasedT-AOC levels, indicative of improved antioxidant defences, correspond with higher BNP levels, reflecting improved cardiac function and reduced oxidative damage.

5.3.3 T-AOC in Heart Tissue

The T-AOC levels in heart tissue were notably higher than in blood (figure 4.5), suggesting a localized effect of the treatments within the myocardium. The combination therapy group exhibited thehighest T-AOC levels in heart tissue, indicating that CBD and Selexipag together provide substantial antioxidant protection directly within the heart. This finding is consistent with Rajesh et al. (2011), who reported that CBD reduces oxidative stress and enhances cardiac function. The significant increase in heart T-AOC in the MCT-CBD group, despite lower blood T-AOC levels, highlights a targeted antioxidant effect of CBD on myocardial tissue. This could be due to CBD's preferential distribution or accumulation in the myocardium, where it can directly counteract oxidative stress associated with PAH. The ability of CBD to elevate T-AOC specifically in heart tissue underscores its potential as a therapeutic agent for cardiovascular conditions where oxidative damage is prevalent.

5.3.4 Comparative Analysis of BNP and T-AOC

The concordant increase in BNP and T-AOC levels observed with the combination therapy emphasizes the interconnectedness of oxidative stress and cardiac function. Elevated T-AOC levels, reflecting enhanced antioxidant capacity, appear to support improved BNP levels, suggesting that reducing oxidative stress can have a beneficial effect on cardiac function and BNP secretion. This relationship is particularly evident in the combination therapy group, where both biomarkers reached their highest levels, indicating that the combined treatment not only addresses oxidative stress but also promotes cardiac health. The higher T-AOC levels in heart tissue compared to blood further reinforce the localized efficacy of the treatments within the myocardium, suggesting that the antioxidant effects of CBD and Selexipag are particularly pronounced in the site of greatest oxidative stress.

5.3.5 Implications and Future Research

The findings from this study suggest that the combination of CBD and Selexipag has the potential to offer a comprehensive approach to managing PAH by targeting both oxidative stress and cardiac function. The synergistic effects of these treatments on BNP and T-AOC levels highlight their potential in improving cardiac health and reducing PAH symptoms. Future research should focus on elucidating the molecular mechanisms underlying the observed synergistic effects of CBD and Selexipag. Understanding how these treatments interact at a biochemical level could provide insights into optimizing their use and developing more effective therapeutic strategies. Additionally, investigating the role of specific antioxidants within the total antioxidant capacity could offer a more detailed understanding of how individual antioxidants contribute to the overall antioxidant defence. Long-term studies are necessary to evaluate the clinical efficacy and safety of this combination therapy, as well as its impact on patient outcomes, quality of life, and progression of PAH. Such research could ultimately lead to improved treatment protocols and better management of PAH in clinical settings.

5.4 TNF-α

The analysis of TNF-α levels across the experimental groups revealed substantial variations, underscoring the differential effects of the treatments on inflammation. The MCT control group exhibited the lowest TNF-α levels, which could indicate a lower inflammatory response compared to other treatments (figure 4.9). This observation is unexpected given the known association between MCT-induced PAH and elevated inflammatory markers (Ghofrani et al., 2012). Thelower TNF-α levels in the MCT control group might be attributed to the timing of sample collection, which may not fully capture the peak inflammatory response. Furthermore, diseaseprogression stages and the variability in disease onset across subjects could also influence TNF-α levels (Reddy et al., 2016).

In contrast, the MCT-CBD group showed the highest TNF-α levels, highlighting a pronounced inflammatory response associated with CBD treatment. Statistical analysis confirmed significant differences in TNF-α levels among the groups, with Welch’s ANOVA indicating statistical significance ($p<0.05$). Unpaired Welch’s T-Tests revealed significant differences between the control group and the MCT control group, as well as between the MCT control group and the MCT-CBD group ($p<0.05$). The lack of significant differences between the MCT+Selexipag group and the control or MCT control groups suggests that Selexipag, whether administered alone or in combination with CBD, did not significantly alter TNF-α levels.

5.4.1 Impact of CBD on TNF-α Levels

The elevated TNF-α levels observed in the MCT-CBD group are noteworthy, particularly considering previous research linking high TNF-α levels to adverse outcomes, including increased mortality (Irwin et al., 1999). The observed mortality rate of four in the MCT-CBD group aligns with this correlation, suggesting that CBD treatment might exacerbate inflammation rather than mitigate it. This paradoxical effect is intriguing given CBD’s known anti-inflammatory properties (Kozlowska et al., 2007). One possible explanation is that CBD's influence on TNF-α may be context-dependent, with its effects being overshadowed by other factors in the PAH model. CBD's complex interaction with inflammatory pathways may result in differential effects based on disease stage or specific inflammatory environments (Carrier et al., 2003).

The unexpectedly low TNF-α levels in the MCT control group prompt further investigation. This outcome could be due to the timing of the experiment or an early phase of disease progression. The lack of significant inflammatory response at this stage might reflect a period when the inflammatory markers have not yet reached their peak. Additionally, individual variability in treatment responses could affect TNF-α levels, with some animals potentially displaying a more pronounced inflammatory response due to genetic or health differences (Rittner et al., 2006).

5.4.2 Role of Selexipag

Selexipag’s lack of significant impact on TNF-α levels suggests that its therapeutic effects in PAH might not involve substantial modulation of inflammatory cytokines. Selexipag is known for its action as a selective prostacyclin receptor agonist, primarily influencing vasodilation and platelet aggregation (Ghofrani et al., 2012). Its mechanism of action might not directly involve the modulation of TNF-α or other inflammatory cytokines, indicating that its therapeutic benefits in PAH are likely mediated through other pathways. This finding aligns with existing literature, which has highlighted Selexipag’s role in improving hemodynamic and exercise capacity without markedly affecting inflammatory markers (Ghofrani et al., 2012).

5.4.3 Implications for Research and Treatment

The observed increase in TNF-α levels in the MCT-CBD group, despite CBD's known anti-inflammatory properties, suggests that the application of CBD in PAH may require careful reconsideration. This finding underscores the need for further research to elucidate the mechanisms underlying CBD's effects on inflammatory markers. Investigating the specific inflammatory pathways influenced by CBD and how these interact with PAH pathology could provide valuable insights. Additionally, understanding Selexipag’s role in managing PAH and its interaction with other treatments can inform therapeutic strategies and optimize patient outcomes.

Overall, these results contribute to a more nuanced understanding of inflammatory responses in PAH and highlight the importance of evaluating treatment effects on inflammatory biomarkers. Future studies should aim to address these issues, explore alternative therapeutic strategies, and investigate the interactions between different treatments to manage PAH and associated inflammatory responses effectively.

Chapter 6

Synthesis

With the increased prevalence and mortality rate of PAH, there isa case to be made with regards to finding a more effective, cheaper, and easily accessible treatment than the current gold standard treatment, Selexipag (Kaufmann et al., 2015). The ease on cannabis legislation globally for both recreational and medicinal use allows for more research to be conducted on a variety of diseases, particularly cardiovascular diseases, given CBD's known immunomodulatory, anti-inflammatory, and analgesic properties (Lu & Mackie, 2016).

As of recent times, there is substantial evidence that exists within the literature that shows CBD's efficacy in treating and managing various symptoms of hypertensive conditions, however not enough with regards to PAH, and co-treatment with Selexipag. This study therefore aimed to show how CBD, alone and in combination with Selexipag, affected key biomarkers Brain Natriuretic Peptide and TNF-α of PAH, as well as the Oxidative Stress and key haematologic factors, haemoglobin, and platelets, in a rat model of PAH. All these factors together helped paint a picture of PAH progression within the heart and vascular system and how the treatments affected the overall disease state and management of symptoms upon analysis of biological samples.

6.1 Comparative Analysis to Existing Literature

6.1.1 CBD and PAH

6.1.1.1 Combination Therapy Efficacy

The principal finding of this study indicates that CBD exhibits enhanced therapeutic efficacy when used in conjunction with Selexipag rather than as a standalone treatment. The co-administration of CBD and Selexipag demonstrated superior results compared to each agent used in isolation, underscoring the potential benefits of adjunctive therapy. Specifically, this combination therapy effectively normalised levels of BNP and T-AOC, aligning these markers with control levels. This outcome suggests that the synergistic effects of CBD and Selexipag may offer a more robust approach for managing PAH.

Emerging literature supports the role of CBD in cardiovascular modulation. Studies have indicated that CBD can influence cardiovascular health by affecting oxidative stress and inflammation, key factors in PAH (Izzo et al., 2009). Selexipag, a selective prostacyclin receptor agonist, has been well-documented for its efficacy in reducing pulmonary vascular resistance and improving PAH outcomes (Galiè et al., 2016). The combination of these two agents leverages their distinct mechanisms to achieve a more comprehensive therapeutic effect.

6.1.1.2 Mechanisms and Biomarker Implications

The combination therapy's impact on BNP and T-AOC levels is consistent with previous research highlighting the importance of these biomarkers in assessing cardiac function and oxidative stress. BNP is a well-established biomarker for heart failure and PAH, reflecting ventricular strain and pressure overload (Hsu et al., 2015). Elevated BNP levels are indicative of disease severity and treatment efficacy. Our findings that combination therapy elevated BNP levels suggest a beneficial effect on cardiac function in PAH. T-AOC is a measure of the overall ability of antioxidants to neutralise free radicals and reduce oxidative stress. Increased T-AOC levels, as observed with the combination therapy, reflect improved antioxidant defence mechanisms (Rajesh et al., 2011). This is supported by literature suggesting that enhanced T-AOC is associated with reduced oxidative stress and better cardiovascular health (Vannacci et al., 2012). Leong et al. (2016) emphasised the value of integrating BNP with markers of oxidative stress and inflammation, such as TNF-α, to enhance the detection of ventricular dysfunction and early-stage heart failure. Their research aligns with our findings, suggesting that increased BNP levels and T-AOC are indicative of improved cardiac function and reduced oxidative stress in PAH management. This reinforces the utility of these biomarkers as diagnostic and monitoring tools in PAH.

In conclusion, the combination of CBD and Selexipag provides a promising therapeutic approach for PAH, as it effectively normalises BNP and T-AOC levels, indicating improved cardiac function and reduced oxidative stress. This finding aligns with emerging research on the cardiovascular benefits of CBD and the established efficacy of Selexipag. Future studies should continue to explore the mechanisms underlying these effects and evaluate the long-term benefits of combination therapies in PAH and other cardiovascular conditions.

6.1.2 TNF-α Levels and Inflammation

6.1.2.1 Elevated tumour necrosis factor- α (TNF-α) Levels in the MCT-CBD Group

The study revealed elevated levels of TNF-α in the MCT-CBD group, suggesting a potential exacerbation of inflammation due to CBD treatment. This finding contrasts with the broader literature, which generally supports the anti-inflammatory properties of CBD. For instance, Kozlowska et al. (2007) and Mechoulam et al. (2014) have documented CBD's efficacy in reducing inflammation in various preclinical models. The observed increase in TNF-α levels may reflect specific interactions between CBD and the inflammatory pathways in the context of PAH. CBD's effect on inflammation might be influenced by the disease model, dosage, and duration of treatment (Campos et al., 2012) (VanDolah et al., 2016).

6.1.2.2 Influence of CBD on Haematological Parameters

In addition to TNF-α, haematological parameters such as platelet count, and haemoglobin levels can provide insights into disease state and progression. Elevated TNF-α levels are associated with increased inflammatory responses, which can affect haematological markers and contribute to disease severity. Although CBD, in isolation, restored heart T-AOC and normalised TNF-α levels to control values, its overall impact on inflammation in PAH appears paradoxical. Higher TNF-α levels are indicative of severe disease and increased mortality risk, as demonstrated by Irwin et al. (1999), who found a significant correlation between elevated TNF-α levels and increased mortality in PAH models. The increased mortality observed in the MCT-CBD group underscores the need to carefully assess CBD's role in modulating inflammatory markers and its potential as a therapeutic agent.

6.1.2.3 Potential Screening Methods

The elevated TNF-α levels in the MCT-CBD group suggest that CBD may be useful in screening for PAH, where increased TNF-α levels are indicative of severe disease and higher mortality risks. Irwin et al. (1999) emphasized that TNF-α levels could serve as a biomarker for assessing disease progression and mortality risk in PAH. However, the apparent discrepancy between the expected anti-inflammatory effects of CBD and the observed increase in TNF-α necessitates further investigation. This includes exploring CBD's effects in different experimental models and evaluating its potential role in PAH management.

6.1.3 T-AOC and Cardiovascular Health

6.1.3.1 CBD's Role in Enhancing Antioxidant Defence

The results of this study highlight the significant potential of CBD in enhancing antioxidant defences and mitigating oxidative stress in the cardiovascular system, especially in the context of PAH. Rajesh et al. (2011) conducted a study on CBD's influence on oxidative stress and cardiac dysfunction in a hypertensive model and reported that CBD notably increased antioxidant capacity in cardiac tissues. This finding supports our observation that CBD administration improves T-AOC, reflecting its protective role against oxidative damage in the heart (Rajesh et al., 2011).

Further research by VanDongen et al. (2016) also demonstrated that CBD has a potent antioxidant effect, which can help in reducing oxidative stress and subsequent tissue damage in various models of cardiovascular disease. This complements our study's findings that CBD enhances antioxidant status, providing additional evidence of its therapeutic potential.

6.1.3.2 Mechanisms of Vascular Remodelling and Antioxidant Defence

The impact of CBD on vascular remodelling and its potential to reverse damage is significant. CBD's role in reducing oxidative stress and enhancing antioxidant defences is crucial for vascular health. A study by Moreira et al. (2015) highlighted that CBD attenuates vascular inflammation and oxidative stress, leading to improved vascular function. This aligns with the results, which suggest that CBD contributes to reversing vascular damage through its effects on antioxidant pathways.

Similarly, a study by Di Marzo et al. (2004) explored the effects of cannabinoids on vascular function and found that CBD plays a role in reducing oxidative stress and inflammation, which can be beneficial in treating cardiovascular disorders. This reinforces our observation that

CBD’s increase in T-AOC might protect against oxidative stress-induced vascular remodelling in PAH.

6.1.3.3 Influence of TNF-α on Platelet Function

The relationship between antioxidant defences and inflammatory markers, such as TNF-α, is critical in understanding the broader implications of CBD treatment. Elevated TNF-α levels have been associated with platelet hyperfunction, exacerbating cardiovascular conditions. Pignatelli et al. (2005) investigated the impact of TNF-α on platelet activation and found a strong correlation between elevated TNF-α levels and increased platelet aggregation. This connection is relevant to our study, where increased platelet count was observed in the MCT-Selexipag-CBD group, possibly linked to elevated TNF-α levels.

In addition, research by Jang et al. (2012) showed that TNF-α can enhance platelet activation, which could be an underlying factor in the increased platelet count observed in our study. Understanding these interactions is crucial for optimizing CBD’s therapeutic effects and addressing potential inflammatory contributors to PAH.

6.1.3.4 Clinical Implications and Future Research

The findings from this study, coupled with existing literature, suggest that CBD holds considerable promise in enhancing antioxidant defences and reversing vascular damage in PAH. However, the role of inflammatory markers like TNF-α and their impact on treatment outcomes requires further investigation. Future studies should focus on the interactions between CBD, oxidative stress, and inflammatory markers to refine therapeutic strategies for PAH. Additionally, exploring long-term effects and potential interactions with other treatments will be essential to fully understand CBD’s therapeutic potential and optimise its use in managing PAH.

6.1.4 Haematological Profile

In comparison to the broader literature, the results underscore a complex interaction between CBD and haematological parameters- platelets and haemoglobin. While Atsma et al. (2012) and Bonderman et al. (2005) highlight elevated haemoglobin as a marker of hypertension and vascular stress, the increased platelet counts observed here further align with research by Chiu et al. (2014), who discuss platelet activation as a critical factor in cardiovascular diseases.

The discrepancy between the expected anti-inflammatory effects of CBD and the observed increase in platelet counts and haemoglobin levels could be attributed to differences in the experimental models or dosages used. Rajesh et al. (2011) reported that CBD increased antioxidant capacity in cardiac tissues, supporting a potential role for CBD in reducing oxidative stress. However, the observed increase in platelet counts and haemoglobin levels in this study might suggest a more nuanced role of CBD, where it could contribute to vascular stress or inflammatory processes in PAH, contrary to its anti-inflammatory effects (Kozlowska et al., 2007).

6.1.4.1 Impact of CBD on Platelet Count and Haemoglobin Levels

The observed increase in platelet counts and haemoglobin levels in the MCT-CBD group suggests a potential impact of CBD on haematological parameters, which may be relevant to PAH pathophysiology. Elevated haemoglobin levels in this context align with the findings of Atsma et al. (2012), who demonstrated that elevated haemoglobin is frequently associated with hypertension. This study's results indicate a possible compensatory response to increased vascular stress or hypoxia, commonly observed in PAH, where erythropoiesis is stimulated to enhance oxygen transport (Bonderman et al., 2005).

6.1.4.2 Platelet Activation and PAH

The increase in platelet count observed in the MCT-CBD group corresponds with the literature highlighting the role of platelet activation in PAH progression. Zanjani (2012) emphasised that platelet activation contributes significantly to PAH by promoting endothelial dysfunction and inflammation, which exacerbates vascular remodelling and thrombotic events. The findings in this study reinforce these observations by showing elevated platelet counts, suggesting that CBD treatment might inadvertently enhance platelet hyperfunction, potentially worsening PAH outcomes.

McLaughlin et al. (2006) further support this association, demonstrating that platelet activation is a critical factor in PAH development, leading to increased thrombotic risk and vascular inflammation. Their study indicates that heightened platelet activity contributes to the pathogenesis of PAH, aligning with the results showing increased platelet counts in the MCT-CBD group.

6.1.4.3 Haemoglobin Levels and Disease Mechanisms

The increase in haemoglobin levels in the MCT-CBD group is also consistent with findings from van den Berg et al. (2010), who linked chronic hypoxia with elevated haemoglobin levels as an adaptive response. This adaptation aims to enhance oxygen delivery but might also indicate underlying disease severity. Elevated haemoglobin in PAH often reflects the body's response to prolonged hypoxic conditions, which are prevalent in PAH patients (Gulati et al., 2016). The findings suggest that CBD may influence this adaptive response, potentially affecting disease progression and severity.

6.2 Limitations

A significant limitation of this study is the non-pressure measurement by right heart catheterisation, an invasive procedure to establish an increased pressure in the right ventricle due to PAH. Use of this kind of procedure is not particularly cheap or easy to do as one would need a substantial amount of funding, equipment, and microsurgical training. Furthermore, obtaining ethical clearance for this highly invasive procedure would come with its own challenges and limitations, however a case could be made in future studies forthe use of this procedure to be done as it would yield incredibly accurate results and higher quality research in any cardiovascular study by monitoring blood pressure directly in the right, or left, ventricle of the heart and in real time, whilst comparing a range of diseases and drugs at different dosages.

As mentioned previously, a shortcoming during the experimental protocol was the continued loss of animals due to the nature of MCT’s physiological effects on the cardiopulmonary system. This could be addressed in future studies by means of possibly lowering MCT dosages and/or increasing Selexipag/CBD dosages until a balanced optimal dosage is found that prevents intense adverse effects on the cardiopulmonary system.

6.3 Recommendations for Future Research

Based on the findings of this study, several recommendations for future research emerge. First, while this study demonstrates the potential of CBD in conjunction with Selexipag for treating

PAH, further investigation is needed to elucidate the underlying mechanisms by which CBD affects biomarkers like TNF-α and BNP, particularly given the observed increase in TNF-α levels. Future studies should explore different dosages and treatment regimens of CBD and Selexipag to optimise therapeutic outcomes and minimise potential adverse effects. Additionally, research should extend to human clinical trials to validate the efficacy and safety of CBD and its combination with Selexipag in PAH patients. Investigating the long-term effects and potential interactions of CBD with other PAH therapies could also provide valuable insights. Finally, examining the impact of CBD on other biomarkers and pathways involved in PAH, as well as its effects on different animal models or patient populations, could further refine our understanding and application of this potential treatment.

Conclusion

From the results generated it is clear to see both the positive and negative effects that CBD can have on the expression on TNF-α, BNP, and oxidative stress. CBD's efficacy lies in whereother drugs are taken in conjunction with it, in this case Selexipag. There is promise with regards to CBD and Selexipag being taken in combination to increase BNP levels, by activation of the natriuretic peptide system which will ameliorate pulmonary hypertension owing to the direct vasodilatory properties of BNP. Further research would therefore need to be conducted to investigate the synergistic relationship that CBD and Selexipag has. In contrast to that and in this case, CBD seems to increase TNF-α and further drive PAH and inflammation which can be supported by the platelet count and haemoglobin levels, as well as mortality rate in that group. By increasing/decreasing amounts of MCT and/or CBD, as well as further prolonging the experiment itself, we could potentially see a decrease in TNF-α levels. Research would need to be done to investigate why CBD contributed to a rise in TNF-α, and why the lowest levels were recorded in the MCT control group. Interestingly, T-AOC in the heart, specifically in the MCT-CBD group, was the highest, owing to CBD's potential to attenuate oxidative stress and counteract the increased stress and inflammation the heart was under during PAH.

The study showed promise overall with regards to CBD being used, not necessarily as an alternate form of treatment, but rather in conjunction with Selexipag to treat PAH by way of decreasing BNP levels. However, there is promise for CBD to be used to decrease oxidative stress in PAH patients and potentially monitor PAH progression by looking at TNF-α levels, platelet counts, and haemoglobin levels. However, it should be worth taking into major consideration, by looking at the platelet and haemoglobin level, that taking CBD and Selexipag in combination with each other shows thrombogenic characteristics that could potentially increase viscosity of the blood making it an unviable option for patients with haematological diseases. The study could in future be further extrapolated with different parameters, as discussed earlier, to potentially give us an overview of understanding PAH pathogenesis, particularly with CBD.

In summary, this study highlights the promising role of CBD when used in combination with Selexipag for the treatment of PAH. The combination therapy proved superior to individual treatments, effectively increasing BNP levels and T-AOC, which are critical for managing PAH. These findings suggest a synergistic interaction between CBD and Selexipag that enhances the natriuretic peptide system and improves oxidative stress markers, potentially offering a novel adjunctive treatment for PAH. However, the study also identified concerns, such as the increase in TNF-α levels with CBD alone, which may exacerbate inflammation and could undermine the treatment's effectiveness. The observed limitations, including the absence of pressure measurements and the loss of animals during the study, underscore the need for further research. Future investigations should aim to explore optimal dosing strategies, validate these findings in human clinical trials, and assess long-term effects and interactions with other therapies. By addressing these gaps, subsequent studies could refine therapeutic approaches, enhance understanding of CBD’s mechanisms, and provide new insights into PAH management. Overall, this research contributes to the growing body of evidence supporting the use of cannabinoids in cardiovascular health and suggests significant potential for future therapeutic developments.

The findings of this study have significant implications not only for cardiovascular diseases but also for the broader application of medicinal cannabinoids in treating various conditions. With the increasing legalisation and the established therapeutic benefits of cannabinoids, there is a promising opportunity for extensive research in this field. This research could pave the way for the development of natural, cost-effective treatment options that are both accessible and commercially viable. Moreover, the potential societal impact is substantial, as it could offer a more affordable alternative to conventional medications and alleviate the financial burden on global healthcare systems.

The hypothesis that combining CBD with Selexipag enhances therapeutic outcomes in PAH is partially supported by the findings. While the combination therapy improved BNP and T-AOC levels, indicating better cardiovascular function and oxidative stress reduction, the unexpected increase in TNF-α levels with CBD alone suggests a potential pro-inflammatory effect. Regarding the research questions, CBD lowered BNP but increased TNF-α, while Selexipag reduced BNP without affecting TNF-α. Their combination normalised BNP but had no significant impact on TNF-α levels, suggesting a balancing effect. In terms of oxidative stress, CBD significantly reduced T-AOC, while Selexipag moderately preserved it, the combination brought T-AOC levels closest to the control group, indicating a protective role. These results highlight both the potential and complexity of using CBD and Selexipag in PAH treatment, emphasising the need for further investigation into dosage optimisation and inflammatory responses.

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Appendices

Table A1. 1 Full haematological profile of Control group (Group 1)

	1.1	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**1.7**	**1.8**	**Average**
WBC (10^3/µL)	9,3	8,4	6,1	5,9	6,4	8,5	7,3	6,9	**7,4**
RBC (10^6/µL)	8,81	9,62	8,79	7,44	8,59	9,02	9,19	9,46	**8,9**
HGB (g/dL)	15,4	16,4	15,3	13,2	15,1	15,6	15,9	16	**15,4**
HCT (%)	48	51,6	47,6	40,8	47,4	48,4	51	50	**48,1**
MCV (fL)	54	54	54	55	55	54	55	53	**54**
MCH (pg)	17,5	17,1	17,4	17,7	17,6	17,3	17,3	16,9	**17,4**
MCHC (g/dL)	32,1	31,9	32,1	32,3	31,8	32,3	31,2	32	**32**
RDW (%)	9,2	9,8	9,3	9,3	9,7	9,8	9,3	9,6	**9,5**
PLT (10^3/µL)	736	739	742	783	760	803	921	797	**785**
MPV (fL)	5,9	6,3	5,6	5,9	5,9	5,9	5,9	6	**5,9**
PCT (%)	0,433	0,464	0,418	0,461	0,447	0,473	0,542	0,48	**0,465**

Table A1. 2 Full haematological profile of Monocrotaline Control group (Group 2) *Indicates animal that was lost.

	2.1	**2.2***	**2.3**	**2.4***	**2.5**	**2.6***	**2.7**	**2.8**	**Average**
WBC (10^3/µL)	9,9		5,2		6		4,4	7,6	**6,6**
RBC (10^6/µL)	8,18		8,68		8,99		8,49	7,49	**8,4**
HGB (g/dL)	14,2		13,1		14,9		14,2	12,7	**13,8**
HCT (%)	44,1		46,1		49,1		47	39,2	**45,1**
MCV (fL)	54		53		55		55	52	**54**
MCH (pg)	17,4		17,4		17,6		17,9	16,9	**17,4**
MCHC (g/dL)	32,3		32,7		32,3		32,3	32,3	**32,4**
RDW (%)	9,6		9,2		9,3		9,1	10,6	**9,6**
PLT (10^3/µL)	508		749		863		769	344	**647**
MPV (fL)	5,9		5,4		5,8		5,5	6,3	**5,8**
PCT (%)	0,299		0,403		0,497		0,423	0,216	**0,368**

Table A1. 3 Full Haematological Profile of Monocrotaline-Selexipag group (Group 3) *Indicates animal that was lost.

	3.1	**3.2**	**3.3***	**3.4**	**3.5**	**3.6**	**3.7**	**3.8**	**Average**
WBC (10^3/µL)	7,8	8,4		8,6	4,3	4,7	9,5	5,1	**7,0**
RBC (10^6/µL)	6,63	9,46		9,49	7,78	8,01	6,43	9,31	**8,16**
HGB (g/dL)	11,4	17		15,8	12,7	15	11,2	17,4	**14,4**
HCT (%)	34,5	52,2		49,3	38,8	46,4	34,7	53,5	**44,2**
MCV (fL)	52	55		52	50	58	54	57	**54**
MCH (pg)	17,1	18		16,7	16,4	18,7	17,4	18,7	**17,6**
MCHC (g/dL)	32,9	32,6		32,1	32,9	32,2	32,3	32,6	**32,5**
RDW (%)	10,2	9,1		10,2	11,3	9,1	9,1	8,7	**9,7**
PLT (10^3/µL)	210	819		901	798	856	208	837	**678**
MPV (fL)	6	5,9		6,1	5,9	6,1	6	5,6	**5,9**
PCT (%)	0,126	0,482		0,553	0,47	0,526	0,125	0,471	**0,393**

Table A1. 4 Full Haematological Profile of Monocrotaline-CBD group (Group 4) *Indicates animal that was lost.

	4.1	**4.2***	**4.3**	**4.4**	**4.5***	**4.6***	**4.7***	**4.8**	**Average**
WBC (10^3/µL)	6,1		6,1	5,4				6,4	**6**
RBC (10^6/µL)	7,61		8,13	9,32				9,54	**8,65**
HGB (g/dL)	13,7		15,2	15,9				18,5	**15,8**
HCT (%)	42,4		46,5	48,7				52	**47,4**
MCV (fL)	56		57	52				55	**55**
MCH (pg)	18		18,7	17				18,5	**18,1**
MCHC (g/dL)	32,4		32,8	32,6				32,9	**32,7**
RDW (%)	9,5		8,4	10,5				9,8	**9,6**
PLT (10^3/µL)	842		862	791				848	**836**
MPV (fL)	5,4		6	5,8				6,4	**5,9**
PCT (%)	0,452		0,518	0,438				0,481	**0,472**

Table A1. 5 Full Haematological Profile of Monocrotaline-Selexipag-CBD group (Group 5) *Indicates animal that was lost.

	5.1*	5.2	5.3*	5.4	5.5	5.6*	5.7	5.8	Average
WBC (10^3/µL)		9,3		5,3	9		5,1	8,7	**7,5**
RBC (10^6/µL)		8,88		8,37	8,81		8,6	7,96	**8,5**
HGB (g/dL)		15,7		14,7	14,9		14,6	13,9	**14,8**
HCT (%)		48,1		45,1	46,6		45	42,5	**45,5**
MCV (fL)		54		54	53		52	53	**53**
MCH (pg)		17,7		17,6	16,9		17	17,5	**17,3**
MCHC (g/dL)		32,7		32,7	32		32,4	32,7	**32,5**
RDW (%)		9,3		9,9	9,6		9,5	9,5	**9,6**
PLT (10^3/µL)		851		858	798		617	780	**781**
MPV (fL)		5,8		5,6	5,5		5,9	5,4	**5,6**
PCT (%)		0,49		0,483	0,448		0,363	0,419	**0,441**

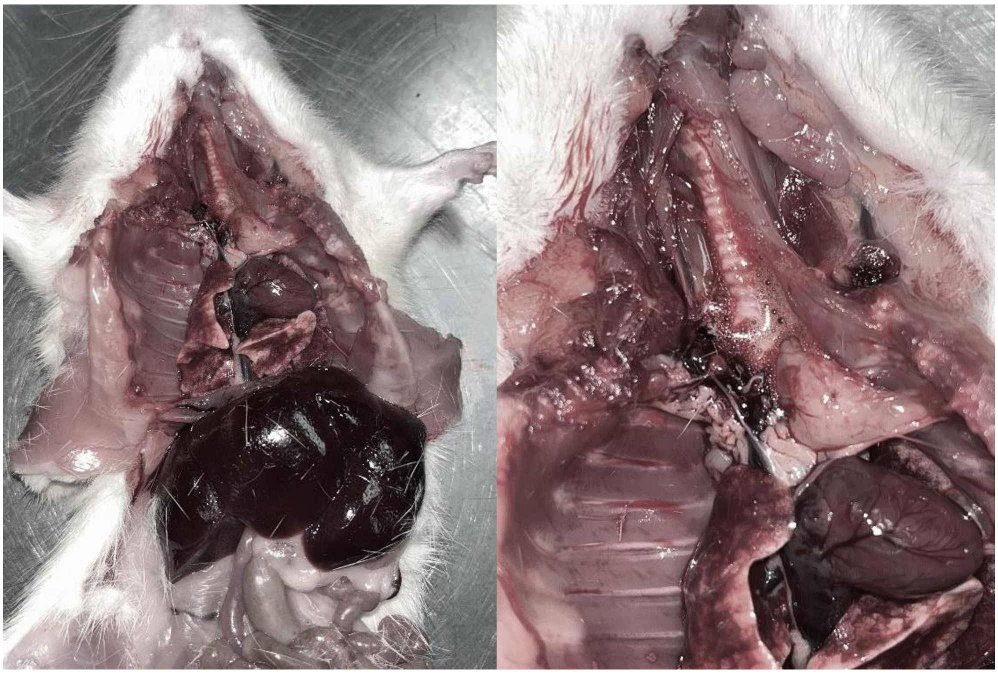

Figure A1. 1 Photograph showing pulmonary oedema and frothing in a rat that prematurely succumbed to the effects of MCT. The image highlights the fluid accumulation in the lung tissue, evident from the engorged and fluid-filled lungs. Notably, frothing/foaming is observed within the lungs, indicative of severe pulmonary distress. This manifestation of pulmonary oedema, coupled with frothy fluid, underscores the acute cardiopulmonary damage caused by MCT. The inset provides a close-up view of the foaming and oedema, illustrating the severity of the pathological changes. This observation emphasizes the critical impact of MCT on lung function and reinforces the importance of evaluating therapeutic interventions for PAH.