The potential for repurposing α_2-adrenoceptor agonists and noradrenaline uptake1 inhibitors in the management of septic shock.

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Abstract

Background: Septic shock is a life-threatening condition characterised by consistent hypotension caused by vasoplegia, endothelial dysfunction, and abnormal nitric oxide (NO) metabolism, leading to multiple organ failure. It affects more than 48 million people globally each year and is associated with approximately 11 million deaths. The guidelines from the Surviving Sepsis Campaign (SSC) describe early fluid resuscitation and antibiotics as the fundamental treatments of sepsis. However, a large number of patients with sepsis still progress to septic shock with unstable haemodynamic responses, necessitating the use of vasopressors to enhance organ perfusion. Noradrenaline (NA) is the first choice to be used in intensive care units (ICUs), but high doses of NA might lead to adverse cardiac complications such as tachyarrhythmia, peripheral ischaemia, and immunosuppression. A strategy of combining NA with other vasoactive agents has been recently proposed to reduce the required doses or the time spent on NA infusion and to improve the outcomes of sepsis patients in ICU.

Methods: Porcine isolated blood vessels were used in vascular contractility studies of α_2-adrenoceptor agonists and NA uptake1 inhibitors, which were dissected and set up in organ baths containing Krebs-Henseleit solution. The effect of dexmedetomidine on endothelial cell permeability was demonstrated using Human Umbilical Vein Endothelial Cells (HUVECs) in tissue culture experiments. In a clinical retrospective cohort study, data from 426 ICU patients at Nottingham University Hospitals (NUHs) were collected to examine the association between clonidine infusion and changes in neutrophils, white blood cells (WBCs), and platelets.

Results: In addition to the central actions of α_2-adrenoceptor agonists, they have various peripheral effects including immunosuppressive actions and vasoconstriction. The administration of dexmedetomidine as a sedative agent in ICU patients can potentially improve peripheral vascular resistance and subsequently maintain blood pressure through peripheral vasoconstriction. The present results showed that dexmedetomidine, clonidine, brimonidine, guanfacine, and mivazerol exhibit vasoconstrictive actions in peripheral and central porcine isolated blood vessels. The splenic vein showed higher sensitivity to α_2-adrenoceptor agonists compared to the splenic and tail arteries. The combination of NA with dexmedetomidine or guanfacine enhanced NA mediated contractions in porcine splenic arteries. The potency and maximum contraction induced by therapeutic related concentrations of NA (1 nM - 200 nM) were enhanced in the presence of 10 nM dexmedetomidine or 100 nM guanfacine. The -Log $EC_{30\ KCl}$ of NA increased from 7.06 ± 0.07 to 7.40 ± 0.09 (p-value < 0.0001), and from 6.92 ± 0.06 to 7.63 ± 0.10 (p-value < 0.001) in the presence of dexmedetomidine and guanfacine, respectively. Dexmedetomidine (50 µM) showed protective action on LPS induced endothelial cell hyperpermeability in HUVECs. Therapeutically relevant concentrations of NA uptake1 inhibitors such as atomoxetine, reboxetine, and desipramine enhanced the EFS induced contraction by increasing the time duration to 50% relaxation in porcine splenic and renal arteries. The combination of NA with atomoxetine 100 nM enhanced NA mediated contractions, where the Log $EC_{30\ KCl}$ of NA increased from 7.06 ± 0.06 to 7.43 ± 0.07 (p-value < 0.01). The retrospective cohort study demonstrated that there was no significant difference in the levels of neutrophils, WBCs, and platelets between the clonidine and control groups.

The hospital survival rate was significantly higher in the clonidine group compared to the control group (p-value < 0.05).

Conclusion: The vasoconstrictive effects of α_2-adrenoceptor agonists suggest that using dexmedetomidine and clonidine as sedatives in ICU might have additional non-sedative actions involving the immune system and cardiovascular system, potentially benefiting the management of sepsis. Dexmedetomidine and guanfacine demonstrated NA-sparing effects by reducing the required NA dosage by 50%. NA uptake1 inhibitors enhanced the duration of vasoconstriction induced by endogenous NA, and coadministration of atomoxetine with exogenous NA produced NA-sparing effects. In ICU patients, clonidine infusion was associated with increased hospital survival despite no changes in the levels of neutrophils, platelets, and WBCs.

Publications

Thesis related abstract publications:

Julian Benyamen, Paul Smith, Martin Beed, Vincent Wilson. Do selective α_2-adrenoceptor agonists have a role in the management of septic shock? Pharmacology 2020. British Pharmacological Society. Virtual Conference.

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Awards

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Abbreviations

α	Alpha
β	Beta
^{0}C	Temperature degrees in Celsius
ADHD	Attention Deficit Hyperactivity Disorder
AC	Adenylyl Cyclase
ADP	Adenosine Diphosphate
AGTR	Angiotensin Receptor
APACHE	Acute Physiology and Chronic Health Evaluation
AVPR	Arginine Vasopressin Receptor
BBB	Blood Brain Barrier
BP	Blood Pressure
cAMP	Cyclic Adenosine Monophosphate
CCDMS	Critical Care Minimum Dataset
cGMP	Cyclic Guanosine Monophosphate
COMT	Catechol-O-methyltransferase
CNS	Central Nervous System
CRP	C-Reactive Protein
DAG	Diacylglycerol
DBPS	Dulbecco's Phosphate Buffered Saline
DMSO	Dimethylsulfoxide

DW	Distilled Water
ECM	Endothelial Cell Medium
ECGS	Endothelial Cell Growth Supplement
eNOS	Endothelial Nitric Oxide Synthase
EFS	Electrical Field Stimulation
FBS	Fetal Bovine Serum
GDPR	General Data Protection Regulations
GPCR	G-protein Coupled Receptors
HBMVECs	Human Brian Microvascular Endothelial Cells
HMGB1	Human Mobility Group Box 1
HPMVECs	Human Pulmonary Microvascular Endothelial Cells
HUVECs	Human Umbilical Vein Endothelial Cells
Hz	Hertz
ICAM-1	Intracellular Adhesion Molecule 1
ICU	Intensive Care Unit
IG	Information Governance
IL	Interleukin
IMS	Industrial Methylated Spirit
iNOS	Inducible Nitric Oxide Synthase
IP3	Inositol Triphosphate
IV	Intravenous

LOS	Length of Stay
LPS	Lipopolysaccharide
MAP	Mean Arterial Pressure
MAPK	Mitogen-activated Protein Kinase
MAO	Monoamine oxidase
mA	Milliampere
MD2	Myeloid-2 Differentiation Factor
MIMVEC	Mouse Intestinal Microvascular Endothelial Cells
MLC	Myosin Light Chain
MLCP	Myosin Light Chain Phosphatase
MLCK	Myosin Light Chain Kinase
ms	Millisecond
NA	Noradrenaline
NAT	Noradrenaline Transporter
NF-kB	Nuclear Factor-kB cells
NHS	National Health Service
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NUH	Nottingham University Hospital
P/S	Penicillin/Streptomycin
P	Passage
PCA	Porcine Coronary Artery

PIP2	Phosphoinositol-bis-Phosphate
PKA	Protein Kinase A
PLC	Phospholipase
PR	Pulse Rate
PSA	Porcine Splenic Artery
PRA	Porcine Renal Artery
PTA	Porcine Tail Artery
REC	Research Ethics Committee
sec	Second
SIRS	Systemic Inflammatory Response Syndrome
SSC	Surviving Sepsis Campaign
SNP	Sodium Nitroprusside
SP	Substance P
TEER	Transepithelial Electrical Resistance
TLR-4	Toll-Like Receptor-4
TNF-α	Tumour Necrosis Factor-α
VE-cadherin	Vascular Endothelial Cadherin
VCAM-1	Vascular Cell Adhesion Protein 1

1. Chapter One: General Introduction

1.1. General Introduction

Septic shock is the most severe form of sepsis and considered as a major cause of death in intensive care units (ICUs) worldwide (Vincent *et al.,* 2019). It is characterised with persistent hypotension, compromised organ perfusion, loss of peripheral vascular resistance and vascular leakage as a response to the infection. Patients with septic shock require vasopressor therapy to obtain a targeted mean arterial pressure (MAP) (≥65 mmHg) after adequate fluid resuscitation (Shankar-Hari *et al.,* 2016). Noradrenaline (NA) has been identified as the first line vasoactive agent to support arterial pressure and organ function in critically ill patients. Sepsis induces immune paralysis, which may prevent patients from clearing primary infections and increase the risk of secondary infections. The high infusion of NA might cause further immunosuppression and subsequently worsen the hemodynamic instability (Stolk *et al.,* 2020). The usage of multi vasopressor therapies could be useful to avoid adverse effects of high doses of monotherapy. Therefore, this study hypothesised that the reduction in the dosage of NA by combining it with vasopressors that have NA-sparing activities might improve the outcomes of ICU patients.

1.2. Introduction on sepsis and septic shock

The American College of Chest Physicians/Society consensus conference has distinguished between sepsis, severe sepsis, and septic shock. Accordingly, sepsis was defined as a host's systemic inflammatory response syndrome (SIRS) to infection, where the SIRS referred to four variables: temperature, heart rate (HR), respiratory rate, and white blood cell (WBC) count. Sepsis associated with organ dysfunction was termed severe sepsis, which could then progress to the septic shock, defined as "sepsis-induced hypotension persisting despite adequate fluid resuscitation." (Bone *et al.,* 1992). In 2001, the International Sepsis Definitions conference suggested that

the previous definitions of the sepsis and related conditions should be updated through expanding the list of diagnostic criteria, however no evidence exist to support the change in the sepsis definitions (Levy *et al.,* 2003). It has been reported that the presence of SIRS, infection, organ dysfunction within the initial 24 hours in the ICU are required to meet the criteria of severe sepsis (Padkin *et al.,* 2003). The guidelines of sepsis definitions have remained mostly unchanged for more than two decades. Nonetheless, the SIRS definition is no longer used as screening criteria but it is relevant to the identification of the infection raised from burn or trauma and can be used to define uncomplicated sepsis (Daniels, 2022). Figure 1.1 summarises the pattern of progression stages of the sepsis.

Despite of immediate activation of inflammatory process, coagulation, and microbial clearance; sepsis should not be defined as the host response to an invasive infection, or inflammation. Rather, sepsis is a deleterious, non-resolving inflammatory response to infection that leads to the organ dysfunction. It is usually reserved for patients whose condition is severe enough that they need to be admitted to ICU and need to be monitored more carefully. The response that predominates in the clinical phenotype varies across patients and over time in each patient (Vincent *et al.,* 2013). According to the 3rd International Consensus Definitions of sepsis and septic shock, “sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection”, and if it is not recognized early and managed promptly, it can lead to septic shock, in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of multiple organ failure and death (Singer *et al.,* 2016).

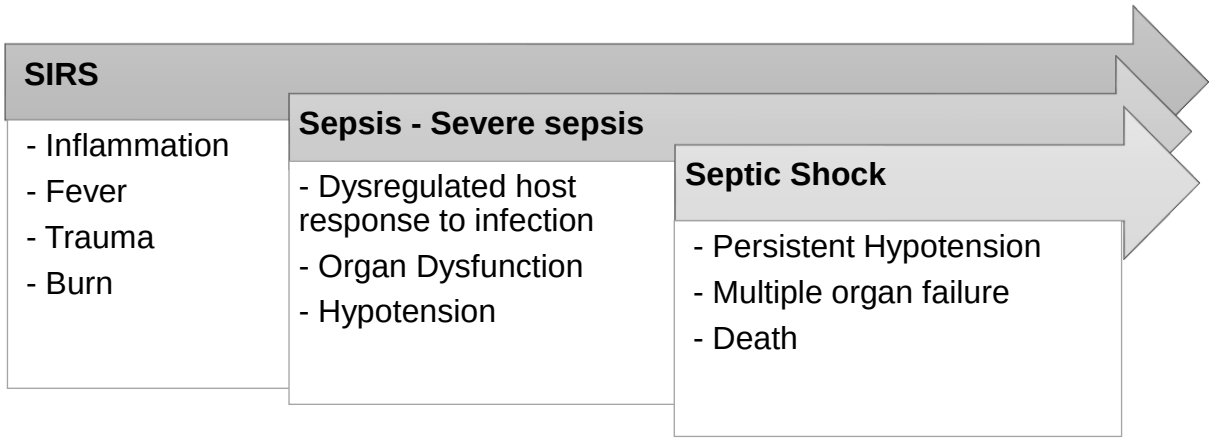


Figure 1.1: The progression stages of sepsis. SIRS: systemic inflammatory response syndrome.

1.3. Epidemiology of sepsis

Sepsis is a major healthcare problem and one of the leading causes of death around the world (Vincent *et al.,* 2019). It affects more than 48 million people worldwide every year and potentially 11 million sepsis related deaths, which represented about 20% of the global deaths. Sepsis incidence and mortality varied across regions with highest rate in low- and middle-income countries such as sub-Saharan Africa, south Asia, and east Asia (Rudd *et al.,* 2020). According to the UK Sepsis Trust 2018, the conservative estimates suggested 226,000 emergency sepsis admissions to hospitals in the UK for the year 2017/2018. A 2001 study in United States suggested high incidence of about 300 cases per 100,000 population, while 2016 population-based study from Sweden identified an incidence of 780 per 100,000 per year. In 2020, the Global Burden of Disease team reported that the UK cases of sepsis reached 245,000 with 48,000 deaths, while the Academy of Medical Royal Colleges 2022 reported that there are 918,000 sepsis admissions to hospitals across the UK each year (Daniels, 2022).

Although sepsis might affect any age or sex, the data have shown that neonates and pregnant women are at higher risk of sepsis. In 2018, about 20 million of all estimated sepsis cases occurred in children under 5 years old, where 15% of all neonatal deaths worldwide were due to sepsis (Rudd *et al.,* 2020)

Data from a large European study showed that severe sepsis accounted for 29.6% of ICU admissions and mortality rates of patients with severe sepsis and septic shock were 32.2% and 54.1%, respectively (Khwannimit and Bhurayanontachai, 2009). In addition, the average annual incidence of sepsis varied by as much as 3.5 fold depending on the method of database abstraction used, accordingly, it has been recommended to use a uniform and consistent method in national registries to facilitate accurate assessment of clinical interventions and outcome comparisons between hospitals and regions (Gaieski *et al.,* 2013).

So far epidemiological data have reported that the ethnicity is an important risk factor for the development of sepsis and a predictor of sepsis outcomes. Racial inequalities are important because they may reflect differences in biological response, genetics, economic factors, and health behaviours. The proportion of the population living in the poverty is one of the significant mediating community factors on the relation of the region and sepsis. An epidemiological analysis of sepsis in the USA from 1979 through 2000, revealed that sepsis was more common among men than among women and among non-white persons than among white persons. Black ethnicity is likely associated with higher rates of severe sepsis and hospitalization mortality (Martin *et al.,* 2003), and particularly those living in the regions with greater economic poverty (Barnato *et al.,* 2008).

In 2011, sepsis was economically considered as a major public health concern, accounting for more than $20 billion of total US hospital costs (Singer *et al.,* 2016). In 2014, the mortality rates in the hospitals of the USA were as high as myocardial infarction and stroke. The data obtained from large retrospective studies in high-income countries indicate that the admissions of patients with sepsis exceed those of patients who have suffered myocardial infarction or stroke (Fleischmann *et al.,* 2016).

Based on the infection rate and risk of acute organ dysfunction, the severe sepsis is more prevalent in black than in white individuals (Mayr *et al.,* 2010). However, a cohort study of the Reasons for Geographic and Racial Differences in Stroke (REGARDS) has shown that black participants were less likely than white participants to experience infection and sepsis events, while there were no racial differences among the patients acquired infections after hospitalization (Moore *et al.,* 2015). According to York Health Economics Consortium (YHEC), the estimated number of cases of sepsis is at least 250,000 cases in the UK each year, with at least 46,000 deaths. The sepsis costs the National Health Service (NHS) about £1.5 - 2 billion and costs the society of the UK Sepsis Trust about £15.6 billion every year (Daniels, 2022). Even though the incidence of sepsis in developed countries has been reported to be increasing, an European study in which sepsis was defined according to the latest guidelines proposed that there is a relative stability in the rate of sepsis from 2002 to 2012 (Vincent *et al.,* 2019).

1.4. Pathophysiology of sepsis:

Sepsis initiates when the host response to an infection grows and becomes dysregulated. The clinical features include fever, mental confusion, transient hypotension, diminished urine output or unexplained thrombocytopenia. If the patient does not receive an appropriate treatment, the symptoms might develop to the respiratory or renal failure, abnormalities of coagulation, and/or severe unresponsive hypotension (Cohen, 2002). The most common causal agents of sepsis are Gram-negative and Gram-positive bacterial infections. The sepsis due to Gram-negative bacteria has accounted for 25-30%, where Gram-positive and polymicrobial infections accounted for 30-50% and 25% of cases, respectively. Viruses and parasites are identified in only 2-4% of cases, but their frequency could be underestimated (Annane *et al.,* 2005).

Lipopolysaccharide (LPS), also called endotoxin, has a dominant role in initiating of the septic cascades. It is an outer membrane component of Gram-negative bacteria, composed of toxic lipid A-structure and an O-polysaccharide chain, and varies highly between species. There is no endotoxin in Gram-positive bacteria, but thick peptidoglycan layer without lipid membrane. The cell walls contain peptidoglycan, lipoteichoic acid, and teichoic acid (Figure 1.2). A collection of the physiological responses to the infections will be the start of a process known as inflammation, which includes the immune system and the coagulation process. The defence response of the immune system in sepsis produce deregulated cascade of reactions (Cohen, 2002).

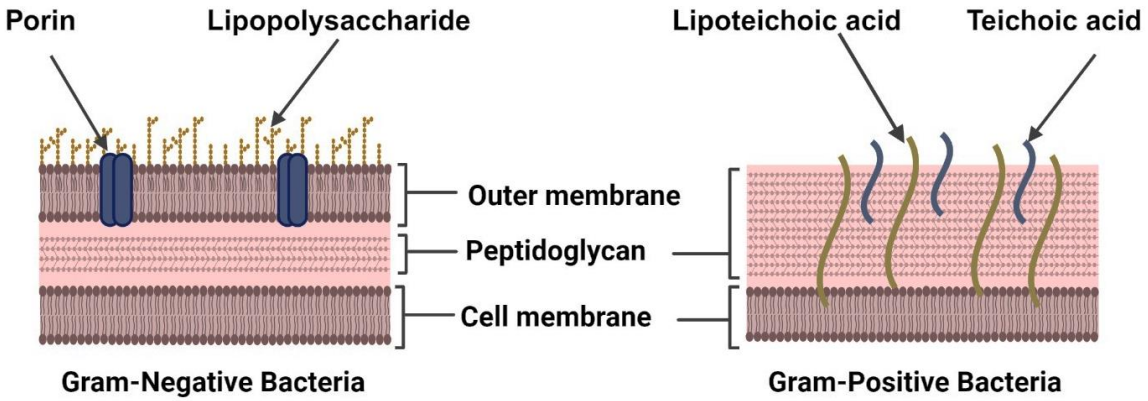


Figure 1.2: Representative diagram showing the components of the outer layers of Gram-negative and Gram-positive bacteria (created **in Biorender.com**).

The activation of the host cells by infections depends on the presence of two accessory proteins known as CD14 receptors and myeloid-2 differentiation factor (MD-2) on the surface of the immune cells. The stimulation of toll-like receptor TLR-4 by LPS-MD2 complex activates reaction with myeloid differentiation primary-response protein 88 (MyD88), resulting in activation of the serine and tyrosine kinases and activation of nuclear factor-κβ (NF-κβ) (Solov'eva *et al.,* 2013). The binding sites of NF-κβ regulates the expression of target genes to encode pro-inflammatory mediators, cytokines, clotting components, complement, nitric oxide synthase (NOS), chemokines, and acute phase proteins genes. After exposure to LPS, the inflammatory cytokines; such as tumour necrosis factor-α (TNF-α), interleukins; IL-1, IL-2, IL-8, IL-12 and IL-18, produce uncontrolled inflammation with diffuse microvascular thrombosis, tissue injury, organs failure, and ultimately septic shock (Mallat *et al.,* 2019)(Figure 1.3).

A study on healthy volunteers has shown an early protein catabolic response through determining the time course of the synthesis and breakdown of these proteins in early sepsis. The plasma growth hormone, TNF-α and IL-6 concentrations significantly increased and peaked 120 minutes after LPS administration, and the alterations persisted for up to 480 minutes. The plasma cortisol concentration, as well as the C-reactive protein (CRP) concentration, increased significantly from 360 minutes after LPS administration, whereas there were no changes in plasma insulin or glucagon levels were observed during this period (Khan *et al.,* 2015).

Vasodilatation is the first step in inflammation to increase blood flow and to mobilise white blood cells (leukocytes), fibrin and platelets. High circulating levels of nitric oxide (NO) found in patients with septic shock, contributes to a reduction of vascular tone and capillaries leakage (Boyle *et al.,* 2000). The other essential part of the response process is that the blood capillaries become permeable, where the inflammatory mediators and cytokines (Table 1.1) enter the interstitial tissues to fight the infection. This causes the symptoms of patients such as swelling, oedematous, runny nose, dizziness, diarrhoea and/or vomiting. In sepsis, the balance between the pro-inflammatory and anti-inflammatory becomes disordered leading to rapid progression and poor prognosis (Daniels, 2022).

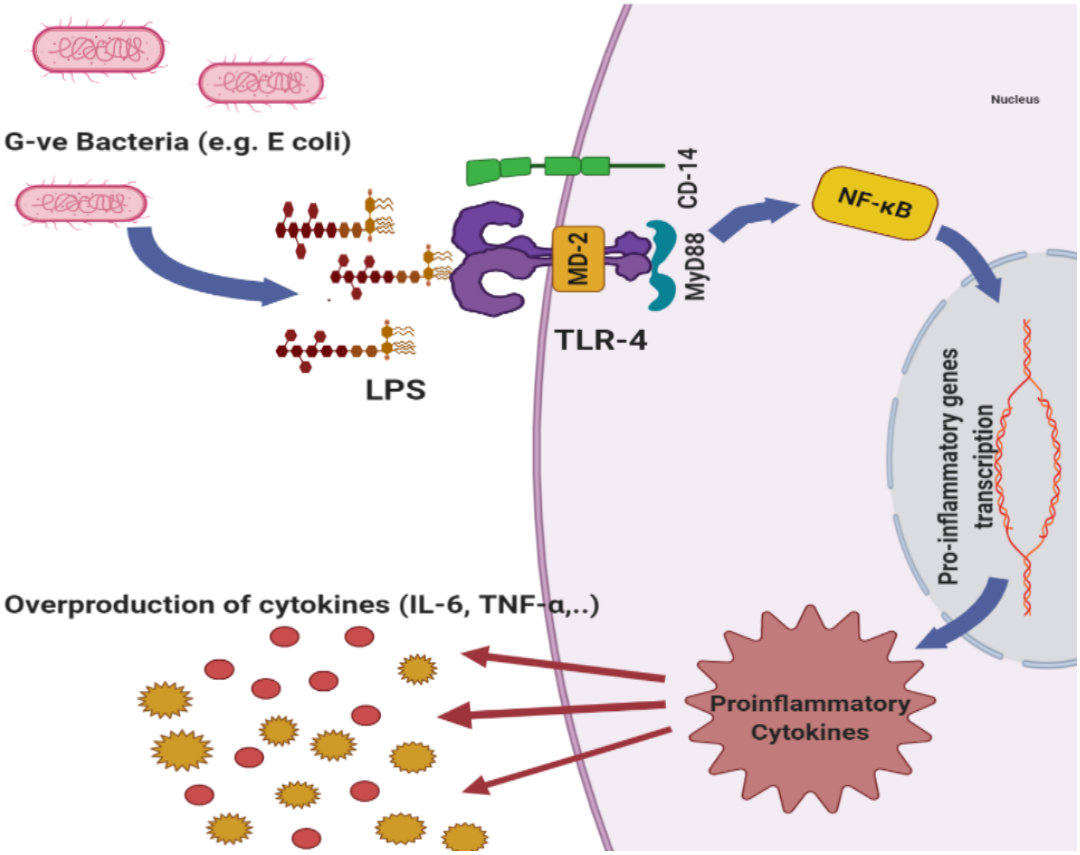


Figure 1.3: The induction of sepsis by stimulation of TLR-4 and the subsequent pathophysiological steps of sepsis (created **in Biorender.com**).

Table 1.1: Key inflammatory mediators implicated in sepsis and their functions (adopted from Daniels, 2022).

Inflammatory Mediator	Function
Bradykinin	It is responsible for the pain at the site of inflammation, in addition to the vasodilatation and vessel leakage.
Thrombin	It helps the formation of the clot by converting fibrinogen into fibrin.
Interleukins	They are a group of proteins that are involved in the function of leukocytes to modulate inflammation.
Tumour Necrosis Factor (TNF)	It is a pro-inflammatory cytokine
Complement proteins	They act directly to neutralise the pathogens, mobilise the leukocytes and amplify the immune system.

1.5. Management of sepsis and septic shock

There is currently no specific drug for the treatment of sepsis and administration of antibiotics is considered the best option despite numerous issues, and the development of drugs to control the pathogen-induced inflammatory responses associated with sepsis is essential (Lee *et al.,* 2019). In general, treatment of septic shock can be divided into those directed against bacterial components, those directed against host-derived inflammatory mediators and those designed to minimise organ damage. The early identification and appropriate management in the initial hours after sepsis develops an improvement in the outcomes (Rhodes *et al.,* 2017).

1.5.1. Antibiotics as initial treatment:

Effective antimicrobial administration within the first hour of documented hypotension has been associated with 79.9% survival rate, and each hour of delay over the early six hours resulted in 7.6% decrease in the survival rate. Despite of an increase in the risk of mortality due to delay in the antibiotic treatment, only 50% of septic shock patients received effective antimicrobial therapy within 6 hours of documented hypotension (Kumar *et al.,* 2006). CRP is synthesised and rapidly released by the liver in response to the inflammation and tissue damage to fight the infection. Thus CRP has been shown to be a valuable marker in the diagnosis of infection and in monitoring its response to antibiotics, where it has been found that changes in CRP levels over the first 48 hours of antibiotic therapy can help to evaluate the response to initial antimicrobial therapy in septic patients (Schmit and Vincent, 2008).

It has been reported that combination antibiotic therapy is more effective in improving the survival and clinical response of high-risk, life-threatening infections, especially those associated with septic shock. The combination therapy demonstrates a significant advantage over monotherapy when the risk of mortality rate exceeds 25% (Kumar *et al.,* 2010). However, clinical trials have shown no beneficial effects from combination therapy of aminoglycoside such as gentamicin and beta lactam such as penicillin compared to beta lactam monotherapy. The use of short courses of gentamicin in critically septic patients is still prevalent (Ong *et al.,* 2017).

As previously mentioned, early management of sepsis plays an effective role in reducing mortality rates. A strategy of sepsis treatment has been placed under the name the sepsis six (Table 1.2) to be given within first hour in order to control the

source of infection, and to measure and restore circulation and oxygen delivery (Daniels *et al.,* 2011).

Table 1.2: Treatment strategy (Sepsis Six) required in the management of sepsis (adopted from Daniels *et al.,* 2011).

Initial Management	Purpose
Oxygen Ventilation	To improve the oxygen content of the blood and its supply to the tissues.
Blood cultures	To identify the source of infection and guide antimicrobial therapy.
Broad spectrum antibiotics	To control the underlying infection and to eliminate the trigger for immune overreaction.
IV fluids	To improve preload to the heart by correcting hypovolemia, improving cardiac output and blood pressure.
Check serial lactates	High in lactate level indicates that the patient has perfusion disorder.
Measurement of urine output	Decrease in urine output indicates that the patient is hypovolemic.

1.5.2. Fluid Resuscitation

In addition to administration of antimicrobial therapy and targeted source control, fluid resuscitation is a fundamental step in sepsis therapy. Patients with sepsis experience hypoperfusion and hypovolemia. Hypoxia would eventually cause the multi-organ failure commonly seen in patients with sepsis. Intravenous (IV) fluid therapy is recommended to increase preload, which increases cardiac output, resulting in improved oxygen delivery to organs (Brown and Semler, 2019). The Surviving Sepsis Campaign (SSC) was established in 2002 to reduce sepsis-related mortality by producing a series of care bundles spanning 3 hours, 6 hours, and 24 hours of management. The SSC recommended the use of crystalloids in early fluid resuscitation to improve hypotension and the most used is the isotonic crystalloid 0.9% saline which has a high concentration of chloride compared to human plasma. In high volumes, this can have a negative impact on kidney function due to the overload of chloride (Daniels, 2022). Alternatively, colloids such as albumin can be used but studies have shown no significance in the primary outcome of 28-day mortality (Lee and Levy, 2019). An increase in serum lactate levels is associated with an increased mortality rate from 35% to 70%, it is considered a serious sepsis marker and is indicative of hypoperfusion. In addition to fluid resuscitation, the administration of oxygen via a mask or early endotracheal intubation is recommended to optimise and reduce oxygen consumption (Rello *et al.,* 2017).

1.5.3. Vasopressors

Vasopressors activate vascular smooth muscle receptors such as: NA and adrenaline that act on α_1, β_1, and β_2-adrenoceptors; vasopressin on arginine vasopressin receptors (AVPR1a, AVPR1b, and AVPR2, AVPR2); angiotensin II on angiotensin receptors (AGTR1 and AGTR2); and dopamine on DA1, DA2 (Russell, 2019).

Hypotension is the first clinical sign of hypo-perfusion, and the rapid restoration of perfusion is achieved with the initial energetic fluid intake. After circulatory volume is recovered, the administration of high doses of catecholamine such as NA and dopamine is critical in maintaining the arterial pressure (Levy *et al.,* 2010). Blood transfusion is recommended for patients with a haemoglobin level below 7 g/dL. The late stage of the treatment should begin when the septic patients presented with multi-organ dysfunctions. Vasopressors are added if fluid resuscitation does not restore the organ hypo-perfusion, but this is limited by excessive vasoconstriction and possibility of organ ischaemia. Vasopressors such as NA, vasopressin, angiotensin II, are used to raise the MAP and improve the cardiac output. Dobutamine is recommended in patients with decreased ventricular contractility, while dopamine is recommended in bradycardic patients (Russell, 2019). NA is the first-line vasopressor in critically septic patients. According to the sepsis guidelines, the initial target MAP of septic patients on vasopressors is 65 mmHg. Targeting a higher MAP is reasonable in patients with chronic hypertension. In cases of refractory hypotension, increasing the dose of the NA could be the option, but the sepsis guidelines suggest to combine NA with other vasopressors such as vasopressin or adrenaline to raise the MAP to target or to decrease the dosage of NA (Shi *et al.,* 2020, Evans *et al.,* 2021).

1.5.4. Adjunct therapy and promising therapeutic agents under investigation

The use of corticosteroids might be necessary in the septic patients who require high doses of the vasoactive drugs and have no improvement in the lactate level during the first hours of the treatment. There are many factors that induce an increase in the glucose level causing hyperglycaemia, hence the insulin infusion therapy is required but it should be controlled to avoid hypoglycaemia (Rello *et al.,* 2017). There are

several investigated supportive therapies in sepsis including thrombosis prophylaxis and renal replacement therapy, and immunomodulatory therapy. However, none of them were approved and further investigations are needed to be therapeutically used (Table 1.3).

Table 1.3. Mechanisms of action and clinical outcomes for some promising therapies in sepsis.

Treatment	Mechanism of action	Investigated results	Reference
E5564 (Eritoran)	Inhibitor of TLR4 (LPS analogue)	No reduction in mortality rate (phase II clinical trial).	(Opal *et al.,* 2013)
CytoSorb	Elimination of cytokines	Better clinical outcomes but further randomised clinical trials are needed.	(Mehta *et al.,* 2020)
Afelimomab	Monoclonal antibody	Potential improvement in mortality rate further investigation is needed	(Kotsaki and Giamarellos-Bourboulis, 2012)
Selepressin	Selective vasopressin VI agonist	Many issues remain unsolved and further investigation is needed	(Maybauer *et al.,* 2014, Antonucci *et al.,* 2022)
Interlukin-7 (IL-7)	Prevents secondary immune suppression (Pro-inflammatory cytokines)	Promising therapy for septic patients with impaired immunity	(Francois *et al.,* 2018)

1.6. The role of adrenergic nervous system in sepsis

The arterial pressure and cardiac output are generally maintained by vasoconstrictors such as NA and adrenaline, which stimulate the adrenoceptors in sympathetic nerves (Rello *et al.,* 2017). Adrenergic receptors are distributed on nearly all cell type in the

body and are the targets for adrenaline and NA in sympathetic nervous system. They are members of the cell surface receptors, and they carry the signals via binding to G-proteins. They have an essential role to maintain the body homeostasis in normal physiological functions and to response the pathological conditions. Hence, they are considered as targets for many therapeutically agonists and antagonists (Small *et al.,* 2003).

1.6.1. Historical background on adrenoceptors

Langley (1905) was the first who define the adrenotropic receptors as the hypothetical structures or systems that located in, on or near the muscle or gland cells affected by adrenaline and he also introduced the concept of a receptive mechanism through the effect of curare on skeletal muscles. Afterwards, Dale (1906) made a significant use of the receptor concept in connection with the sympathetic nervous by studying the influence of ergot alkaloids on adrenaline actions and sympathetic nerve stimulation. Dale was the first who considered the adrenoceptors to be of two classes, those whose action results in excitation and those whose action results in inhibition of the effector cell. He also recognised that ergot alkaloids could produce sympatholytic actions by blocking the excitatory actions of adrenaline. According to Ahlquist (1948), two pharmacological experiments were used to differentiate the types of the adrenergic receptors; first, by plotting the dose-response curves of adrenaline and some commonly used sympathomimetic amines that structurally related to adrenaline. Second, by using drugs that antagonise the responses of the effector system to one or more of these agonists. The amines were extensively studied to compare their quantitative actions on different isolated tissues from animal models including

cardiovascular, intestine, uterus, iris and nictitating membrane, and ureter. The study has estimated that there are two distinct types of adrenotropic receptors (Alpha and Beta receptors) based on the order of the relative activity of the amines; adrenaline, NA, α-methyladrenaline, α-methylenoradrenaline (or phenylephrine) and isoproterenol. The order of the potencies of these amines for the responses mediated through alpha receptors is adrenaline > NA > α-methyladrenaline > isoproterenol, while the order of the potencies for the responses mediated through the beta receptors is isoproterenol > adrenaline > NA > α-methyladrenaline. The alpha (α) receptor is associated with excitatory functions such as vasoconstriction, uterine contraction, and stimulation of the nictitating membrane, ureter, and dilator pupillae and one important inhibitory function which is intestinal relaxation. Conversely, the beta receptor is mostly associated with inhibitory functions such as vasodilatation, and inhibition of the uterine and bronchus, and the only excitatory action in myocardial contraction (Ahlquist, 1948).

A subsequent study by Lands (1949) has classified adrenergic receptors in heart as undifferentiated (Acr) receptors and the other receptors into two groups of the receptors as the excitatory receptors (Ac) or the inhibitory receptors (Ar) of smooth muscle (Furchgott, 1959). The classification of adrenergic receptors was further supported by the evidence that the responses mediated through adrenoceptors can be blocked using adrenergic blocking agents. The α-adrenoceptors can be antagonised by several natural and synthetic compounds such as ergot alkaloids, phentolamine and some β-haloalkylamines, and β-adrenoceptors can be selectively antagonised by dichloroisoproterenol, pronethalol and propranolol (Furchgott, 1967).

The β-adrenoceptors antagonists were studied in human and animal models, and the antagonism to catecholamine induced vasodilatation and cardiac stimulation has been reported. Accordingly, pronethalol was described as a selective sympathetic β-adrenoceptor antagonist. Afterwards, propranolol was discovered in 1965 to be a more effective β-adrenoceptor blocker than pronethalol, as it lacks any intrinsic sympathomimetic activity of pronethalol (Black *et al.,* 1965). The β-adrenoceptor is generally classified into two sub-classification, β_1 and β_2 supported by the development of selective agonists and antagonists and their therapeutic applications (Lands *et al.,* 1967).

According to Starke (1975), the noradrenergic nerves also contain presynaptic α-adrenoceptors that mediate the negative feedback mechanism to inhibit the release of NA induced by nerve impulses. The pre and postsynaptic adrenoceptors differ in their sensitivity to the drugs and the presynaptic agonist and postsynaptic agonists produced opposite effects on the vasoconstrictor response to nerve stimulation (Starke *et al.,* 1975). In 1970, two α-adrenoceptor agonists (xylazine and clonidine) were studied for their effect on presynaptic adrenoceptors, and it was reported that these agonists can lower the arterial blood pressure by their actions on the central nervous system causing a reduction in the release of NA (Starke, 1977).

Unlike clonidine, the activity of the α-adrenergic agonists such as oxymetazoline, naphazoline, methoxamine and phenylephrine are more active at post-synaptic than pre-synaptic α-adrenoceptors. Additionally, some α-adrenergic antagonists; such as thymoxamine, piperoxan, yohimbine and tolazoline show their equal potency in

antagonism of vasodilatation induced by clonidine, suggesting their blockage activity on presynaptic α-adrenoceptors (Drew, 1976).

The α-adrenoceptors have been sub-classified as α_1 and α_2 -adrenoceptors according to the order of potency of agonists and antagonists for pre- and post-junctional α adrenoceptors (Starke, 1977). The presence of adrenoceptor subclasses contribute to the therapeutic importance; for instance agonists at α_2-adrenoceptor induce antihypertensive effects such as clonidine and methyldopa (Starke, 1981). However, the subsequent studies reject the concept that suggested the sub-classification of α-adrenoceptor into: α_1-adrenoceptor located on smooth muscle and mediate contraction, and α_2-receptors located on nerve terminals and mediate inhibition of transmitter release (McGrath, 1982).

With the development of more pharmacological techniques for studying drug-receptor interactions including radioligand-binding tests, the α- and β-adrenoceptors were further divided into $\alpha_{1A/D}$, α_{1B}, and α_{1C} and into α_{2A}, α_{2B}, and α_{2C}, and three β-receptor subtypes β_1, β_2 and β_3 (Figure 1.4), based on their affinity for many synthetic agonists and antagonists (Bylund *et al.,* 1994). The α_2-adrenoceptor binding sites have been sub-classified into α_{2A} and α_{2B} sites based on their affinity for prazosin, where the α_{2B} sites have shown relatively high affinity for prazosin, suggesting that the α_2-presynaptic auto-receptors are obviously different from the α_{2B}-subtype (Limberger *et al.,* 1991). The drugs shown in Table 1.4 that interact with these subtypes have been proven to be therapeutically effective in a variety of major diseases include hypertension, angina, congestive heart failure, cardiac arrhythmia, asthma, depression, prostatic hypertrophy, and glaucoma (Rang *et al.,* 2016).

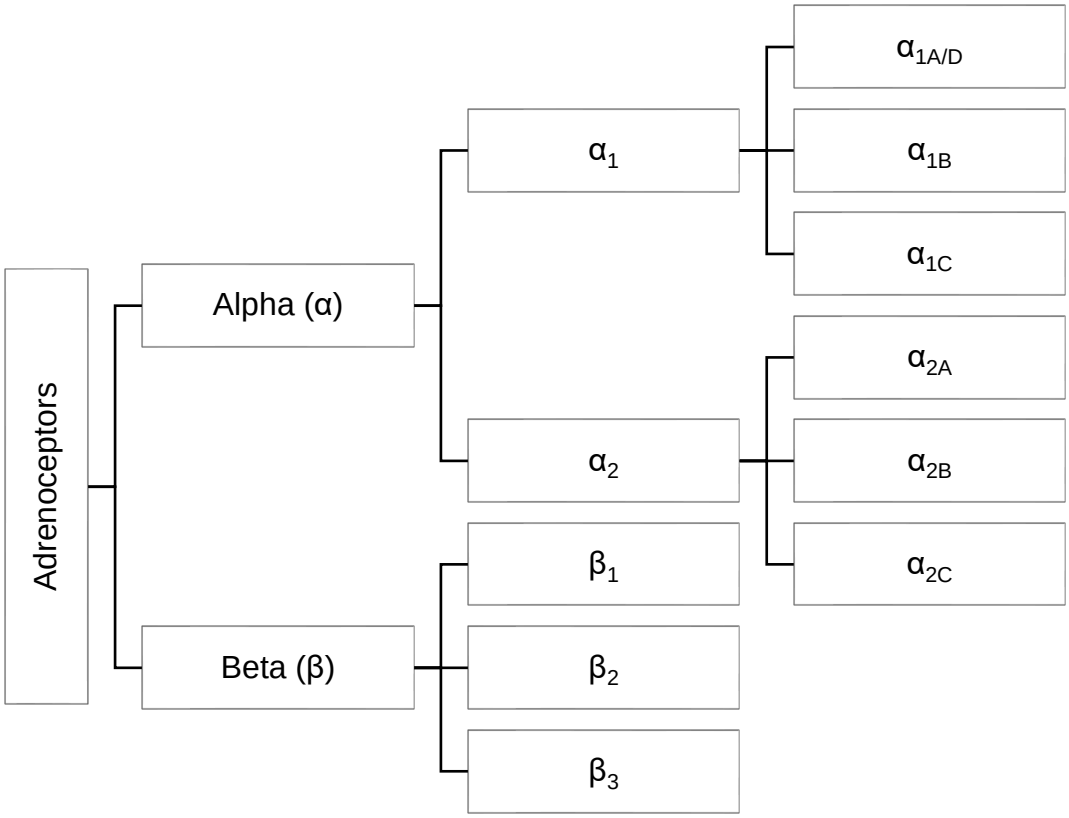


Figure 1.4: Classification and subclassifications of adrenoceptors (Bylund *et al*., 1994).

Table 1.4: The selectivity of agonistic and antagonistic drugs to α- and β-adrenoceptors (Rang *et al.,* 2016).

Agonists	Receptor	Antagonists	Receptor
NA	Mixed α/β	Labetalol	Mixed α/β
Adrenaline		Carvedilol	Mixed β/α_1
Isoprenaline	B	Propranolol	B
Dobutamine	β_1	Alprenolol	
Salbutamol	β_2	Timolol	
Terbutaline		Metoprolol	β_1
Salmeterol		Atenolol	
Mirabegron	β_3	Nebivolol	
Methoxamine	α_1	Butoxamine	β_2
Phenylephrine	α_1	Phenoxybenzamine	α
Clonidine	α_2	Phentolamine	
Lofexidine		Prazosin	α_1
Guanfacine		Tamsulosin	α_{1A}
		Yohimbine	α_2

1.6.2. Mechanism of action of adrenoceptors

All adrenoceptors are prototypical G-protein coupled receptors (GPCRs), classified into several transmembrane subtypes that can couple with more than one types of G-proteins. These receptors can differentially activate the signalling pathways of G-protein groups; Gq (stimulation of phospholipase C), Gi (inhibition of adenylyl cyclase) and Gs (stimulation of adenylyl cyclase). The β-adrenoceptors couple mainly to Gs, and α_1-adrenoceptors to Gq, although β_2- and α_1-subtypes can also couple to Gi (Figure 1.5) (Ahles and Engelhardt, 2014).

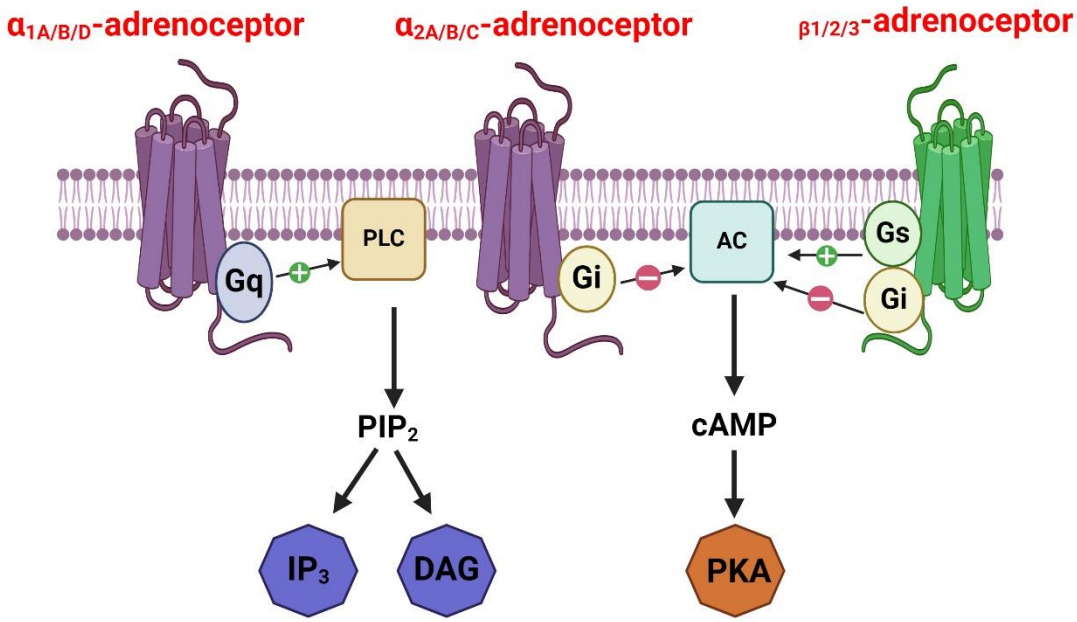


Figure 1.5: Signal transduction of adrenoceptors. AC: adenylyl cyclase, DAG: diacylglycerol, PIP2: phosphoinositol-bis-phosphate, PKA: protein kinase A, PLC: phospholipase C, IP_3: inositol triphosphate (created **in Biorender.com**).

1.6.3. Distribution and pharmacological actions of adrenoceptors

The specific functions and locations of the adrenoceptor subtypes are not yet fully appreciated and additional subtypes might be discovered. However, the extended classification of nine distinct subtypes of adrenoceptors is necessary for understanding the distribution of receptors and selectivity of some drugs. The organs innervated by adrenergic nerves might have both types of receptors but typically one subtype is more predominant. Adrenergic receptors play an important role in coronary artery blood flow and cardiac functions. Both α and β subtypes have been demonstrated in cardiac muscle, but β_1 receptors are more predominant. In coronary arteries, α-adrenoceptors cause vasoconstriction, whereas β-adrenoceptors cause vasodilatation. It is generally known that post-junctional α_1- and α_2-adrenoceptors coexist on the vascular smooth muscle. Both α_1 and α_2-adrenoceptors can mediate contraction in vascular smooth muscle by utilising the intracellular and extracellular Ca^{++} as support sources of vasoconstriction (Daly *et al.,* 1990). A study on porcine ciliary arteries has shown that the α_{2A}-adrenoceptors subtypes mediate contraction and selective α_2-adrenoceptors agonists such as brimonidine is a potent vasoconstrictor (Wikberg-Matsson and Simonsen, 2001). Furthermore, these receptors induce vasoconstriction in human saphenous vein through their α_{2C}-adrenoceptor subtypes (Rizzo *et al.,* 2001).

The α_{1D}-adrenoceptors primarily constrict epicardial coronary arteries and arterioles, but α_2-adrenoceptors act mostly on the coronary microcirculation (Jensen *et al.,* 2009). The expression of the α_{1D}-and α_{2C}-adrenoceptors in the mesenteric arteries and veins contributes to the increased sensitivity of mesenteric veins to the constrictor effects of NA (Sporkova *et al.,* 2010). The vasculature of the lung smooth muscle and skeletal

muscle mainly contain β_2 receptors. The α_{1A} receptors are found primarily in the urinary tract and prostate gland based on the affinity of antagonist tamsulosin to α_{1A} that is used to treat benign prostate hyperplasia (Harvey, 2012). It is important to organise the most predominant physiological functions of these receptors according to the receptor type and their distribution in the body. Figure 1.6 summarises the major effects of adrenoceptor subtypes (Rang *et al.,* 2016). The $\alpha_{2A/D}$ receptors have been demonstrated primarily on the endothelium of large arteries and coronary, renal, and mesenteric microcirculation (Long and Kirby, 2008). Currently, the development of adrenergic-specific drugs shows therapeutic potential and enables more precise pharmacologic targeting of diseases in critical illness.

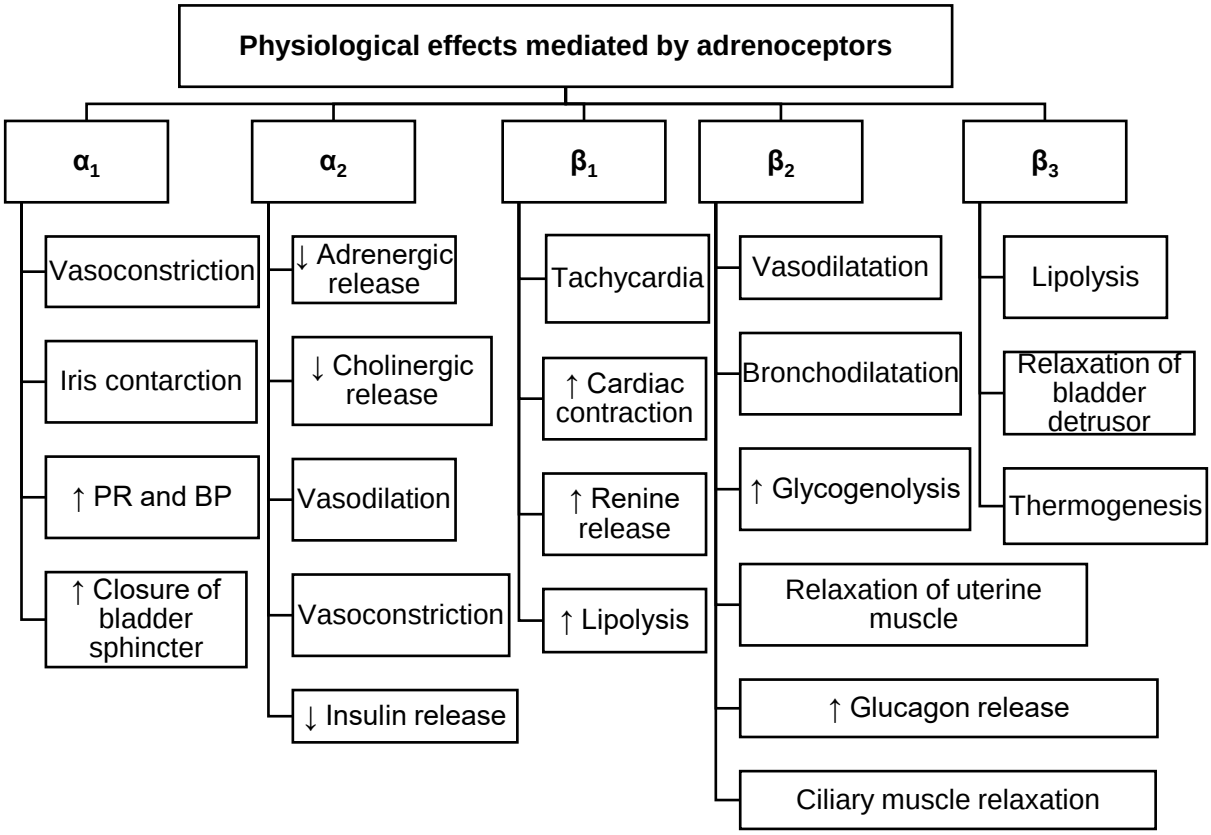


Figure 1.6: The main pharmacological actions of adrenoceptors (adopted from Rang *et al.,* 2016). BP: blood pressure, PR: pulse rate.

1.6.4. The role of NA and NA-sparing agents in the management of sepsis:

NA, an endogenous neurotransmitter, is synthesised and stored in the presynaptic terminal of adrenergic nerves. It is then released into the synaptic in response to the action potential and Ca^{++} influx. In synaptic cleft, NA binds to either pre or post synaptic GPCRs to exhibit its excitatory or inhibitory effects. NA could be removed via the NA transporter back into the presynaptic terminal, where it can either undergo degradation or storage in vesicles (Hussain *et al.,* 2022). It has a high affinity to α_1-adrenergic receptors to induce vasoconstriction and modest β-agonist activity causing

vasodilatation. It is vasoconstriction that primarily increases systolic and diastolic pressure with minimum net impact on cardiac output. It has minimal chronotropic effects and, therefore is preferably used in critical patients when cardiac stimulation is undesirable (Overgaard and Dzavik, 2008). NA might also bind to α_1- and β_2-adrenoceptors on leukocytes to differentially modulate immune responses in sepsis (Russell, 2019). As mentioned before, NA is a first-line therapy that can be used as an adjunct in the management of vasodilatory shock, when fluid therapy does not improve hypotension. The administration of NA is typically through continuous infusion because of its relatively short half-life (2.5 minutes). The recommended dose is to start the infusion at 8 to 12 µg/minute, and the average maintenance dose is 2 to 4 µg/minute (Smith and Maani, 2023).

The maximum tolerable dose of NA to achieve the target MAP is still undefined. The high dose of NA is defined as ≥1 µg/kg/minute which might be used as rescue therapy in severe hypotension (Shi *et al.,* 2020). High exposure to NA has several drawbacks, where it could result in down-regulation of α_1-adrenoceptors and subsequently reduces the sensitivity to NA (Russell, 2019). It may compromise the host immune system and promote bacterial growth (Stolk *et al.,* 2020). Several retrospective studies reported that the mortality rates increased by about 80-90% in patients who received high NA doses (Brown *et al.,* 2013, Martin *et al.,* 2015). Some clinical trials have shown that the usage of other vasopressors such as vasopressin and angiotensin II could reduce the required dose of NA dose (Rhodes *et al.,* 2017) (Russell, 2019). A recent retrospective observational cohort study on septic shock patients showed that the initiation of vasopressin with low doses of NA reduced the 28-day mortality rate and

shortened the duration of required NA infusions compared to the patients with high doses of NA (Xu *et al.,* 2023).

It has been reported that sympathetic activity is strongly related to the level of blood pressure, where the plasma level of NA was higher in the patients with essential hypertension patients than the normal volunteers (Louis *et al.,* 1973). The plasma concentration of catecholamine in hypertensive patients was 0.370 ± 0.032 ng/ml which was significantly higher than the concentration of catecholamine in healthy volunteers (0.218 ± 0.014 ng/ml) which was constant regardless of difference in age and sex (de Champlain *et al.,* 1976). There is considerable inter-patient variability in the plasma level of catecholamine during the shock. The mean plasma level of catecholamine was 10.64 ng/ml, and the NA level was 7.34 ng/ml in shocked patients, which was higher in comparison to the normal level (2.35 ng/ml). This condition could result in vasoconstriction of the blood vessels supplied with α-adrenoceptors due to the release of catecholamine from the adrenal medulla and the sympathetic endings in the early stage of circulatory distress (Hanquet *et al.,* 1970). However, the free radicals such as superoxide produced during the septic shock might deactivate the catecholamine and decrease its concentrations (Macarthur *et al.,* 2000).

The pharmacokinetics of NA could vary in septic patients. Thus, the pressor responses and other effects of immune modulation of NA might be affected due to the differences in the pharmacokinetics of NA in septic patients. It is known that the sympathetic nervous system is activated and the NA release at the sympathetic nerve increases during acute hypoxia, however, the plasma level of NA attenuated due to an increase in NA clearance (Leuenberger *et al.,* 1991). The half-life of NA was reported as 2

minutes in less severe septic patients, which increased to 6.8 minutes in most severe septic patients (Beloeil *et al.,* 2005). The variability in the clinical responses to NA depends on both pharmacokinetic and pharmacodynamic factors. In healthy volunteers, the infusion of NA ranged from 0-56 ng/kg/minute resulted in the serum catecholamine concentration of 3.00 ±0.23 and 1.35 ±0.12 nmol/L in arterial and venous circulation respectively (Chang *et al.,* 1988). There is a linear relationship between the dosing of NA and plasma concentrations, where the infusion of NA at the rate of 0.01 - 0.2 µg/kg/minute resulted in 0.199 - 7.5 ng/ml (1.18 - 44.18 nmol/L) (Ensinger *et al.,* 1992).

However, it was hard to observe any relationship between the dosing and plasma concentrations of NA in septic patients due to the variability in the volume of distribution and clearance of NA among septic patients. The plasma concentrations of NA ranged from 0.99 to 186 ng/ml (average of 16.2 ng/ml) for the septic/trauma patients who received the doses of NA at the rate of 0.093 to 6.3 ng/kg/minute to raise the blood pressure (Beloeil *et al.,* 2005) (Figure 1.7). Furthermore, the plasma concentration of NA at baseline in septic patients was reported as 0.99 to 56.7 nmol/L (average 8.8 nmol/L), which was significantly reduced to 0.30 to 38.9 nmol/L (average 4.5 nmol/L) when the patients received a fixed cumulative dose of adrenaline infusion (Abboud *et al.,* 2009).

As mentioned previously, the high doses of NA could increase the risk of lethal complications in septic patients in the ICU (Stolk *et al*., 2020, Bode *et al*., 2024). Therefore, the clinical study suggested that the combination of NA with vasoactive agents might reduce the required doses of NA and subsequently improve the patient's

outcomes. For instance, methylene blue, an inhibitor of NOS, has been used as adjuvant therapy for patients with refractory septic shock. The randomised trial on 91 septic patients, has reported that administration of methylene blue within the first 24 hours could reduce the time to vasopressor discontinuation and increase vasopressor-free days., and reduce the length of stay in ICU (Ibarra-Estrada *et al.,* 2023).

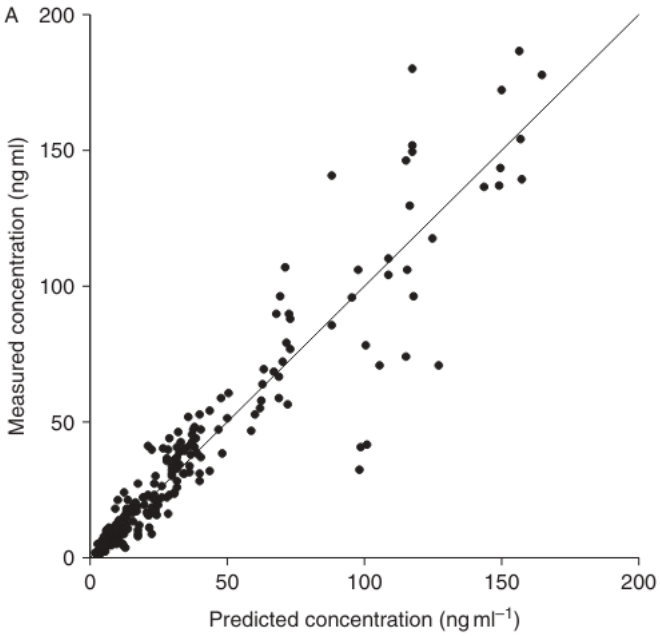


Figure 1.7: The plasma concentrations of NA (predicted versus measured) in septic patients (adopted from Beloeil *et al*., 2005).

1.6.5. α_2-adrenoceptor agonists and their potential role in the management of septic shock

Dexmedetomidine, a highly selective α_2-adrenergic agonist, has been examined in several studies as a sedative agent that suppresses inflammatory reactions and protects organs in both animals and humans. It has been approved clinically for short-term mechanical ventilation in ICU patients (Xu *et al.,* 2013). A clinical study showed that dexmedetomidine was used to induce sedation in septic patients (n= 201)

undergoing ventilation, where the results have shown that there is no significant improvement in mortality or ventilator-free days. However, the mortality rates after 28 days of treatment for the dexmedetomidine group and control group were 9.5% and 14%, respectively (Kawazoe *et al.,* 2017).

Chang *et al*, 2013 have shown that dexmedetomidine can reduce the expression of mobility group box 1 (HMGB1); the critical factor related to mortality in patients with sepsis, through the NF-κB pathway and α_2-adrenoceptors (Chang *et al.,* 2013). Moreover, a clinical case of an infant presented with septic shock has shown that clonidine was effective in improving circulation. The addition of clonidine to the treatment led to about 90% reduction in NA requirements over 13 hours and about 67% reduction in procalcitonin plasma concentration over 24 hours (Leroy *et al.,* 2017). A recent study has suggested that α_2-adrenoceptor agonists play an important role in reversing the effects of liver oxidative stress and apoptosis, and they also exert antioxidant and antiapoptotic effects (Sha *et al.,* 2019).

1.6.6. NA-uptake1 inhibitors and their potential vasoconstriction role in the management of septic shock

Drugs of abuse, such as cocaine, and antidepressants have a high affinity for noradrenaline transporter (NAT) proteins and potentiate the activation of postsynaptic receptors. These psychiatric medications include atomoxetine, which is licensed to be used for the treatment of attention deficit hyperactivity disorder (ADHD) and depression due to its central nervous system (CNS) effects (Zhou, 2004). However, atomoxetine, a potent and selective noradrenergic uptake1 inhibitor currently

increases the blood pressure in patients with central autonomic impairment. The neuronal reuptake inhibitors could enhance the vascular actions of NA at a lower infusion rate by elevating the concentration of endogenous NA in peripheral sympathetic neurons (Shibao *et al.,* 2007).

In the present study, the role of therapeutically approved NA uptake1 inhibitors; atomoxetine, reboxetine, and desipramine was examined on neurogenic contractions of porcine isolated splenic and renal arteries. This could potentially support the hypothesis that an increase in sympathetic activity by co-administration of NA uptake1 inhibitors might lower NA dose requirement and enhance vascular resistance in septic shock. To investigate the effect of NA uptake1 inhibitors on endogenous NA of rat tail artery, the voltage pulses delivered by electrical stimulators were used to stimulate the perivascular sympathetic nerves via microelectrode or bipolar stimulator electrodes. The release of NA induced by the electrical stimulation diffuses across the nerve junction and binds to α_1-adrenocetors on vascular smooth muscle cells producing contraction (Msghina *et al.,* 1999). Hence, the neuroeffector junctions at sympathetic nerves are influenced by diffusion, neuronal uptake, enzymatic degradation, and receptor desensitisation. This would clear the NA leading to the relaxation of the vascular smooth muscles (Park *et al.,* 2006).

An in vitro study on rat mesentery reported some differences in the kinetics of NA, arrangement of perivascular nerve fibres, sensitivity to the endogenous NA, function of α_2-adrenoceptor and NAT, as well as sympathetic neuroeffector mechanisms between veins and arteries. The results showed that veins are more sensitive to NA than arteries, where the NA oxidation current and vascular constriction of the

electrically stimulated mesenteric vein was detected at a lower frequency (0.5 Hertz (Hz) and 0.2 Hz) than in the mesenteric artery (1 Hz and 0.5 Hz). The NA oxidation currents in the veins were bigger than those of arteries, and the frequency response curve of constriction in the veins was left-shifted in comparison to that in the arteries. The rise time of the constriction was shorter in the veins, but the decay time was longer than that in the arteries. The vascular contraction in the veins was blocked by yohimbine but unchanged in the arteries suggesting that α_2-adrenoceptors contributed to the NA-induced constriction in veins. NA overflow was greater in the arteries than that of veins at all frequencies, but the yohimbine increased the contractions at frequencies up to 7 Hz. Above this frequency, the contraction was the same with and without yohimbine. This could be due to the saturation of the post-synaptic receptors (Park *et al.,* 2006, Park *et al.,* 2007).

1.7. Sepsis model

LPS macromolecules are the most important toxin involved in the development of septic shock, where the injection of small amounts of LPS in experimental animals can activate the biochemical cascade of acute systemic inflammation. LPS appears to be greatly present in patients with standard clinical criteria of sepsis with Gram-negative bacteria cultures. The LPS activity was also considered as one of the early indicators of Gram-negative bacterial infection, sepsis severity and risk factor to develop severe sepsis (Bottiroli *et al.,* 2017).

The vasculature of tissues isolated from animals was used to study the influence of the *E. coli* LPS on the responsiveness of the blood vessel to the vasoconstrictors. The

smooth muscle contractility induced by catecholamine in the isolated thoracic aorta was greatly depressed after three hours of exposure to endotoxin via unclear mechanisms suggested to involve endothelium and vascular smooth muscle (Beasley *et al.,* 1990). The LPS also enhances the release of IL-1 and TNF that are involved in *de novo* protein synthesis, and possibly the products of cyclooxygenase activity (McKenna, 1990). However, many models have shown that endotoxin-induced vascular hyporeactivity is associated with the production of NO within the blood vessel and activation of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) isoforms of NOS. The presence of L-arginine (a precursor of NO) during the incubation with LPS can decrease vascular responsiveness, which can be restored by using L-NAME (non-selective NOS inhibitor). Accordingly, the LPS induce the production of the vascular NO resulting in the relaxation through extracellular L-arginine (Schott *et al.,* 1993). The long-term incubation (20 hours at 37ºC) of isolated intrapulmonary arteries of piglets with group B streptococcus and E. coli LPS causes an induction of NOS activity, resulting in a decrease of the vascular responsiveness to NA (Villamor *et al.,* 1995).

A model of small vasculatures from rat superior mesenteric artery demonstrated in vitro vascular hyporeactivity largely mediated through NO-cyclic guanosine monophosphate (GMP) pathway (O'Brien *et al.,* 2001). In an *ex vivo* study on mesenteric arterioles of mice, LPS treatment significantly reduced the NA induced contractions suggesting that iNOS expression is critical for developing NA hyporesponsiveness (Boyle *et al.,* 2000). Nevertheless, L-NAME could not improve the survival rate of endotoxemic animals despite causing an increase in blood pressure and a decrease in cardiac output (Levy *et al.,* 2010). In contrast to the

expected actions of an NOS inhibitor, clinical studies have shown that treatment with L-NAME produced adverse cardiac and pulmonary effects resulting in higher mortality in sepsis (Bakker *et al.,* 2004).

Sepsis continues to be a leading cause of death, but early detection and recognition of symptoms could improve the outcomes. An early administration of appropriate antibiotics is crucial for survival. In the ICU, septic patients receive support with fluids vasopressors, and other sedative agents such as dexmedetomidine to manage the delirium. Preliminary data indicates that dexmedetomidine may potentially have additional non-sedative actions involving the immune system and cardiovascular systems that could benefit the management of sepsis.

1.8. Aims of the study:

1. To compare the peripheral and central actions of α_2-adrenoceptor agonists along with their lipophilicity properties, and subsequently to demonstrate the better agonists with potential role in raising the peripheral resistance in septic patients.

2. To design a sepsis model using sodium nitroprusside (SNP) or LPS, and hence to investigate the vasoconstriction actions of α_2-adrenocepetor agonists under normal and septic conditions. Additionally, to study the vasoconstriction actions of α_2-adrenoceptor agonists and their NA-sparing effects in different blood vessels.

3. To study the influence of α_2-adrenoceptor agonists on LPS-induced endothelial hyperpermeability in human umbilical vein endothelial cells (HUVECs).

4. To investigate the effect of NA uptake1 inhibitors on endogenous released NA using the electrical field stimulation in porcine splenic and renal arteries and to investigate the NA sparing effects of atomoxetine in porcine splenic artery.

5. To identify any association between the use of clonidine as a sedative agent and the changes in the levels of neutrophils, white blood cells, and platelets of the ICU patients.

2. Chapter Two: Is there an optimal α_2-adrenoceptor agonist clinically available for the management of sepsis and septic shock?

2.1. Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic shock is a subset of sepsis with a high mortality rate, in which there are profound abnormalities in the circulation and cellular metabolism leading to acute circulatory failure (Rhodes *et al.,* 2017). Over the last few years, the acute respiratory syndrome raised from COVID-19 has fundamentally changed the global infection landscape. This might potentially affect the epidemiology of sepsis, where the mortality rate for COVID-19 patients who meet the sepsis criteria was more than 45%, which is much higher than the estimated mortality for non-COVID sepsis (Shappell *et al.,* 2023). Sepsis has also a profound economic effect on healthcare systems worldwide. In the USA, the annual cost of sepsis patients admitted to hospitals is about US $20 billion, with septic shock requiring longer ICU stays and higher hospitalization costs. In the UK, data suggested that the total hospital costs is over £1 billion for patients with sepsis each year (Vincent *et al.,* 2019).

There is no specific drug for the treatment of sepsis and administration of antibiotics is considered the first choice (Lee *et al.,* 2019). However, the early identification and appropriate management in the initial hours of sepsis might develop an improvement in the outcomes. The most common causal agents of sepsis are *Escherichia coli* (*E. coli*), a Gram-negative bacterium about 25-30%, and group B streptococcus, a Gram-positive bacterium about 30-50%. Viruses and parasites are identified in only 2-4% of cases (Annane *et al.,* 2005). The infection will activate the host cell receptors called Toll-like receptor 4 (TLR4) via two accessory proteins known as CD14 receptors and myeloid-2 differentiation factor (MD-2) on the surface of the immune cells such as macrophages. This activation increases the release of pro-inflammatory cytokines and

late inflammatory mediators, NO and pro-coagulant molecules leading to the disruption of the endothelial barrier with diffuse microvascular thrombosis, changes in the vascular tone, tissue injury, organ failure, and ultimately septic shock (Shukla *et al.,* 2014).

The adrenergic system is greatly linked to endotoxin induced vascular hyporeactivity, where the adrenergic receptors play a vital role in the pathophysiology and therapy of hypoperfusion, and hypotension associated with septic shock. Identifying the potential factors contributing to the therapeutic approaches of septic shock is critical. An increase in the sympathetic outflow is essential to modulate the vascular tone and maintain organ perfusion. NA has been used as the first-line vasopressor to restore MAP (Rhodes *et al.,* 2017), and its administration is associated with a better hemodynamic profile and reduced adverse effects in comparison to other vasopressors (Avni *et al.,* 2015). However, the beneficial vasoconstrictor actions of NA on α-adrenoceptors achieved by high infusion rates might cause further immunosuppression and subsequently worsen the hemodynamic instability (Stolk *et al.,* 2020). Also, catecholamines including NA might enhance the growth of several species of Gram-negative bacteria by improving the iron intake because the catechol rings are considered siderophores in bacteria (Kinney *et al.,* 2000). Co-administration of NA with α_2-adrenoceptor agonists that are used as sedative agents could lower vasopressor requirements in surgical settings in ICU patients. In experimental sepsis on various animal models such as septic rats and ovine, clonidine and dexmedetomidine increased the pressor responses to NA and improved the blood pressure. NA restored the MAP but worsened medullary hypoperfusion in septic ovine. The coadministration of NA with dexmedetomidine (0.5 μg/kg/h) significantly reduced

the required dose of NA from 0.8 µg/kg/minute to 0.4 µg/kg/minute (Geloen *et al.,* 2013, Lankadeva *et al.,* 2015, Lankadeva *et al.,* 2019).

Dexmedetomidine is a highly selective α_2-adrenoceptor agonist that acts centrally in the locus coeruleus to inhibit NA secretion. It is used as an anaesthetic adjuvant and sedative drug for mechanically ventilated patients and is considered a potentially ideal sedative drug in ICU as its administration is associated with less complications than other sedative agents (Gertler *et al.,* 2001). Dexmedetomidine provided safe and effective sedation with reduced usage of other sedative agents and NA (Herr *et al.,* 2003). Its administration to the patients requiring intensive care resulted in the same quality of sedation as propofol without any respiratory depression. Hence dexmedetomidine has a protective action against myocardial ischaemia (Venn and Grounds, 2001), and its administration to the ICU septic patients without shock resulted in less hypotension in comparison to the propofol in a retrospective cohort study (Benken *et al.,* 2020). In addition, the continuous infusion of dexmedetomidine with low-dose NA can effectively prevent hypotension in the supine position (Zhang *et al.,* 2023). Furthermore, studies have shown that dexmedetomidine reduces NA requirements in the ICU. It was reported that less NA was required in postoperative patients treated with dexmedetomidine, with less problem of systolic hypotension in comparison to those treated with morphine (Shehabi *et al.,* 2009). A clinical trial on intubated patients in the ICU who need long-term sedation (more than 24 hours) showed a reduction in the ICU costs of the dexmedetomidine patients than midazolam patients and this is due to less ICU stay (Dasta *et al.,* 2010). This review will highlight the important roles of α_2-adrenoceptor agonists which could potentially help in the management of sepsis and septic shock in humans.

2.2. α_2-adrenoceptors and their pharmacological actions:

The α-adrenoceptors were initially referred to as prejunctional and postjunctional α-adrenergic receptors (Langer, 1974, Starke *et al.,* 1975), and were classified into α_1-adrenoceptors and α_2-adrenoceptors based on their responses to different adrenergic agents. The α_1-adrenoceptors on vascular smooth muscle cells have been suggested as a prototype of the postsynaptic excitatory receptors and the α_2-adrenoceptor on the adrenergic nerve endings as a prototype of the presynaptic inhibitory receptors (Starke, 1977) (Berthelsen and Pettinger, 1977). Three subfamilies of α_2-adrenoceptors have been characterised in mammalian species and they were identified as α_{2A}, α_{2B} and α_{2C}-adrenoceptors (Bylund *et al.,* 1994). They are cell surface G-protein coupled receptors (GPCRs) that act through coupling with at least four different G proteins, including guanine nucleotide binding proteins Gi_{1-3} and G_o proteins. Through the inhibitory coupling proteins, they resulted in the inactivation of adenylyl cyclase and decreased intracellular cyclic adenosine monophosphate (cAMP). The decreased cAMP attenuates the stimulation of protein kinase and the phosphorylation of target regulatory proteins. In addition to the reduction of cAMP, the efflux of K^+ through calcium activated channel can provide an effective means to hyperpolarize the excitable membrane suppressing the neuronal firing and inhibiting the secretion of neurotransmitters (Hayashi and Maze, 1993) (Flordellis *et al.,* 2004).

Alpha$_2$-adrenoceptors are targets for different therapeutically administered agonists and antagonists. All three α_2-adrenoreceptor subtypes may have presynaptic inhibitory effects to control the release of catecholamine from sympathetic nerve endings, but the pharmacological effects of α_2-adrenoreceptor agents could be assigned to specific receptor subtypes. The α_{2A} and α_{2C} subtypes are found mainly in the central nervous

system. Stimulation of α-$_{2A}$ resulted in hypotension, sedation, and analgesia, whereas negative feedback control of catecholamine release is mediated via α_{2C} (Buerkle and Yaksh, 1998, Knaus *et al.,* 2007).

The adrenergic system plays an important role in the microvascular coronary vasoconstriction that could be mediated by α_2-adrenoceptors (Gregorini *et al.,* 2002). The α-$_{2B}$ receptors are found more frequently on vascular smooth muscle and have been shown to mediate vasopressor effects. However, the vascular contraction could be also mediated through α_{2A}-adrenoceptors subtypes in the ciliary arteries (Wikberg-Matsson and Simonsen, 2001), and α_{2C}-adrenoceptors subtypes in human saphenous vein (Rizzo *et al.,* 2001). A study on rabbit isolated saphenous vein suggested that there is the interaction of the effects of selective α_1 and α_2 -adrenoceptor agonists at both adrenoceptor subtypes (Daly *et al.,* 1988). Similarly, α_2-adrenoceptors potentiate constrictions mediated by α_1-adrenoceptors in mesenteric veins suggesting that there is a synergistic interaction between α_1- and α_2-adrenoceptors (Sporkova *et al.,* 2010). The insulin secretion from pancreatic islets is inhibited by the activation of α_{2A}-adrenoceptors in β cells and an increase of the α_{2A}-adrenoceptors expression can impair insulin release, while several candidate gene studies have tested for the association of the α_{2B}-adrenoceptor with cardiovascular disorders (Ahles and Engelhardt, 2014).

Furthermore, NA induces platelet aggregation through stimulation of α_{2A}-adrenoreceptors coupled with Gzα-protein of the Gi family on the platelet membrane surface (Yang *et al.,* 2002). It has been demonstrated the presence of α_{2A}- and α_{2C}-adrenoceptors in renal tubules, and nephron segments, including proximal convoluted

tubules and cortical and medullary collecting ducts. The NA mediated stimulation of these receptors promotes renal inflammation and interstitial fibrosis in the progression of chronic kidney diseases (Jang *et al.,* 2019).

The pharmacological agents either agonists or antagonists of α_2-adrenoceptors could result in multiple responses due to their counteracting mechanisms in the central and peripheral nervous systems. Several α_2-adrenoceptor agonists are therapeutically approved to be used for various medical indications. Table 2.1 has shown that most α_2-adrenoceptor agonists shared similarities in their imidazoline chemical structures such as clonidine, dexmedetomidine, brimonidine, guanfacine, tizanidine, and lofexidine. However, they differ in their therapeutic actions which might be due to their multiple mechanisms of action. The ligand binding studies on the affinities and efficacies of α-adrenoceptor agonists at adrenergic receptor subfamilies found that clonidine, DEXMEDETOMIDINE, brimonidine, guanfacine, and tizanidine have α_2-adrenoceptor selectivity in comparison to α_1, β_1 and β_2 adrenoceptors. However, these agonists showed some variation in the affinities and potencies at different α-adrenoceptor subfamilies (Proudman *et al.,* 2022, Proudman and Baker, 2021) (Figure 2.1).

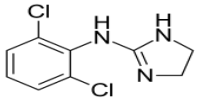
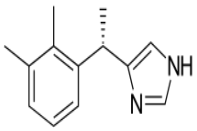
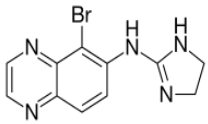
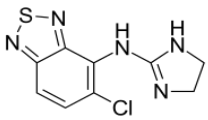
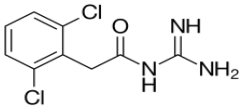
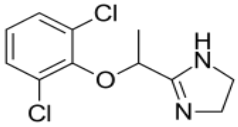
All the agonists showed greater affinity to α_2-adrenoceptors than α_1-adrenoceptors. The affinity of dexmedetomidine to both α_1 and α_2-adrenoceptors appeared within the range of its plasma concentrations, supporting the fact that increasing the dose of dexmedetomidine could result in α_1-adrenoceptor mediated vasoconstriction. The inhibition action of forskolin-induced cAMP of brimonidine is higher than dexmedetomidine and clonidine at α_{2A}, while guanfacine is a more potent inhibitor of

forskolin-induced cAMP than brimonidine and clonidine at α_{2C}-adrenoceptors. Unlikely, tizanidine has shown a small agonism action at the cAMP inhibition pathway (Jansson *et al.,* 1998, Parsley *et al.,* 1999, Kukkonen *et al.,* 2001). Dexmedetomidine is more potent than brimonidine, clonidine and guanfacine, but brimonidine has higher efficacy at α_{2}-adrenoceptors that mediate the contractions in the canine saphenous veins, followed by Dexmedetomidine, clonidine and guanfacine (MacLennan *et al.,* 1997). Brimonidine has a greater restoration of platelet function through their pro-aggregatory platelet action, followed by dexmedetomidine and clonidine (Porta Bonete *et al.,* 2020) (Table 2.2).

The α_2-adrenoceptor agonists affect the hemodynamic actions through their presynaptic and postsynaptic properties. The activation of vascular postsynaptic receptors could result in both vasoconstriction and vasodilatation. There is a significant contribution of endothelial NO in the action of α_2-adrenoceptor agonists on the peripheral circulation. Clonidine induced NO mediated vasodilatation via endothelial α_2-adrenoceptors (Figueroa *et al.,* 2001). Also, the inhibition of nitric oxide synthase (NOS) increased dexmedetomidine mediated vasoconstriction, suggesting that patients with impaired vascular endothelium due to diabetes mellitus or atherosclerosis could have a greater increase in peripheral resistance (Snapir *et al.,* 2009). However, the activation of these receptors on vascular smooth muscle cells resulted in vasoconstriction and an increase in blood pressure despite attenuated sympathetic outflow (Talke *et al.,* 2003).

Table 2.1: The chemical structures, dosages, and therapeutic indications of α_2-adrenoceptor agonists (Kim *et al.,* 2023) (NICE, 2023). (++++) high sedation action used for therapeutic

purposes, (+++) sedation as a common side effect, (++) drowsiness and dizziness as a common side effect.

α_2-adrenoceptor agonists	Dose and administration	Clinical indications	Sedation action
Clonidine 	By mouth 50-100µg 3 times/day (maximum dose 1.2mg) IV injection	Hypertension Prevention of recurrent migraine, vascular headache, and menopausal symptoms Sedation in ICU	+++
Dexmedetomidinemedetomidine 	IV injection 0.2–1.4µ/kg/hour	Maintenance of sedation in ICU	++++
Brimonidine 	Topical use (eye and skin) 3.3mg/day	Intraocular pressure in glaucoma Facial erythema	++
Tizanidine 	By mouth 24mg in 3-4 divided doses/day (Maximum dose 36mg)	Spasticity associated with sclerosis and spinal cord injury.	++
Guanfacine 	By mouth Initial dose: 1mg/day for 1week Maintenance dose: 0.05–0.12mg/kg/day	Attention deficit hyperactivity disorder in children (ADHD)	++
Lofexidine 	By mouth Initial dose: 800µg/day in divided doses (Maximum dose 2.4mg/day)	Management of symptoms associated with opioid withdrawal.	++

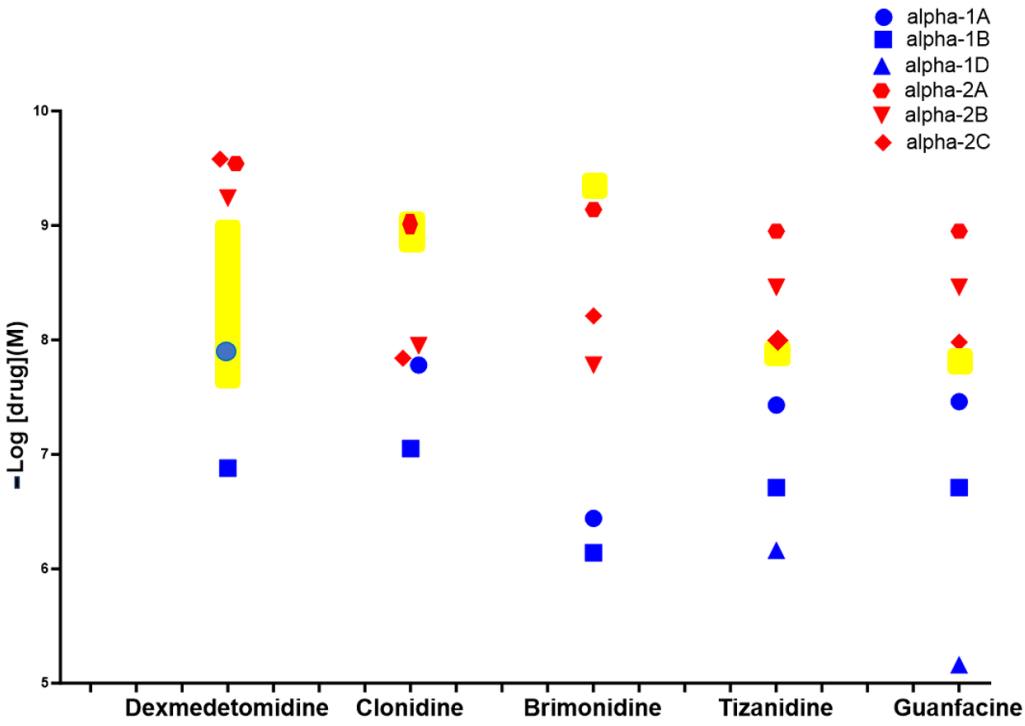


Figure 2.1: The selectivity of α_2-adrenoceptor agonists at α-adrenoceptor subfamilies. The red symbols indicate the affinity of the agonists to α_2-adrenoceptor subtypes and the blue symbols to α_1-adrenoceptor subtypes (Proudman and Baker, 2021, Proudman *et al.*, 2022). The yellow shapes indicate the plasma concentrations of these agonists as reported in Table 2.5.

Table 2.2: Comparison of different pharmacological pathways of α_2-adrenoceptor agonists in *in vitro* models. cAMP: cyclic adenosine monophosphate, HECs: human erythroleukemia cells, ADP: adenosine diphosphate. ‡ The values are estimated from the figures in original references.

Study Model	Pharmacological actions	α_2-adrenoceptor agonists					Reference
		Clonidine	Dexmedetomidine	Brimonidine	Tizanidine	Guanfacine	
In vitro study using HECs (n=3)	% of the inhibition of Forskolin-stimulated cAMP on α_{2A}-adrenoceptors	18.75 ±4.6 ‡	42.18 ±10.2 ‡	65.62 ±15.6 ‡	0.78 ± 1.6 ‡	7.8 ±8.0 ‡	(Jansson *et al.,* 1998)
In vitro study using HECs. (n=3-6)		22.0 ±1.0	49.1 ±10	73.2 ±2.0	1.0 ±2.0	8.0 ±8.0	(Kukkonen *et al.,* 2001)
In vitro study using Human neuroblastoma SH-SY5Y cells. (n=3-5)	% of the inhibition of Forskolin-stimulated cAMP on α_{2C}-adrenoceptors	31.1 ±3.1		50.1 ±2.0		53 ±1.0	(Parsley *et al.,* 1999)
In vitro study using blood samples from human. (n=7)	% of the increase of ADP-stimulated platelet aggregation	34-53	34-52	34-65			(Porta Bonete *et al.,* 2020)
In vitro study using canine saphenous veins. (n=3-5)	pA_{50} (mean±SEM)	7.36 ±0.13	8.12 ±0.08	7.64 ±0.09		6.82 ±0.06	(MacLennan *et al.,* 1997)
	Intrinsic Activity (mean±SEM)	0.56 ±0.04	0.67 ±0.04	0.95 ±0.06		0.41 ±0.06	

2.2.1. The effect of α_2-adrenoceptor agonists on haemodynamic responses:

The α_2-adrenoceptor agonists could alter the systemic haemodynamic resulting in the reduction of the heart rate, myocardial contraction, and oxygen consumption. This might stabilise the activity of the sympathoadrenal system during trauma, anaesthetics, and surgical procedures. An in vivo study on anaesthetised dogs showed that dexmedetomidine decreased the blood flow in the venous system, spleen, and skin, but preserved the perfusion or slightly reduced the blood flow in the heart, brain, and kidneys. This data suggested the considerable effect of α_2-adrenoceptor agonists on the redistribution of the cardiac output and blood flow of the less vital organs (Lawrence *et al.,* 1996). In addition, it has been demonstrated that clonidine attenuated the tachycardia and hypertension during the induction of anaesthesia, providing a rationale for using these agents for anaesthetised coronary and hypertensive patients (Bruandet *et al.,* 1998). Guanfacine has direct peripheral actions that produce dose-dependent vasoconstriction resulting in a transient increase in blood pressure and a decrease in heart rate (Ehringer *et al.,* 1980).

Mivazerol has not been therapeutically approved but it demonstrated some beneficial effects such as anti-ischemic actions in animals and patients with myocardial ischaemia. The results from in vivo micro-dialysis of rats showed that, unlike clonidine, IV administration of mivazerol does not affect the level of NA release in the hippocampus (Zhang *et al.,* 1995). Mivazerol also showed some protection actions in patients undergoing vascular surgery from further coronary problems (Oliver *et al.,* 1999).

Administration of clonidine during surgery could stabilise the haemodynamic responses by reducing the blood pressure and heart rate without resulting in hypotension. Moreover, it attenuates stress-induced sympathoadrenal responses to painful stimuli, improves intraoperative hemodynamic stability, reduces the incidence of perioperative myocardial ischemic episodes in patients with suspected or documented coronary artery disease, and decreases anaesthetic requirements during surgery (Matot *et al.,* 2000b). The preoperative use of dexmedetomidine is associated with reduced incidences of delirium and cardiac surgical complications, as well as the reduction in hospital stay and improvement in the survival rate (Ji *et al.,* 2014). It also decreased arterial blood pressure and heart rate in postsurgical patients (Bhana *et al.,* 2000) and caused a sustained hypotension in surgical patients (Barends *et al.,* 2020). Table 2.3 summarises some clinical studies that studied the effects of α_2-adrenoceptor agonists on hemodynamic changes.

Table 2.3: The changes in haemodynamic responses; mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) of the patients after receiving α_2-adrenoceptor agonists. (↑) increase, and (↓) decrease.

α_2-adrenoceptor agonists	Dose and route of administration	MAP	SBP	DBP	HR	Reference
Clonidine	100 µg (PO)		↓ 18 mmHg (Mean reduction over 6h)	↓ 8.7 mmHg (Mean reduction over 6h)	↓ 44.4 bpm (mean reduction over 6h)	(Simpson *et al.,* 2001)
	300 µg (PO)		166 ±17 mmHg (control) 125 ±23 mmHg during the surgical procedure	97 ±14 mmHg (control) 80 ±13 mmHg (clonidine group) during surgical procedure	86 ±13 bpm (control) 69 ±13 bpm (clonidine group) during surgical procedure	(Matot *et al.,* 2000a)
	150 µg or 300 µg (PO)		↓ (4 to 13) % at a low dose ↓ (23 to 50) % at high dose during fibreoptic bronchoscopy	↓ 10% at low dose ↓ 38% at high dose during fibreoptic bronchoscopy	↑ 2% at low dose ↓15% at high dose	(Matot *et al.,* 2000b)
Dexmedetomidine	0.5 µg/kg/h (IV infusion)	98.8 ±19.6 mmHg (baseline) 78.7 ±10.5 mmHg during sternotomy			84.9 ±13.7 bpm (baseline) 69.1 ±11.1 bpm (during sternotomy)	(Soltani *et al.,* 2017)
	6 µg/Kg/h Bolus dose followed by controlled infusion	↑ 7% to 19% (by increasing the plasma concentration)			↓ 5.5% to 38% (by increasing the plasma concentration)	(Colin *et al.,* 2017)
Tizanidine	3 mg (PO)	148±21 mmHg (control) 130±15 mmHg (tizanidine) during epidural catheterization				(Imanaga *et al.,* 2004)

Guanfacine	IV infusion 4mg	138 ±15.6 mmHg (control) 103.4 ±4.8 mmHg (guanfacine) at 20 h	19 mmHg (control) 208 mmHg (guanfacine) during the first 5 mins	107.7 mmHg (control) 115 mmHg (gunafacine) during the first 5 mins	No Change	(Magometschnigg *et al.,* 1980)
Mivazerol	IV bolus of 4 mg/kg and then 1.5 µg/ kg/h for up to 72 h		No significant change	No significant change	Bradycardia (<40bpm)	(Oliver *et al.,* 1999)

2.2.2. The effect of α_2-adrenoceptor agonists on inflammation/immune system:

Many animal and human studies have shown that α_{2A}-adrenoceptor agonists have anti-inflammatory/immune actions. The presence of α_{2A}-adrenoceptors on macrophages might contribute to the production of proinflammatory cytokines and increase of tumour necrosis factor (TNF-α) (Miksa *et al.,* 2009). It has been found that dexmedetomidine and clonidine have anti-inflammatory effects in both animal and human studies, and this might contribute to the clinical outcomes in humans of critical conditions such as sepsis (Flanders *et al.,* 2019). In healthy volunteers, dexmedetomidine decreased the concentration of eotaxin, interleukin-18 (IL-18), interleukin-2Rα, stem cell factor, stem cell growth factor and vascular endothelial growth factor (Kallioinen *et al.,* 2019), IL-18, L-selectin, E-selectin, and Granzyme B (Bosch *et al.,* 2020a). The clinical studies on patients undergoing surgical operations have investigated the anti-inflammatory responses of dexmedetomidine at the end of the operation which lasts up to 2 hours after surgery (Table 2.4).

The infusion of a small dose of dexmedetomidine (0.3 µg/kg/h) during cardiac surgery significantly reduced the surgery-induced increase in IL-1, IL-6, TNF-α and INF-gamma (Bulow *et al.,* 2016). In addition, there were no postoperative medical complications in the dexmedetomidine group, and the level of plasma high mobility group box 1 (HMGB 1) was reduced by 34% at the end of the operation (Wu *et al.,* 2018). Some recent randomised clinical trials on dexmedetomidine have compared the cytokines levels in surgical patients at pre and postoperative time points. The patients received a loading dose of 0.5 µg/Kg of dexmedetomidine over 10 minutes

before induction of anaesthesia, then randomised into two post-operative groups and received controlled IV analgesia either fentanyl alone or combined with dexmedetomidine. The results have shown that dexmedetomidine has a better post-operative analgesic action, and it significantly attenuated the plasma levels of IL-4 and IL-6 at 48 hours post-operative only, while no significant changes have been shown in IL-1, IL-10, TNF-α, and IFN-gamma (Wang *et al.,* 2021b) (Figure 2.2). In comparing the dexmedetomidine group that received bolus infusion of 0.5 μg/kg over 10 minutes and 0.4 μg/kg/h continuous infusion, to the control group, the levels of IL-1, IL-6 and TNF-α were lower in dexmedetomidine group at the end of surgery and 2 hours after surgery with no significant changes after 24 hours (Xu *et al.,* 2021).

Table 2.4: The effect of dexmedetomidine on inflammatory mediators during surgical operation; data are reported as the percentage of change compared to the control; T0: baseline, T1: end of operation, T2: 24 h after operation. (-) decrease, (+) increase, and (*) indicate the significant changes as reported in the original resources.

Study model	Participants	Dose	Cytokines	Time point	Approximate % of difference by comparing dexmedetomidine group with control group	Reference
Randomised prospective study	40 patients undergoing radical gastrectomy	0.5 μg/kg initial dose followed by 0.4μg/kg/h maintenance dose.	TNF-α	T1	- 26*	(Wang *et al.,* 2015)
			IL-6		- 30*	
Randomised prospective study	75 patients undergoing laparoscopic cholecystectomy	1 μg/kg over 10 min followed by 0.5μg/kg/h maintenance dose	IL-4	T_0	+ 12	(Lee *et al.,* 2018)
				T_1	- 75	
			IL-10	T_0	+ 26.7	
				T_1	- 76.5*	
Double-blinded prospective study	60 patients undergoing thoracoscopic surgery	0.5μg/kg (IV infusion)	IL-6	T_2	- 34*	(Wu *et al.,* 2018)
Randomised prospective study	80 patients undergoing laparoscopic hysterectomy	0.5 μg/kg over 10 min followed by 0.4 μg/kg/h maintenance dose	IL-1	T_0	+ 8	(Xu *et al.,* 2021)
				T_1	- 11.4*	
				T_2	- 1.3	
			IL-6	T_0	- 8.2	
				T_1	- 14*	
				T_2	- 10.3	
			TNF-α	T_0	+ 3	
				T_1	- 12.3*	
				T_2	- 6.6	

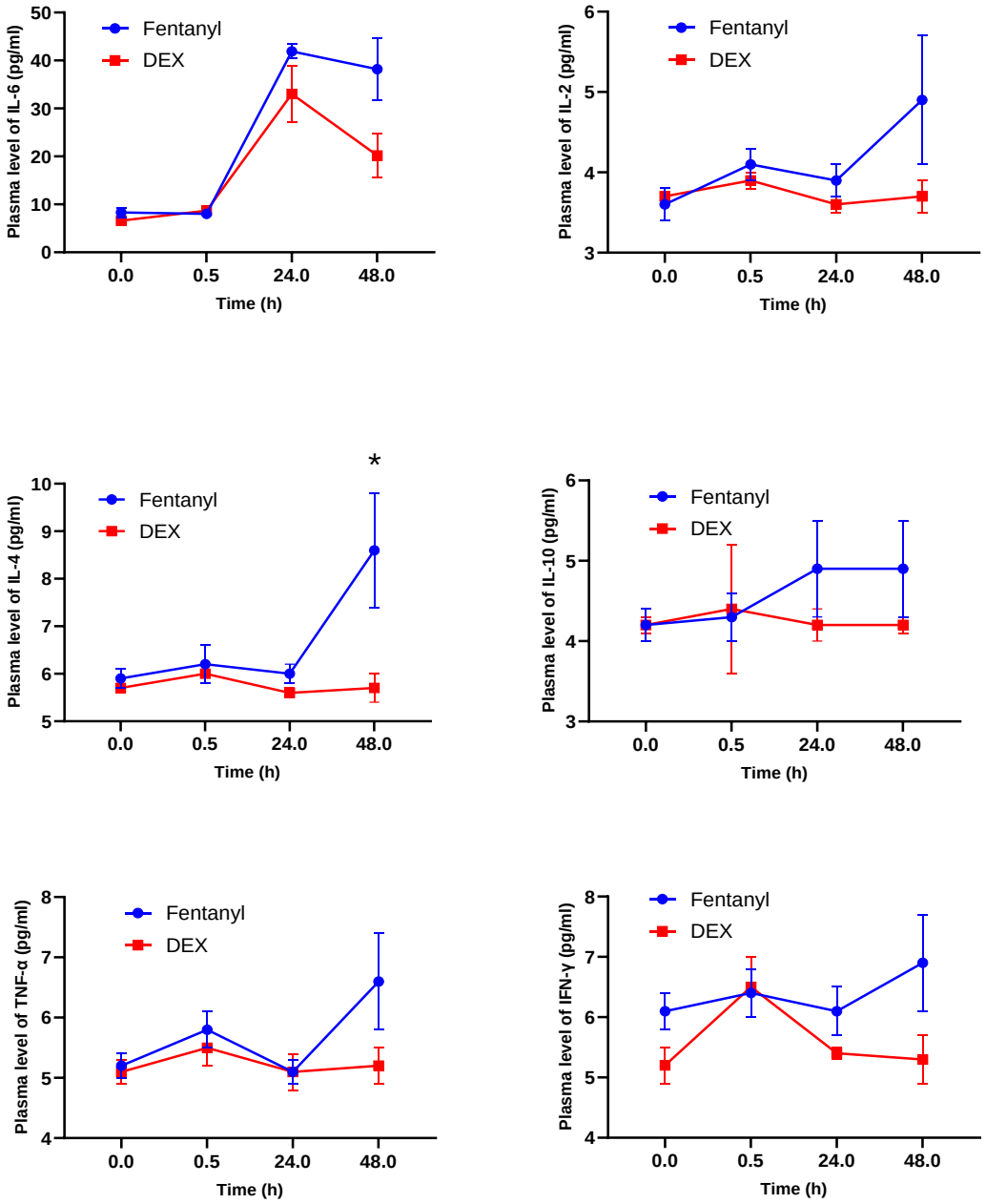


Figure 2.2: Comparison of the plasma levels of cytokines in surgical patients at different time points between the dexmedetomidine group and the fentanyl group. T_0: pre-anaesthesia, T1: 0.5 h after operation, T2: 24 h after operation, T3: 48 h after operation. The data are reported as the mean ± SEM values. (*) represented the significant difference from the fentanyl group (adapted from Wang *et al.,* 2021b).

2.3. α_2-adrenoceptor agonists in sepsis (dexmedetomidine and clonidine):

2.3.1. Anti-inflammatory and protective actions:

Sepsis is characterised by uncontrolled inflammation due to the high release of inflammatory mediators. The activation of α_2-adrenoceptors located on T-lymphocytes might result in suppression of T-cell proliferation, and inflammatory cytokine release (Bao *et al.,* 2007, Su *et al.,* 2018). Also, the stimulation of α_2-adrenoceptors has shown some protective effects on various systems; respiratory, renal, hepatic and intestinal tissues in endotoxemic animals through attenuation of cytokine-mediated inflammation (Chen *et al.,* 2015, Kong *et al.,* 2017, Meng *et al.,* 2018, Liu *et al.,* 2019, Chen *et al.,* 2021). Dexmedetomidine reduced the acute renal injury resulting from hypoperfusion and hypoxia after administration of the high dose of NA in an animal septic model (Lankadeva *et al.,* 2019) and provided possible evidence of renal protection during sepsis (Hsing *et al.,* 2012). The injection of clonidine was able to prevent septic shock and improve the survival rate of the experimental models of sepsis by reducing the pro-inflammatory mediators; TNF-α, IL-6 and IL-1β (Hofer *et al.,* 2009).

The usage of α_2-adrenoceptor agonists in endotoxemic animals has shown some conflicting results, concluding their uncertain therapeutic anti-inflammatory value in sepsis. The α_2-adrenoceptor agonists such as brimonidine and clonidine significantly decreased the plasma level of the IL-10; whereas the α_2-adrenoceptor antagonist such as prazosin increased it (Szelenyi *et al.,* 2000). Furthermore, the blockage of α_{2A}-adrenoceptor by a specific antagonist (BRL-44408 maleate) showed an improvement in sepsis-induced acute lung injury accompanied by depressed HMGB1 expression in

animal models (Ji *et al.,* 2012). It also suppressed pro-inflammatory mediators including TNF-α, IL-6, IL-8 and HMGB1 in the human whole blood treated with lipopolysaccharide (LPS) (Kawasaki *et al.,* 2013).

Dexmedetomidine has been recently studied in several clinical trials with specific interest due to its anti-inflammatory responses during excessive cytokine release in sepsis. It reduced the TNF-α, IL-1β and IL-6 (Memis *et al.,* 2007), HMGB1 (Chang *et al.,* 2013), and improved the CRP, procalcitonin and albumin levels in ventilated septic patients (Ohta *et al.,* 2020). It has been concluded in a meta-analysis of 19 randomised controlled trials, that the use of dexmedetomidine was associated with a significant decrease in the level of TNF-α and IL-6 compared to other sedatives (Zhang *et al.,* 2022).

2.3.2. Circulatory actions:

Sepsis is associated with persistent hypotension due to the peripheral vasodilatation and reduction in the vasopressor responsiveness leading to hypoperfusion and septic shock. Appropriate cardiovascular responses are essential to maintain systemic perfusion and cellular homeostasis for septic shock patients in the ICU. The use of α_2-adrenoceptor agonists might exacerbate sepsis-related hypotension due to their pre-synaptic sympatholytic actions, which could worsen the vascular shock. However, several experimental studies support the hypothesis that α_2-adrenoceptor agonists restored the pressor responses of NA. The reduction in the pressor response to NA was reversed to the baseline levels after administration of clonidine and dexmedetomidine in LPS treated rats (Geloen *et al.,* 2013).

A recent randomised experimental study on early septic pigs has shown that dexmedetomidine stabilised the venous oxygen saturation and alleviated the decreased arterial pressure suggesting its beneficial effects on heart and pulmonary circulation (Aidoni *et al.,* 2020). Furthermore, a prospective clinical study on 38 septic patients showed that dexmedetomidine at a dosage of 0.7 ±0.2 µg/kg/hr nearly halved the amount of NA required to maintain MAP. The dosage of NA was reduced from 0.69 ±0.72 µg/kg/min before dexmedetomidine to 0.30 ±0.25 µg/kg/min after 4 hours of dexmedetomidine infusion (Morelli *et al.,* 2019). Moreover, the subgroup analysis of the SPICE III trial also reported that the concentrations of the vasopressors required to maintain MAP were lower in the dexmedetomidine group (Cioccari *et al.,* 2020).

2.3.3. Other clinical outcomes:

Dexmedetomidine has shown many advantages in the clinical outcomes of septic patients; however, its overall effect remains controversial. In a subgroup analysis of the septic and non-septic patients from the MENDS randomized controlled trial to compare the sedative actions of dexmedetomidine and lorazepam. Dexmedetomidine has shown more beneficial effects in septic patients than non-septic patients. Its administration was associated with more delirium-free days, coma-free days and ventilator-free days in comparison to the septic patients on lorazepam. It also showed a 70% reduction in the risk of death at 28 days (Pandharipande *et al.,* 2010).

In the DESIRE clinical trial, 201 septic patients were randomised to assess their 28-day mortality rate and the ventilator-free days. Although the percentage of change in

the mortality rate was not significantly different in dexmedetomidine patients versus control patients but it was smaller (22.8% vs 30.8%) (Kawazoe *et al.,* 2017). However, a clinical trial of SPICE III stated that early administration of dexmedetomidine was associated with more adverse effects and there was no significant effect on the 90-day mortality rate of critically ill patients in ICU (Shehabi *et al.,* 2019). A post hoc analysis of the DESIRE trial has also shown that dexmedetomidine could be more effective as a sedative agent in the elderly rather than in younger patients (Nakashima *et al.,* 2020, Sato *et al.,* 2021). In another meta-analysis, dexmedetomidine significantly reduced the mortality rate compared with benzodiazepines (Zhang *et al.,* 2022).

The results from two other meta-analysis reviews have shown that septic patients who receive dexmedetomidine didn't show any significant change in 28-day mortality, but it might reduce the length of ICU stays, adverse effects and the score of the sequential organ failure assessment (SOFA) (Huang *et al.,* 2021, Yuan *et al.,* 2022). On the other hand, dexmedetomidine reduced the duration of mechanical ventilation with no effects on mortality and other clinical outcomes (Wang *et al.,* 2021a). As mentioned, the SPICE III trial reported that there was no overall difference in mortality rate; however, a subgroup analysis to quantify the potential heterogeneity of treatment according to age observed an opposite dexmedetomidine effect on mortality. It has been found that early sedation with dexmedetomidine reduced 90-day mortality in older patients (> 65 years) regardless of operative or non-operative patients, but increased 90-day mortality in younger non-operative patients (< 65 years) (Shehabi *et al.,* 2021). Moreover, the clinical studies compared the costs of dexmedetomidine with other sedatives such as midazolam and propofol used for short-term sedation in the ICU.

The results showed that dexmedetomidine is associated with shorter ICU stays and decreased mechanical ventilation times, resulting in more than 20% cost reduction (Aggarwal *et al.,* 2020).

2.4. What are the potential beneficial actions of α_2-adrenoceptor agonists in the management of sepsis:

The multiple pharmacodynamic actions of α_2-adrenoceptors explain further interest in these drugs in critical care settings such as septic shock. Figure 2.3 illustrates the potential central and peripheral mechanisms of α_2-adrenoceptor agonists in septic shock. Vasoplegia results from vascular hyporesponsiveness to the endogenous vasopressors due to various pathophysiological mechanisms such as inappropriate inflammatory reactions, upregulation of iNOS responsible for over-production of NO and downregulation or desensitisation of the vascular smooth muscle receptors; adrenoceptors, vasopressin 1 receptor and angiotensin type 1 receptor (Levy *et al.,* 2018).

Studies have shown that α_2-adrenoceptor agonists could potentially block the immune responses, and this could help in treating the late stage of septic shock. However, the immune suppression caused by decreased monocyte ability to release pro-inflammatory cytokines might lead to failure to eradicate primary infections leading to multi-organ dysfunction and mortality. Activation of α-adrenoceptors by NA has been shown to increase pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 via nuclear factor-κ B cells (NF-κB) mediated by protein kinase C pathway. Conversely, the activation of β-adrenoceptor by high doses of NA exerts an anti-inflammatory effect

by enhancing the cAMP and protein kinase A leading to immunoparalysis (Stolk *et al.,* 2020).

Several clinical trials reported that dexmedetomidine resulted in no significant anti-inflammatory or immune suppressive actions. Dexmedetomidine does not affect the release of cytokines at the end of the surgery up to 48 hours after surgery except for IL-4 and IL-6 which showed the reduction after 48 hours only. Furthermore, the recent clinical trial on septic patients has shown that infusion of dexmedetomidine resulted in no significant difference in the plasma levels of inflammatory biomarkers (CD42a+/CD14+, HLADR+/CD14+), and CRP, IL-6, IL-10 and TNF-α after 12 and 24 hours in comparing to the imidazoline group (Elayashy *et al.,* 2023). The high dose of dexmedetomidine blocked the increase in IL-10 at the end of the surgery (Table 2.4). Accordingly, the α_2-adrenoceptor agonists could have no or less immunosuppressive actions than those associated with high doses of NA.

The receptor desensitisation might result from the excessive release of endogenous catecholamines due to the over activation of the sympathetic system during septic shock (Levy *et al.,* 2018). NA is commonly used as the first-line therapy to restore the MAP in the ICU. However, the low and moderate doses might be insufficient to achieve the target MAP, and the high doses of NA may result in potentially fatal complications (Annane *et al.,* 2018). The recommended therapeutic dose of NA is 0.2 µg/kg/min to maintain the MAP. Nonetheless, higher doses (0.5 to 2 µg/kg/min) could be required in severe septic shock. It has been reported that high doses of NA (more than 1 µg/kg/minute) are associated with a higher mortality rate (Ratnani *et al.,* 2023).

As mentioned, the α_2-adrenoceptor agonists such as dexmedetomidine significantly restore vascular hyperresponsiveness and decrease the dose of NA required in ICU, and this is believed to be due to the reduction in the central sympathetic activity through binding to presynaptic α_2-adrenergic receptors and thereby they increase the sensitivity of post-synaptic adrenergic receptors to the vasopressors. The other potential mechanism of α_2- adrenergic agonists in restoring the pressor responses is by preventing the excessive opening of the K_{ATP} channels resulting in central independent vascular actions. It has been found that the plasma related concentrations of dexmedetomidine (0.1μM) can significantly block the K_{ATP} channels in the vascular smooth muscle cells (Kawano *et al.,* 2012).

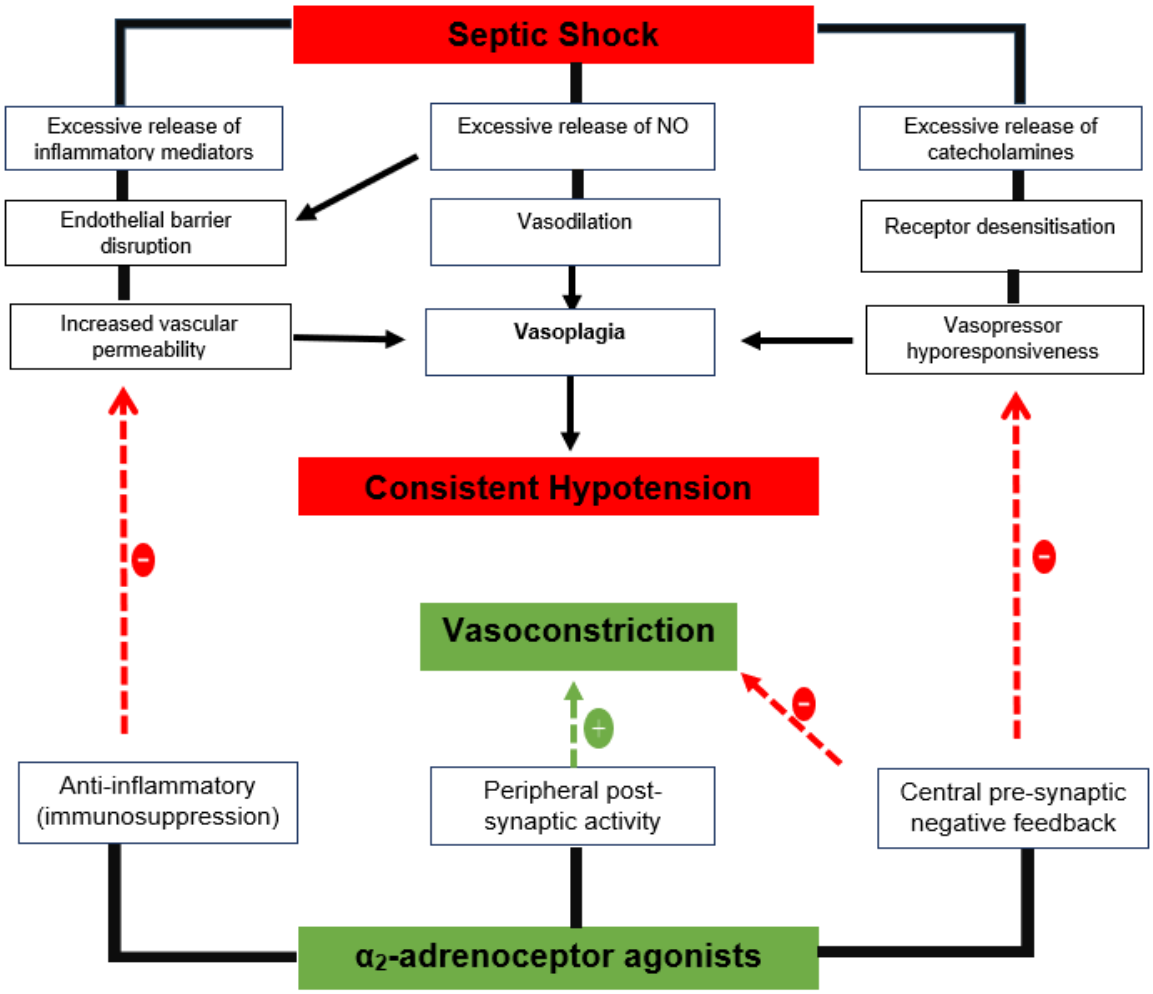


Figure 2.3: The pathophysiological pathways of septic shock and the potential pharmacological actions of α_2-adrenoceptor agonists in the management of septic shock.

2.5. The potential role of plasma concentrations of α_2-adrenoceptor agonists in the management of septic shock:

The plasma drug concentration of α_2-adrenoceptor agonist could identify their central and peripheral actions, which vary over time depending on the dosage, administration duration, and pharmacokinetic properties. It has been found that dexmedetomidine demonstrates immunomodulatory effects in a dose-dependent manner in surgical patients, by blocking the increase in CRP and IL-10 in the dexmedetomidine group receiving a high dose (1µg/kg) in comparison to the group on half of that dose (Lee *et al.,* 2018). The α_2-adrenoceptor agonists also produce concentration-dependent biphasic cardiovascular responses and peripheral vasoconstriction. The small doses of clonidine and dexmedetomidine cause a reduction in sympathetic outflow, peripheral vascular resistance, and blood pressure. The mechanism of vasodilatation is presumably mediated by pre- and postsynaptic α_2-adrenoceptor at the level of the brain and spinal cord and by stimulation of α_2-adrenoceptor on the vascular endothelial cells (Howie *et al.,* 1996, Tulen *et al.,* 1992), The clinical studies showed that IV administration of α_2-adrenoceptor agonists such as clonidine, guanfacine and dexmedetomidine resulted in increased arterial pressure followed by a decrease in blood pressure, heart rate and cardiac output (Carabine *et al.,* 1991, Ehringer *et al.,* 1980, Magometschnigg *et al.,* 1980, Bloor *et al.,* 1992).

Furthermore, it has been reported that during the first 5 minutes of IV infusion of 2 µg/kg dexmedetomidine to healthy volunteers, the MAP increased by 22%, heart rate declined, and continued infusion resulted in the reduction of MAP by 20% and

increased HR (Dyck *et al.,* 1993). The hypertensive effects are more profound than the hypotensive effects by increasing the plasma concentrations of dexmedetomidine. Human studies on dexmedetomidine show decreases in BP and CO after small boluses through activation of presynaptic α_2-adrenoceptors in the central nervous system and through activation of α_2-adrenoceptors in vascular endothelial cells, which causes vasodilatation. The bolus infusion of 0.5 µg/kg for a loading dose followed by the continuous infusion of 0.4 µg/ml/h resulted in a significant reduction in blood pressure of the dexmedetomidine group at the end of the surgery up to 2 h after surgery in comparison to the baseline (Xu *et al.,* 2021). Conversely, the controlled infusion of dexmedetomidine to healthy volunteers increased MAP (Figure 2.4) and declined HR by targeting higher plasma concentrations. This is possibly due to their peripheral vasoconstriction actions via α_2-adrenoceptors in vascular smooth muscle (Ebert *et al.,* 2000, Colin *et al.,* 2017). The stimulation of α_1-adrenoceptors by higher concentrations of dexmedetomidine might also contribute to vasoconstriction (Seyrek *et al.,* 2011). Accordingly, the plasma concentrations of α_2-adrenoceptor play an important role in their central and peripheral actions. Table 2.5 shows the therapeutic outcomes of α_2-adrenoceptor agonists associated with their maximum plasma concentrations. Oral doses of tizanidine, guanfacine and lofexidine produced their pharmacological actions at high plasma levels and this might contribute to their peripheral vasoconstriction.

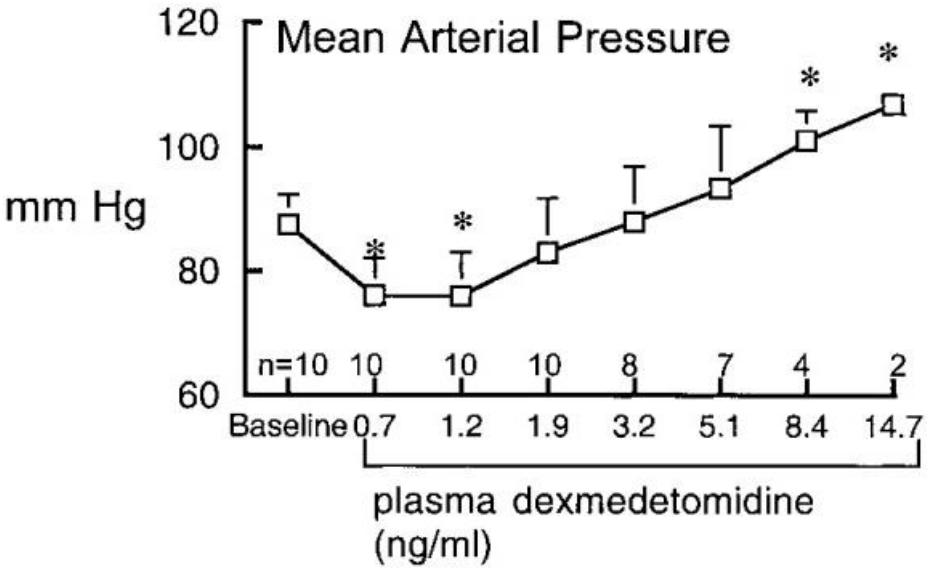


Figure 2.4: Biphasic changes (low then high) in the mean arterial pressure by increasing the plasma concentrations of dexmedetomidine through controlled infusion in healthy volunteers. (*) represents a significant change compared to the pre-infusion baseline (adopted from Ebert *et al.,* 2000).

Table 2.5: The doses, plasma concentrations and therapeutic outcomes of α_2-adrenoceptor agonists. CO: cardiac output, PO: oral, IM: intramuscular, IV: intravenous, QID: four times per day.

α_2-adrenoceptor agonists	Doses	Plasma concentrations (ng/ml) (Approximate Molar Concentrations)	Outcomes	Reference
Clonidine	2 mg/kg (Epidural)	0.45 to 0.77 (2 nM to 3.4 nM)	Post-operative analgesia in paediatrics	(Ivani *et al.,* 1998)
	2 µg/kg or 4 µg/kg	0.28 to 0.81 (1 nM to 3.5 nM)	Sedation in paediatric surgery	(Sumiya *et al.,* 2003)
	75 µg (PO)	0.74 in 3 h (3 nM)	Anti – delirium for elderly patient	(Hov *et al.,* 2018)
Dexmedetomidine	2 µg/ml (IM)	0.3 to 1.3 (1.5 nM to 6.5 nM)	Sedative agent	(Dyck *et al.,* 1993)
	2 µg/ml (IV)	2.5 in 10 mins (12.5 nM)	Severe hemodynamic changes	
	Controlled infusion pump for 40 minutes	0.7 to 1.2 (3 nM to 6 nM)	Sedation, ↓ NA, ↓ BP, ↓ HR, ↓ CO	(Ebert *et al.,* 2000)
		1.9 – 14.7 (9.5 nM to 73 nM)	Sedation, ↑ BP	
Brimonidine	1 drop of 0.5% eye drop (Ocular)	< 0.3 in 1 to 4 h (<1nM)		(Acheampong and Tang-Liu, 1995)
Tizanidine	8 mg (PO)	7.2 in 1.5 h (28.8 nM)	Spasticity in MS	(Nance *et al.,* 1997)
	16 mg (PO)	14.9 in 1 h (59.6 nM)		
Guanfacine	3 mg (PO)	8.4 in 1.5 to 4 h (34 nM)	Sedation, ↓ BP (but slower than clonidine)	(Dollery and Davies, 1980)
Lofexidine	8 mg QID	3.2 in day 7 (12.4 nM)	Opioid detoxification (well-tolerated)	(Yu *et al.,* 2008)

2.6. How the lipophilicity might affect the pharmacological actions of α_2-adrenoceptor agonists:

Lipophilicity is an important factor that potentially affects the pharmacological actions of α_2-adrenoceptor agonists. It is the main predictor of blood brain barrier (BBB) penetration, where the more lipophilic drugs have a higher ability to penetrate the BBB resulting in more central actions (Waterhouse, 2003). It is known that the molecular size and lipophilic properties of drugs contribute to their distribution, elimination, and subsequently pharmacodynamic actions. According to PubChem database 2023, the partition coefficient (LogP) of α_2-adrenoceptor agonists such as dexmedetomidine, lofexidine, and clonidine are 3.1, 2.6 and 1.6 respectively, which are higher than the LogP of other agonists (Kim *et al.,* 2023). However, there are differences in the lipophilicity profile (LogD) of α_2-adrenoceptor agonists at physiological pH 7.4 as well as pH 7.2 and pH 7 (Table 2.6). This difference could affect the pharmacological actions of these agonists. It is known that there is an increased level of lactate concentration causing lactic acidosis (serum pH < 7.3) in severe septic patients and sepsis shock, which is associated with high morbidity and mortality (Suetrong and Walley, 2016). The reduction in the pH due to septic acidosis might affect the lipophilicity and ultimately the permeability of these drugs through BBB. At low pH, brimonidine and guanfacine exhibit smaller partition coefficients, and this might be attributed to their peripheral distribution in sepsis.

Table 2.7 shows the molecular size of α_2-adrenoceptor agonists and the estimated lipophilicity profile from three different databases. The agonists with less lipophilicity might have a better role in redistribution the of blood in the peripheral cardiovascular, platelet aggregation and inflammatory systems. The lipophilicity could define the affinity of these agonists to the peripheral adrenergic receptors presented on the vascular smooth muscle cells and platelets, dexmedetomidine has bidirectional effects; enhancement and suppressive effects on platelet functions, where their platelet enhancement function is through binding to α_2-adrenoceptors (Kawamoto *et al.,* 2015). The therapeutic concentration of dexmedetomidine does not affect the platelet functions in healthy volunteers (Kose *et al.,* 2013). However, brimonidine, with lower lipophilic is a fully α_2-adrenoceptor agonist and can restore platelet aggregation and this is through the inhibition of cAMP (Porta Bonete *et al.,* 2020). The low lipophilic property of brimonidine along with having large molecular weight (Table 2.7) could contribute to their fewer sedative actions than that of dexmedetomidine. Therefore, brimonidine could be suggested as an alternative to dexmedetomidine in treating peripheral cardiovascular functions of septic patients who don't need sedation. On the other hand, guanfacine is therapeutically approved in treating ADHD and has peripheral cardiovascular actions, hence it might be suggested as an alternative sedative agent to dexmedetomidine with potential peripheral vasoconstriction.

In conclusion, despite the conflicting outcomes of the recent clinical trials but many studies support the beneficial role of α_2-adrenoceptor agonists in sepsis. dexmedetomidine is likely to reduce vasopressor requirement, mortality rate, mechanical ventilation, and length of ICU stay. Further clinical investigations are needed to particularly evaluate the role of α_2-agonists in raising the MAP through

peripheral vasoconstriction and to investigate whether brimonidine or guanfacine could be superior to dexmedetomidine in the management of sepsis.

Table 2.6: Predicted LogD of α_2-adrenoceptor agonists at different PH (Chemaxon, 2022).

α_2- adrenoceptor Agonists	**LogD (Distribution Coefficient) at different PH**		
	7	**7.2**	**7.4**
Methyldopa	-1.36	-1.36	-1.37
Alpha-methylNA	-1.59	-1.41	-1.23
Clonidine	1.32	1.49	1.66
Dexmedetomidine	3.27	3.31	3.34
Brimonidine	-0.18	-0.01	0.42
Tizanidine	1.41	1.55	1.68
Guanfacine	0.15	0.32	0.43
Lofexidine	0.63	0.75	0.9
Mivazerol			0.72

Table 2.7: Molecular weight (Mwt), Elimination half-life, and Lipophilicity of α_2-adrenoceptor agonists (Wishart *et al.,* 2018, Kim *et al.,* 2023).

α_2-adrenoceptor Agonists	Mwt (g/mol)	Elimination half-life (h)	Lipophilicity (LogP)			
			Experimental property	Chemaxon	ALOGPS	PubChem
Methyldopa	211.21	1.75 (Oral tablet) 1.30 to 2 (IV injection)		-1.4	-2	-1.9
Clonidine	230.09	6-23 (Oral) 0.5 (Epidural)	1.59	2.49	2.55	1.6
Dexmedetomidine	200.28	2	2.8	3.39	3.28	3.1
Brimonidine	292.13	3		1.37	1.27	0.6
Tizanidine	253.71	2.5	1.72	2.02	1.6	1.1
Guanfacine	246.1	17	0.857	1.74	2.28	2
Lofexidine	259.13	11	5.37	2.66	3.31	2.6
Mivazerol	217.22					1.1

3. Chapter Three: The potential role of vasoconstrictive actions of α_2-adrenoceptor agonists in the management of septic shock.

3.1. Introduction

Vasopressors are classified into sympathomimetics (adrenaline, NA, dobutamine and phenylephrine), vasopressin analogues, and angiotensin II. These drugs are essential in shock management in the ICU (Shi *et al.,* 2020). Sympathomimetics raise blood pressure through α-adrenoceptors induced vasoconstriction and stimulate the β-adrenoceptors resulting in increased inotropy and heart rate. However, administration of these drugs requires dosage monitoring with titration to the minimal efficacious dose due to their narrow therapeutic range, which could expose the patients to lethal complications (Annane *et al.,* 2018). The α_2-adrenoceptor agonists, particularly dexmedetomidine, have attracted attention due to their potential benefit in improving the outcomes of septic patients, where dexmedetomidine improved the renal function (Nakashima *et al.,* 2020), and reduced the mortality rate in patients with septic shock (Kawazoe *et al.,* 2017, Zhang *et al.,* 2022, Zhao *et al.,* 2024). Therefore, demonstrating the role of α_2-adrenoceptor agonists is crucial to finding more effective approaches for treating sepsis.

3.1.1. The pharmacology of vascular smooth muscle contraction and relaxation:

The structure of the blood vessels varies based on their functions. The large blood vessels are composed of three layers: the inner layer consists of the endothelial cells surrounded by a thick layer of smooth muscle cells innervated with the sympathetic nervous system. The outer layer is made of collagen and elastin fibres innervated with the sensory nerves (Harvey, 2012). The contraction of vascular smooth muscle is

controlled by the amount of free intracellular Ca^{++} ion, which increased through the agonist mediated activation of inositol 1,4,5-trisphosphate (IP_3) leading to mobilisation of Ca^{++} into the cells, or activation of voltage-gated or receptor-linked Ca^{++} channels causing an increase in the influx of Ca^{++} (Eckert *et al.,* 2000).

The mechanism of contraction involves the inhibition of the smooth muscle myosin light chain phosphatase (MLCP) preventing the dephosphorylation of the regulatory myosin light chain (MLC) in smooth muscle (Somlyo and Somlyo, 2000). Catecholamines such as NA and other α-adrenergic agonists, angiotensin II, endothelin I, and vasopressin bind to the GPCR on the cell membrane resulting in PLC mediated hydrolysis of phosphoinositol biphosphate (PIP_2) to form two second messengers IP_3 and DAG. These contribute to the activation of the MLCK enzyme through the Ca++/Calmodulin complex and the inhibition of the MLCP enzyme through the Rho-Rho kinase pathway. Protein kinase C activated by diacylglycerol (DAG) can also inhibit MLCP. Subsequently, the phosphorylation of the MLC mediated through the myosin light chain kinase (MLCK) can occur causing vascular smooth muscle contractions (Webb, 2003) (Ahles and Engelhardt, 2014). NA and phenylephrine (PE) induced dose-dependent and fully reversible isometric contractions through the voltage-dependent Ca^{++} channels and intracellular Ca^{++} stores in the adrenergic nerves (Eckert *et al.,* 2000) (Figure 3.1).

Also, the potassium channels regulate the vascular tone based on the action potential at the cell membrane, where the opening of the channels results in the efflux of K^+ ions to the extracellular compartment causing hyperpolarisation and subsequently vasodilatation. In endothelial cells, intracellular Ca^{++} ions play an important role in the

production and release of autacoids such as nitric oxide and prostaglandin (PGI_2), which contribute to the relaxation of the vascular smooth muscle. (Jackson, 2005).

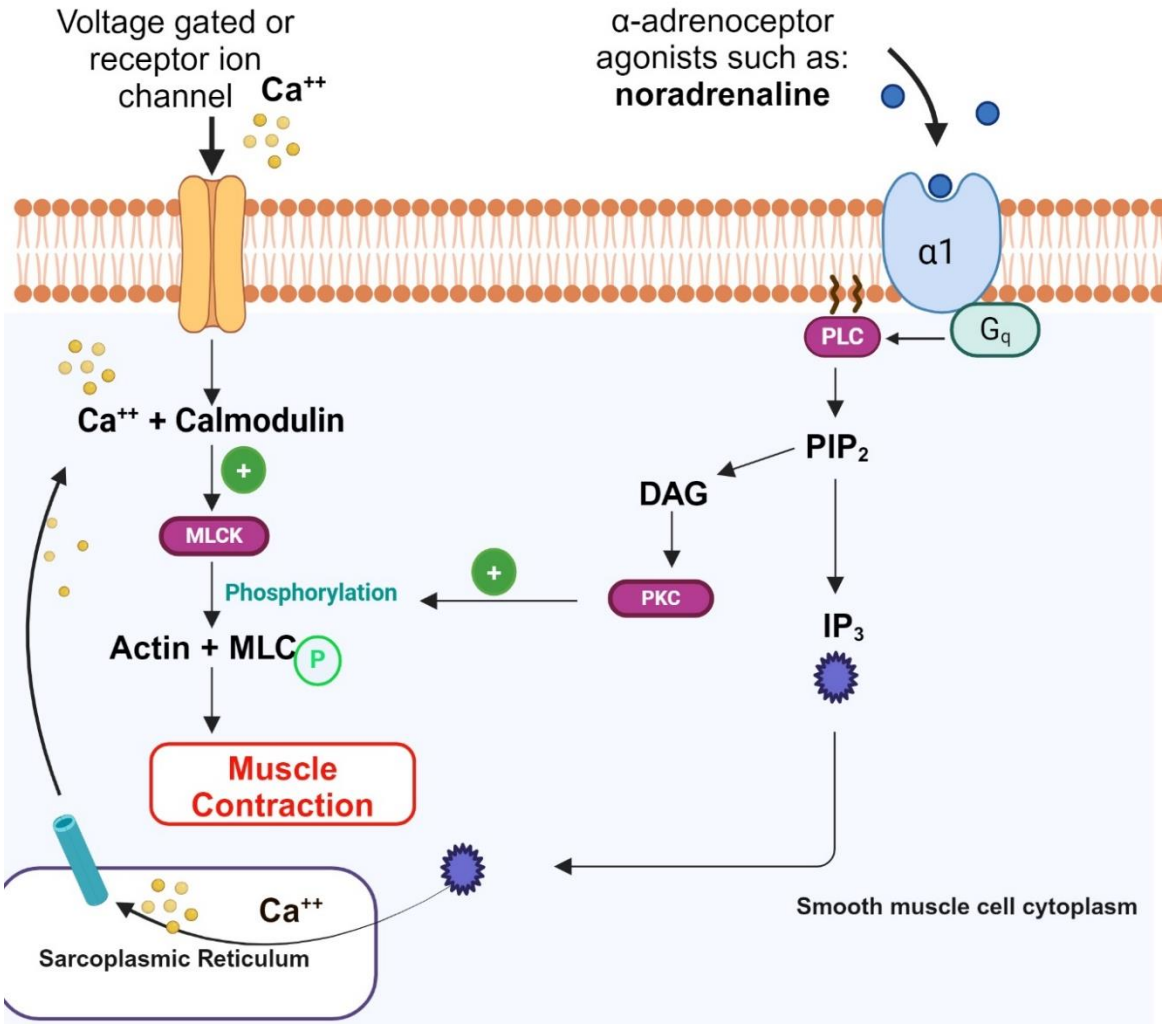


Figure 3.1: The representative diagram showing the mechanism of vascular smooth muscle contraction. PLC: phospholipase C, PIP_2: phophoinositol biphosphate, MLC: myosin light chain, MLCK: myosin light chain kinase, DAG: diacylglycerol, PIP2: phosphoinositol-bis-phosphate, PKC: protein kinase C, IP_3: inositol triphosphate (created **in Biorender.com**).

In addition to the NO, other active substances such as prostacyclin, platelet-activating factor and endothelin-1 are synthesised by endothelial cells to maintain the patency and fluidity of blood vessels. Prostacyclin has antiplatelet and vasodilator actions, in contrast to the thromboxane A_2 which is produced by platelets and induces platelet aggregation and vasoconstriction. The release of these substances is affected by the changes in the concentration of intracellular messengers; cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), in addition to the Ca^{++} ions (Vane *et al.,* 1990). It is known that the principal mechanisms of vascular smooth muscle relaxation are mediated through cAMP and cGMP. An in vitro study on isolated pulmonary arteries reported that vasodilators such as sodium nitroprusside (NO analogue) caused cGMP mediated pulmonary relaxation through the activation of the guanylate cyclase, while isoproterenol (β-adrenoceptor agonist) induced relaxation via cAMP mediated pathways (Fullerton *et al.,* 1994).

3.1.2. The pharmacology of α-adrenoceptor agonists:

The α_2-adrenoceptors are receptors of endogenous catecholamines that mediate physiological and pharmacological actions such as sedation, hypotension, insulin release inhibition, and platelet aggregation. They preferentially couple to the Gi and Go proteins of the GPCRs family and modulate the effector systems including adenylyl cyclase, K^+ and Ca^{++} channels, and mitogen activated protein kinases ERK1 and ERK2 (Flordellis *et al.,* 2004). The α_2-adrenoceptors are divided into presynaptic autoreceptors that inhibit the exocytosis of NA in adrenergic neurons, presynaptic heteroreceptors that reduce the release of other neurotransmitters such as histamine

in non-adrenergic neurons, or postsynaptic heteroreceptors in non-neuronal effector cells (Gilsbach *et al.,* 2011).

The activation of presynaptic α_2-adrenoceptors on sympathetic nerves and CNS induces a sympatholytic effect through the negative feedback, whereas the activation of vascular postsynaptic α_2-adrenoceptors distributed in both arteries and veins might result in vasoconstriction through α_2-adrenoceptors on vascular smooth muscle cells and vasodilatation through the α_2-adrenoceptors on endothelial cells. The activation of the Gi proteins on vascular smooth muscle blocks the adenylyl cyclase and thereby reduces cAMP and enhances vascular smooth muscle contraction (Seyrek *et al.,* 2011). Conversely, the activation of these receptors induced endothelium-dependent relaxation in blood vessels via endothelial α_2-adrenoceptors linked to the L-arginine nitric oxide pathway (Vanhoutte, 2001). Figure 3.2 shows the pharmacological mode of action of α_2-adrenoceptor agonists in vascular smooth muscle.

Three subfamilies of α_2-adrenoceptors were identified as α_{2A}, α_{2B}, and α_{2C}-adrenoceptors. They have various physiological and pharmacological actions and can be activated by endogenous catecholamines; NA and adrenaline that mediate different responses in the central nervous system and peripheral organs (Bylund *et al.,* 1994). A genetic study on the distribution of α_2-adrenoceptor subtypes in rat brains found that mRNA coding for α_{2A}-adrenoceptors can be found throughout the brain, specifically in locus coeruleus. In the human brain, the α_{2B} adrenoceptors were only observed in striatum and globus pallidus, in contrast, the expression of α_{2A}- and α_{2C}-adrenoceptors were intense in most brain regions. In the periphery, the peripheral contraction mediated by postjunctional α_2-adrenoceptors in the human saphenous vein was

investigated to closely resemble the human recombinant α_{2C}-adrenoceptor ligand binding site (Gyires *et al.,* 2009, Gavin *et al.,* 1997).

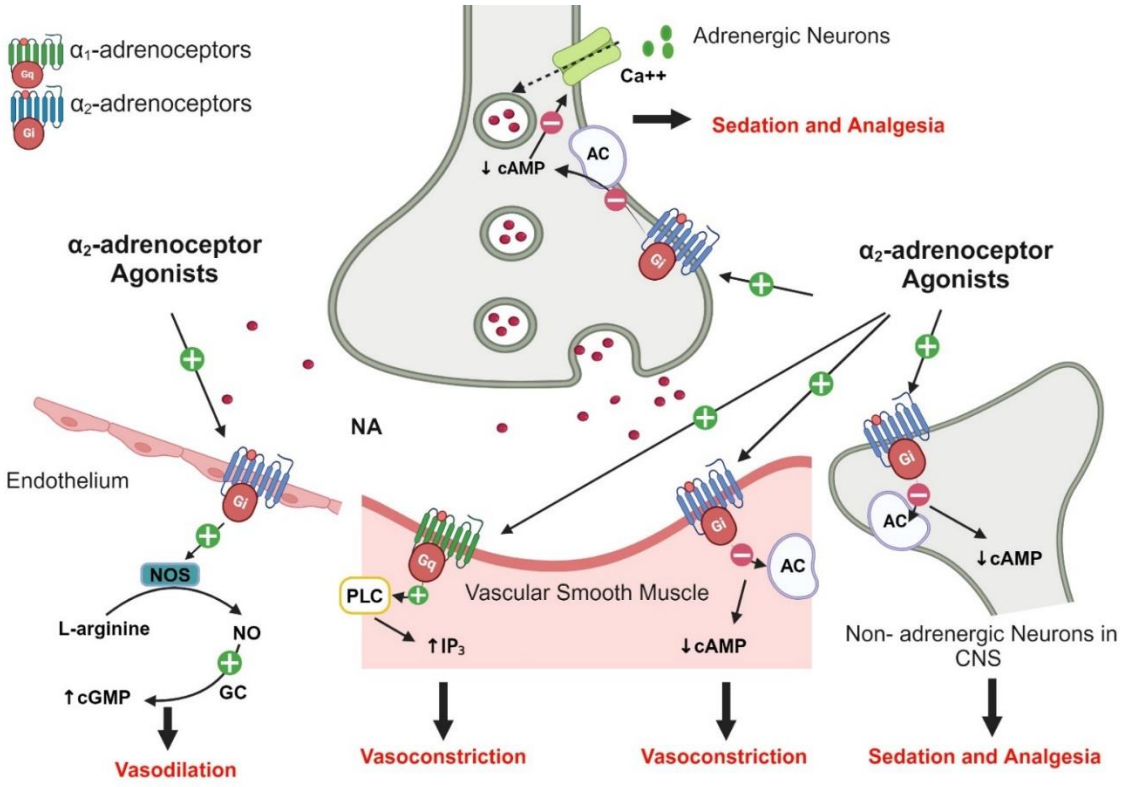


Figure 3.2: Representative diagram illustrating the pharmacological actions of α_2-adrenoceptor agonists in peripheral and central nervous systems. AC: adenylyl cyclase, IP_3: inositol triphosphate, cAMP: cyclic adenosine monophosphate, PLC: phospholipase (created **in Biorender.com**).

Additionally, in vitro studies on porcine coronary arteries have shown that α_{2A}- and α_{2C}-adrenoceptors exist on the vascular endothelium and that the α_{2A}- adrenoceptor subtype predominately mediated the endothelium-dependent relaxation. The rank of the potency for the agonists that resulted in the endothelium relaxation is as follows: p-iodoclonidine > clonidine > UK-14304 > guanabenz > adrenaline > NA (Bockman *et al.,* 1993). Therapeutically approved α_2-adrenoceptor agonists have some differences

in their affinity and potency at α_1 and α_2-adrenoceptor subfamilies. However, the results showed that they are more potent and have higher selectivity at α_2-adrenoceptor subfamilies compared to other α-adrenoceptor agonists such as adrenaline, NA, and phenylephrine. Therefore, precise receptor selectivity is important to understand the diversity in the pharmacological actions of these agonists in peripheral and central systems. In vitro radioligand binding studies on Chinese hamster ovary examined the selectivity of several clinically approved agonists including catecholamines and imidazolines to α-adrenoceptors via whole-cell 3H-prazosin binding and measured their functional responses for calcium mobilization, ERK1/2-phosphorylation, and cAMP accumulation. The results showed that adrenaline, NA, and phenylephrine were highly selective to all three subtypes of α_1-adrenoceptors. While imidazolines were more efficacious at α_2-adrenoceptors subclasses (Figure 3.3). However, there was a difference in their subclass selectivity, and understanding these details helps to clarify their pharmacological actions and minimise side effects (Proudman and Baker, 2021, Proudman *et al.,* 2022). Due to the contraction and relaxation actions of the α_2-adrenoceptor in the vascular smooth muscle, their pharmacological action might be underestimated. The usage of the central blood vessels such as porcine splenic arteries showed that the α_1-adrenoceptor mediated vasoconstriction is more predominant than that mediated by α_2-adrenoceptors (Barbieri *et al.,* 1998). This chapter will investigate the potential enhancement of other vasoconstrictors such as KCl, U46619, L-NAME, or α_2-adrenoceptor agonists on the vasoconstriction mediated by the concentration within the range of human plasma concentrations of NA.

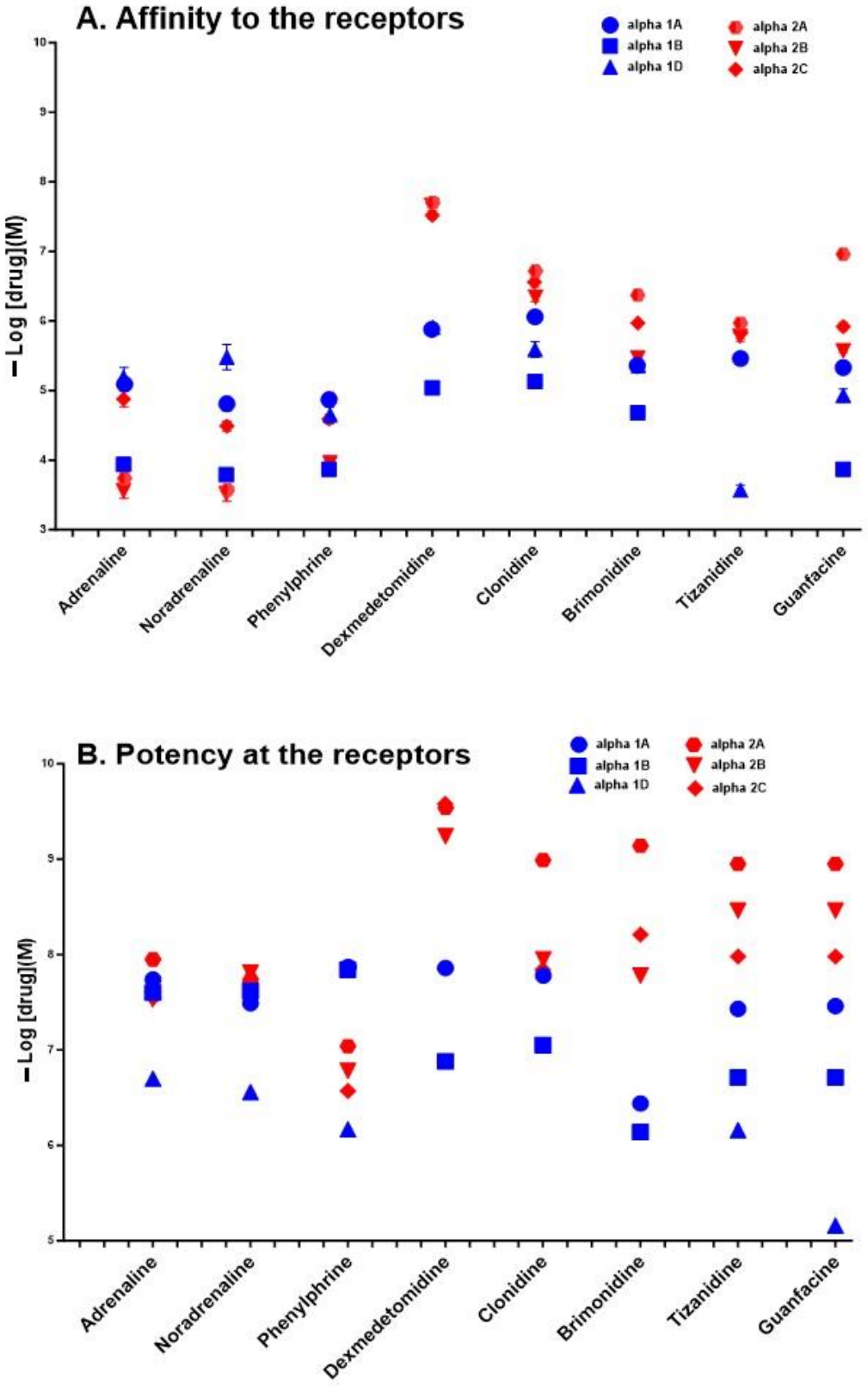


Figure 3.3: The affinities and potencies of α_2-adrenoceptor agonists and other vasoconstrictors (adrenaline, NA, and phenylephrine) at α-adrenoceptor subfamilies. Adapted from (Proudman and Baker, 2021, Proudman *et al*., 2022).

3.1.3. What is the effect of LPS on vascular smooth muscle contraction?

Endotoxin or LPS considerably present in patients with standard clinical criteria of sepsis with Gram-negative bacteria cultures (Singer *et al.,* 2016), and it was considered one of the early indicators of sepsis severity (Bottiroli *et al.,* 2017). Vascular dysfunction characterised by vascular hypo-responsiveness after exposure to LPS was investigated over the past decades. The overnight exposure of isolated porcine coronary arteries to 1 and 100 µg/ml reduced the vascular contractions of KCl and U46619 and impaired the endothelium relaxation mediated by substance P and sodium nitroprusside (Wei *et al*., 2008, Al-Shalmani *et al*., 2011). Several in vitro studies investigated the effect of LPS on isolated blood vessels using a variety of sources of LPS at different concentrations Table 3.1. The vascular contraction induced by catecholamine in the thoracic aorta was significantly reduced after three hours of exposure to endotoxin (Beasley *et al.,* 1990), which also enhances the release of IL-1 and TNF that are involved in *de novo* protein synthesis, and possibly the products of cyclooxygenase activity (McKenna, 1990).

Table 3.1: The effect of LPS on vascular smooth muscle responses in different isolated blood vessels, SP: Substance P, SNP: Sodium nitroprusside, ↑: Increase, ↓: Decrease.

Blood Vessels	Type and concentration of LPS	Incubation time (37 ºC) (h)	Outcomes	Reference
Rat aorta	*E. coli* 055:B5 1, 10, 100 ng/ml	16	↓ E_{max} and ↑ EC_{50} of NA	(McKenna, 1990)
Rat aorta	*E. coli* 055:B5 10 µg/ml	1, 1.5, 3	↓ E_{max} and ↑EC_{50} of phenylephrine	(Beasley *et al.,* 1990)
Rat aorta	*E. coli* 055:B5 30 ng/ml	5	↓ Contraction to NA in endothelium-intact rings.	(Nakaki *et al.,* 1992)
		24	↓ Contraction to NA in endothelium-denuded rings	
Rat aorta	*E. coli* 055:B5 10 µg/ml	4	↓ Contraction ↑ EC_{50} of NA	(Schott *et al.,* 1993)
Rat aorta	1 ng/ml	16	↓ Contraction to phenylephrine	(Sigurdardottir *et al.,* 2010)
Mice aorta	10 µg/ml	24		
Rat mesenteric artery	*Salmonella Typhosa* 1, 10,100 µg/ml	20	↓ E_{max} and ↑ EC_{50} of phenylephrine	(O'Brien *et al.,* 2001)
Rat mesenteric artery	*Salmonella Typhosa* 1 µg/ml	6, 20	↓ E_{max} and ↑ EC_{50} of phenylephrine	(O'Brien *et al.,* 2005)
Rat mesenteric artery	*E. coli* 055:B5 10 µg/ml	5	↓ EFS contraction ↑ Superoxide anions	(Sastre *et al.,* 2012)
Rat mesenteric artery	*E coli* 0127:B8 1 µg/ml	20	↓ Contraction to phenylephrine	(Zhang *et al.,* 2014)
Rat coronary artery	250 ng/ml	24	↑ Sensitivity of contractile response ET_B receptors	(Ghorbani *et al.,* 2010)
Porcine coronary artery	1 µg/ml	16 – 18	↓ Contraction to KCl and U46619, ↓ Relaxation to SP	(Al-Shalmani *et al.,* 2011)
Porcine coronary artery	*E coli* O111: B4 100 µg /ml	16 – 18	↓ E_{max} and ↑ EC_{50} of U46619 ↓ Relaxation to SNP	(Wei *et al.,* 2008)
Human omental artery	50 EU/ml	48	LPS: No significant change in contraction to NA	(Bjorck *et al.,* 2018)

Several animal models showed endotoxin-induced vascular hypo-reactivity due to the activation of iNOS and eNOS isoforms of NOS and thereby production of NO. A study has found that the presence of L-arginine during the incubation of blood vessels with LPS can enhance vascular hypo-responsiveness, which was restored by using L-NAME (Schott *et al.,* 1993). The long-term incubation (20 hours, 37 ºC) of isolated intrapulmonary arteries of piglets with group B streptococcus and E. coli LPS causes induction of NOS activity, resulting in a decrease of the vascular responsiveness to NA (Villamor *et al.,* 1995). A model of small vasculatures from rat superior mesenteric artery demonstrated vascular hyporeactivity largely mediated through NO-cyclic GMP pathway (O'Brien *et al.,* 2001). In an *ex vivo* study on mesenteric arterioles of mice, LPS treatment significantly reduced the NA induced contractions suggesting that iNOS expression is critical for the development of NA hypo-responsiveness (Boyle *et al.,* 2000). Nevertheless, L-NAME could not improve the survival rate of animal models of sepsis, but it produced an increase in blood pressure and a decrease in cardiac output. LPS might reduce the sensitivity to vasopressor agents such as NA, vasopressin, angiotensin II, and serotonin resulting in further arterial hypotension. There are a number of the mechanisms of LPS that mediate vascular hypo-responsiveness in sepsis models as shown in Figure 3.4 (Levy *et al.,* 2010).

As a sequence to the results of the previous studies on porcine isolated coronary arteries conducted under the same laboratory conditions (Wei *et al.,* 2008, Al-Shalmani *et al.,* 2011), we decided to use LPS to induce vascular hyporesponsiveness resembles the sepsis conditions in porcine isolated splenic artery. Also, SNP was used to investigate the influence of NO mediated vasorelaxation on the contractile

responses of α_2-adrenoceptor agonists in the porcine splenic artery. SNP induced vasodilatation by increasing cyclic GMP through the release of NO, causing the relaxation of the vascular smooth muscle in both peripheral arteries and veins (Ranadive *et al.,* 2017).

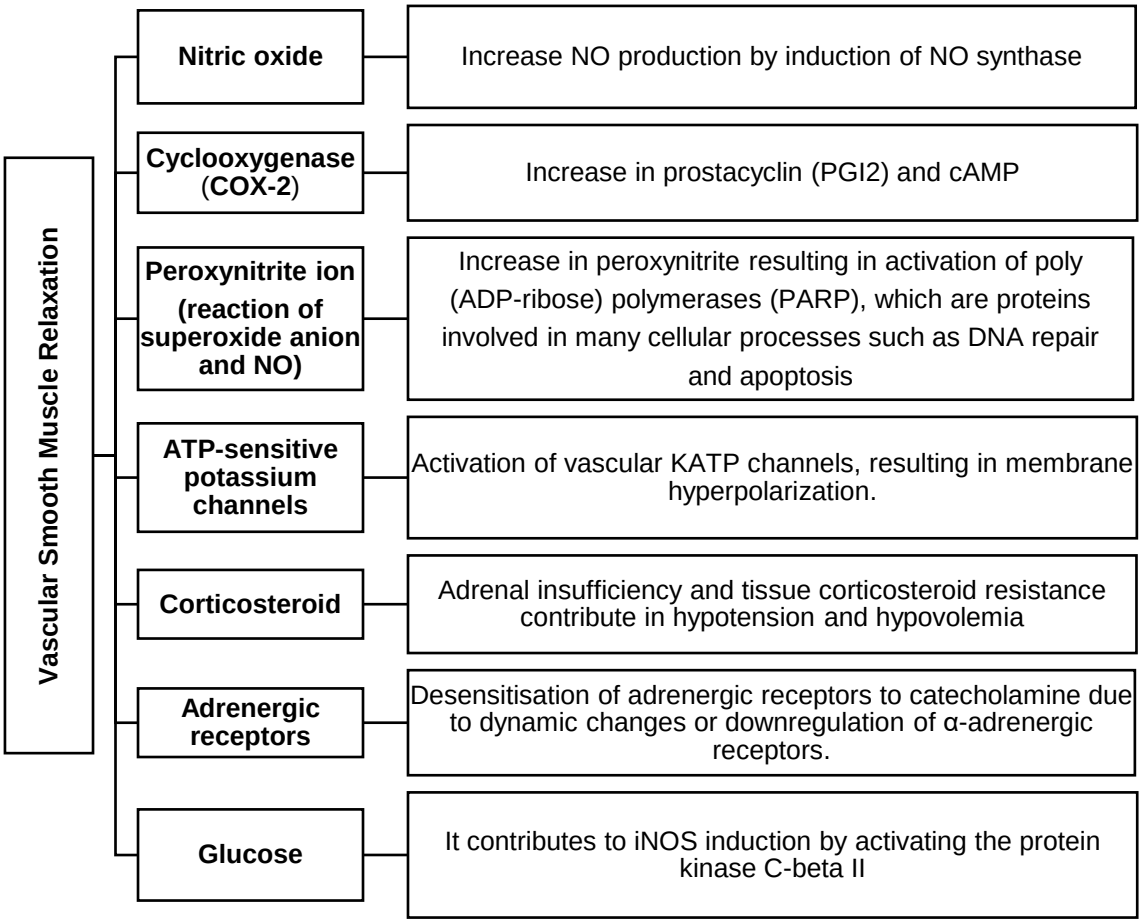


Figure 3.4: The potential mechanisms of vascular smooth muscle relaxation in sepsis (Levy *et al.,* 2010).

3.1.4. Aims of the study:

1. To describe the sepsis model by using LPS in porcine coronary and splenic arteries.
2. To demonstrate the vasoconstrictor actions of various α_2-adrenoceptor agonists in central and peripheral blood vessels.
3. To demonstrate the effect of SNP on vasoconstriction actions of α_2-adrenoceptor agonists.
4. To investigate NA sparing effects of α_2-adrenoceptor agonists in porcine isolated splenic arteries.

3.2. Methods

3.2.1. Tissue collection and dissection

The isolated porcine blood vessels such as splenic arteries and veins, renal arteries, and tail arteries were used in this study (Figure 3.5), which were collected from the local abattoir and placed immediately in ice-cold Krebs-Henseleit buffer to be maintained at 4ºC. The fresh Krebs-Henseleit solution was prepared daily. The blood vessels of about 6-8 cm were dissected to be maintained in Krebs-Henseleit solution overnight at 4ºC. On the following day, the fine dissection was carried out after cleaning the blood vessels from the excess fat and connective tissues and divided into ring segments of 5 mm length and 2 mm internal diameter at rest. The ring segments were mounted between two holders of stainless-steel wires (0.2 - 0.4 mm diameter) in 20 ml organ baths (Figure 3.6).

In the organ bath, the upper wire was attached to the force transducer via cotton thread to record the isometric tension. The transducer was connected to an AD Instruments Quad Bridge pre-amplifier unit linked to a Powerlab via a four-channel unit which in turn connected to a Viglen computer running LabChart 7.0 software to display the traces. The lower wire was fixed to a glass rod (Figure 3.7). The organ bath contained Krebs-Henseleit solution maintained at 37 ºC by a thermocirculator pump and continually gassed for 15 minutes with 95% Oxygen and 5% CO_2 Carbon Dioxide to maintain pH at 7.4. Table 3.2 lists the salts, chemicals and drugs used in organ bath experiments. Distilled Water (DW) was used as a solvent for most of the chemicals and drugs in the contractile study except dexmedetomidine, brimonidine tartrate,

prazosin hydrochloride and U46619 which were dissolved in dimethylsulphoxide (DMSO), and NA was dissolved in Ethylene diamine tetra acetic acid (EDTA).

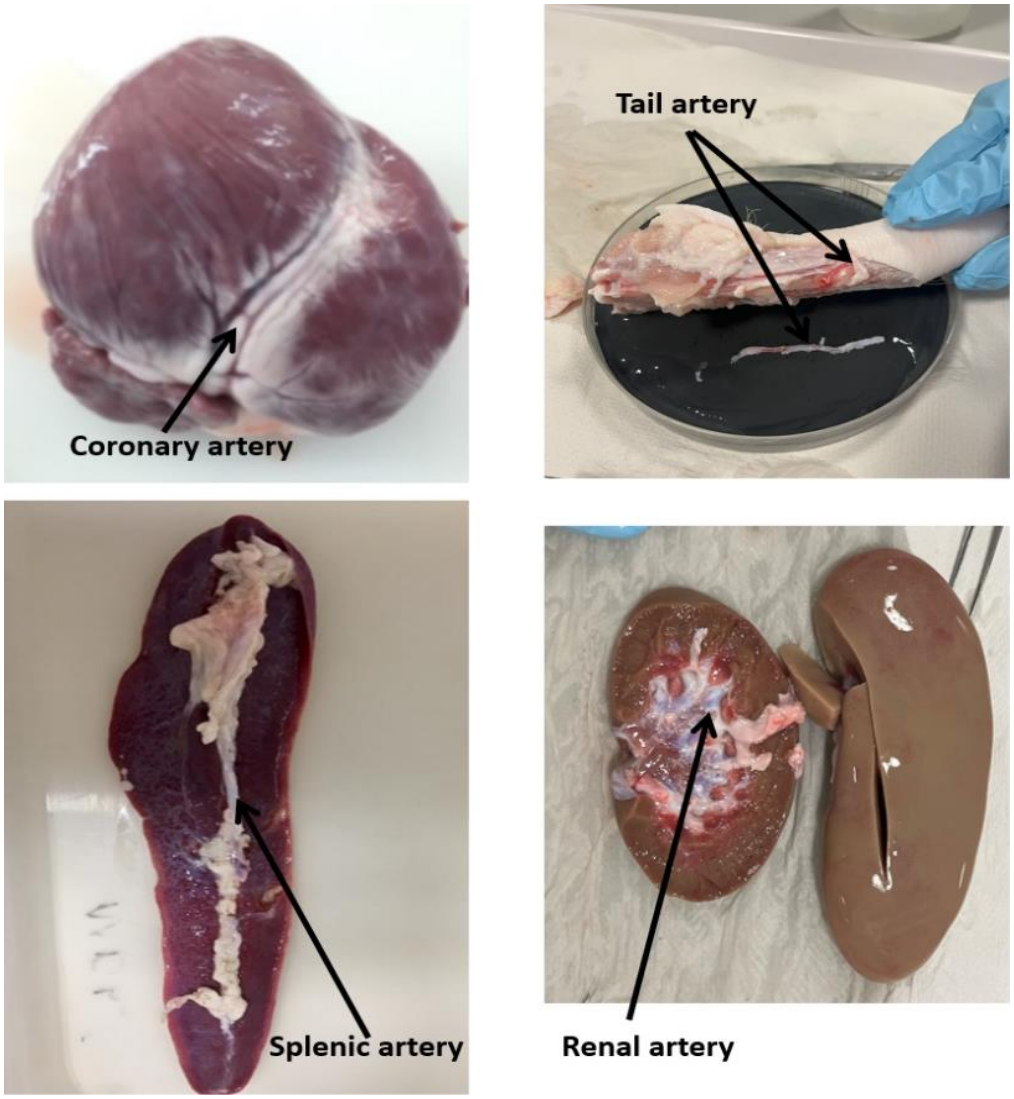


Figure 3.5: Diagram of the isolated porcine organs shows the different blood vessels used in the contractility study: the coronary artery, splenic artery, renal artery, and tail artery.

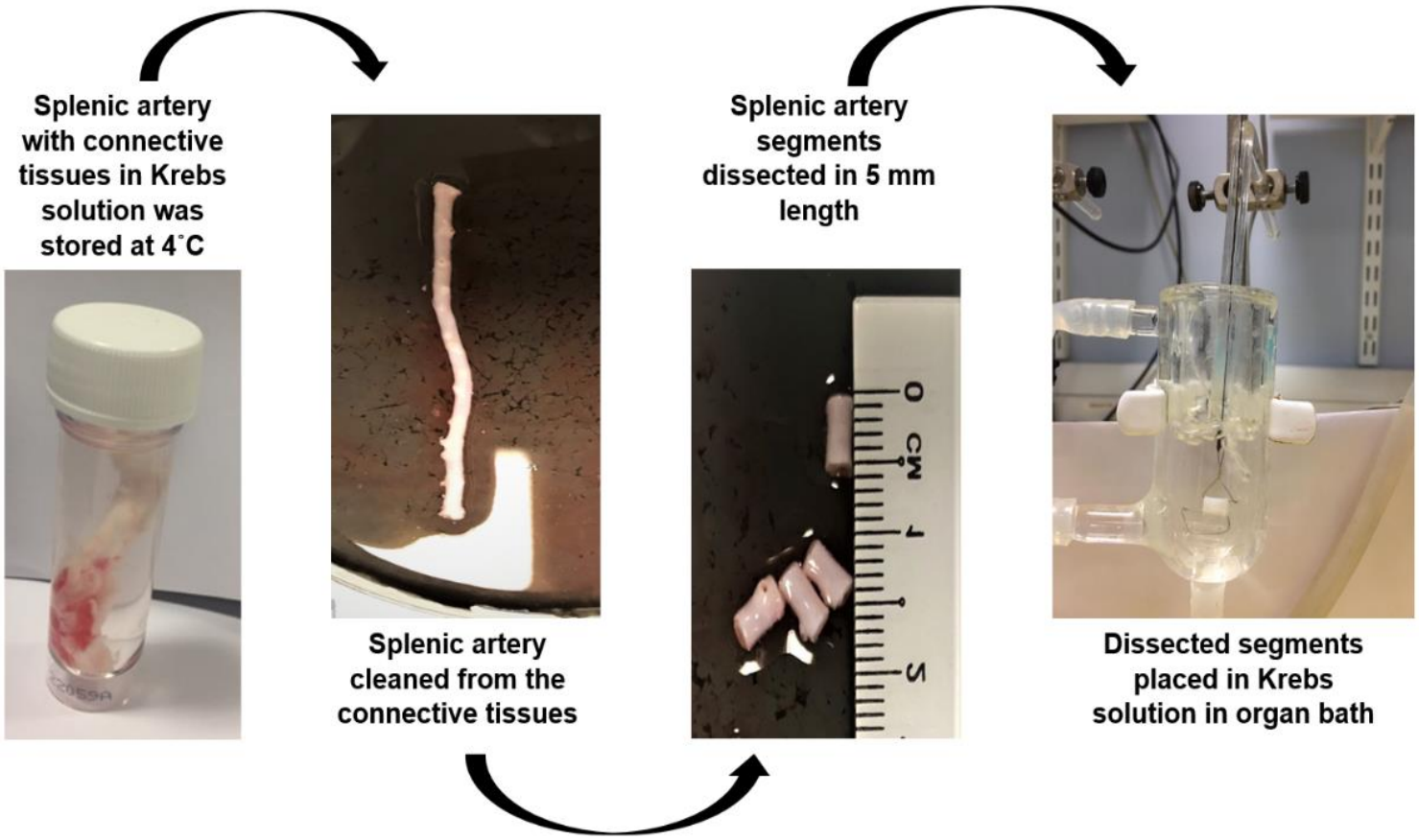


Figure 3.6: Diagram shows experimental steps of dissecting and mounting the porcine splenic artery in an organ bath.

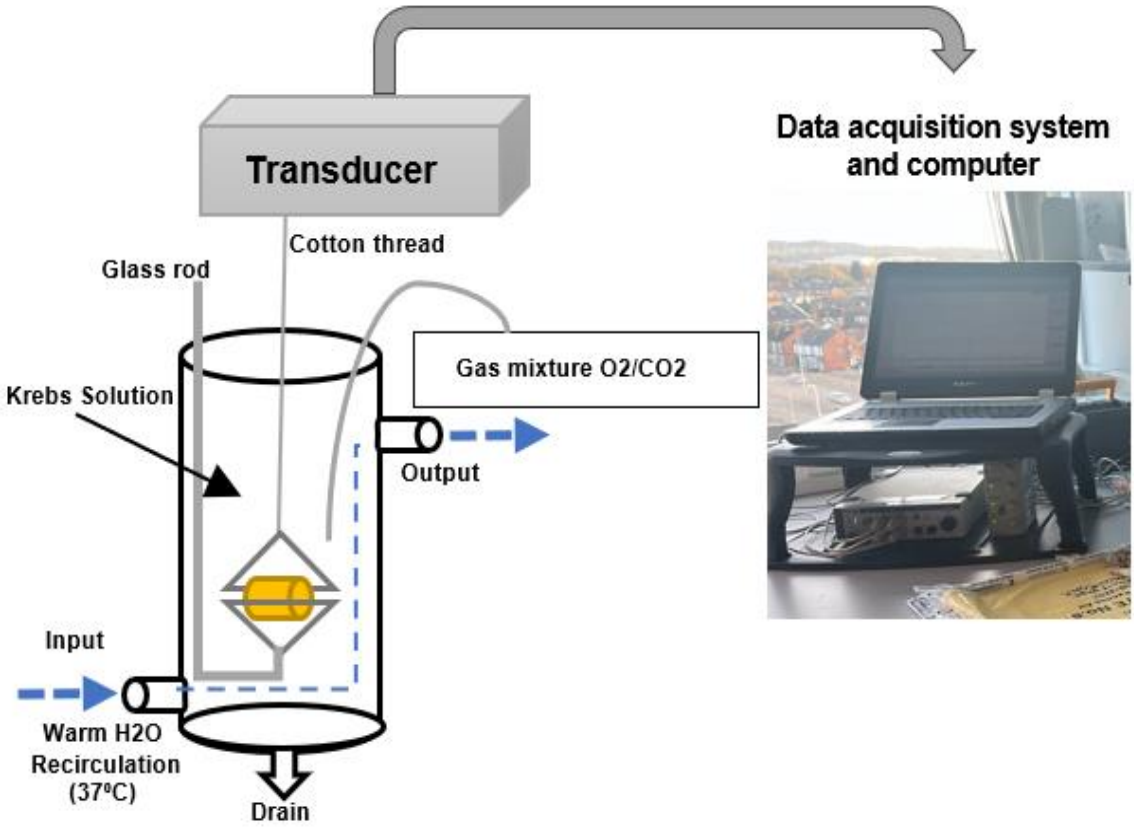


Figure 3.7: Schematic diagram shows the setup of isolated porcine blood vessels in an organ bath connected to a data acquisition system and computer.

Table 3.2: List of the chemicals and drugs used in vascular contractility studies.

	Chemicals and drugs	Solvents	Source/Manufacture
Krebs-Henseleit Buffer	Sodium chloride (120 mM)	DW	Fisher Scientific, Loughborough, UK
	Potassium chloride (4.8 mM)		
	Sodium hydrogen carbonate (25 mM)		
	Glucose anhydrous (11 mM)		
	Calcium chloride (1.25 nM)		
	Potassium dihydrogen orthophosphate (1.2 mM)		
	Magnesium sulfate (1.1 mM)		
	NA bitartrate	EDTA	Sigma Aldrich, UK
	Phenylephrine hydrochloride	DW	
	L-NAME (N^G-nitro-L-arginine methyl ester hydrochloride)		
	Clonidine hydrochloride		
	LPS (lipopolysaccharide) Escherichia coli OIII: B4		
	Sodium nitroprusside (SNP)		
	Substance P		
	U46619 (9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F2α methyl acetate)	Ethanol	Tocris Bio-Techne Abingdon, UK

Cocaine hydrochloride	DW	University of Nottingham, Hospital Pharmacy, UK
Dexmedetomidine	DMSO	
Brimonidine tartrate	DW	TEVA UK limited, UK
Tizanidine		
Guanfacine hydrochloride	DW	Sigma Aldrich, Germany
Lofexidine	DW	Macfarlan Smith Limited, Edinburgh, UK
Mivazerol	DMSO	Evotec Toulouse, France
Ethylene diamine tetra acetic acid (EDTA)	DW	British Drug Houses BDH, Laboratory Chemicals, Poole, UK
DMSO (dimethylsulphoxide)	Used as solvent	Sigma Aldrich, UK
Benzylpenicillin	DW	
Streptomycin sulphate		
Fetal Bovine Serum (FBS) as a liquid		
Endothelial Cell Medium (ECM)		ScienCell Research Laboratories California, USA (Cat. No. 1001)

3.2.2. Experimental protocols

3.2.2.1. Setup of dissected segments in organ bath:

The suspended segment was maintained in an organ bath for approximately 45 minutes before the application of tension. At the end of the equilibration period, an initial tension was slowly applied over 2-minute period. The tension applied for splenic and coronary arteries, renal arteries and splenic veins was 10 g, 8 g and 5 g (wt), respectively. The segments were then allowed to relax for a further 45 - 50 minutes to reach the resting tension. The viability of the tissues was assessed by exposing the segments to 60 mM KCl. The segments were allowed to plateau in 10 - 15 minutes and then washed out twice with the fresh Krebs-Henseleit solution and allowed to reach the baseline in nearly 15 - 20 minutes. The segments were then re-tensioned to nearly half of the initial tension and allowed for a further 15 minutes. The segments were then exposed to one or two more additions of 60 mM KCl to ensure that the reproducible responses were obtained.

3.2.2.2. The contractility study of vasoactive agents in porcine isolated blood vessels:

The preliminary experiments were conducted to demonstrate the consistency and reproducibility of the vascular contraction responses across 8 channels. The segments of the porcine splenic artery of the same animal were exposed to cumulative concentrations of NA (10 nM - 3 µM). The responses were then presented in weight (grams) or as a percentage to 60 mM KCl.

In a second set of experiments, the segments from different porcine isolated blood vessels were exposed to cumulative concentrations of KCl (6 mM - 60 mM). The preparations were then washed and allowed to relax to the baseline, and then exposed to cumulative concentrations of vasoconstrictors; NA (10 nM - 3 µM) or U46619 (1 nM - 200 nM). Cumulative concentrations of SP (1 nM - 100 nM) were added to measure the integrity of vascular endothelium.

3.2.2.3. The effect of LPS on the contractile responses of porcine isolated blood vessels:

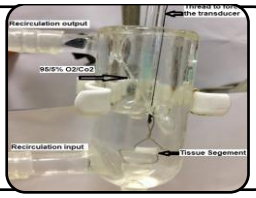
Two different experimental protocols were conducted to build LPS mediated sepsis model. In the first set of the experiments, the segments from splenic and coronary arteries were set up in an organ bath and contracted with 60 mM KCl twice, washed and allowed to relax to the baseline. The segments were then incubated without or with 100 µg/ml LPS for 1 or 3 hours in an organ bath. After adding the cumulative concentrations of KCl (6 mM - 60 mM), the segments were washed and allowed to relax to the baseline. Subsequently, the cumulative concentrations of U46619 (1 nM - 200 nM) were added, followed by 1 nM SP.

In the second set of experiments, the segments were cleaned and dissected into 5 mm long and placed separately in sterile vials containing 2 ml of previously gassed solutions such as Krebs-Henseleit solution, antibiotics solution prepared by mixing Krebs-Henseleit solution with 60 µg/ml benzylpenicillin and 20 µg/ml streptomycin sulphate, 2% ficoll, and 10% FBS, or in endothelial cell medium (ECM) containing 5% FBS, 1% Endothelial Cell Growth Supplement (ECGS) and 1% penicillin/streptomycin solution. The segments in the sealed vials were then incubated overnight without or

with 1 µg/ml or 100 µg/ml LPS at 37ºC. After incubation, the segments were removed from the solution and set up in an organ bath filled with fresh Krebs-Henseleit solution. Figure 3.8 describes the protocols used in this study to incubate the tissues for sepsis models. The preparations were contracted with two additions of 60 mM KCl followed by cumulative concentrations of KCl (6 mM - 60 mM). Subsequently, the cumulative concentrations of U46619 (1 nM - 200 nM) were added followed by 1 nM SP.

Incubation in organ bath

- PCA and PSA were incubated in Krebs-Henseleit solution containing LPS for 1 or 3 hours in organ bath.

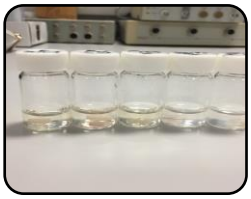


Incubation in vials overnight

- PCA and PSA were incubated in vials containing either Krebs-Henseleit or Antibiotics solutions.

Incubation in vials containing LPS overnight

- PCA and PSA were incubated in Antibiotics solutions containing LPS overnight.



Incubation in a well-plate containing LPS overnight

- PSA was incubated in culture media (ECM) containing LPS overnight.

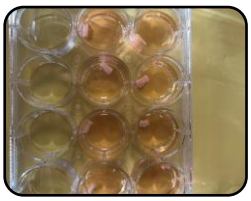


Figure 3.8: The experimental protocols used to incubate the tissue segments at 37C for sepsis models. PCA: porcine coronary artery, PSA: porcine splenic artery.

3.2.2.4. The effect of the incubation period on contractile responses of KCl and U46619 in porcine splenic artery:

In the first set of the experiments, the segments of the porcine splenic artery were cleaned from the connective tissues and dissected into 5 mm long and placed separately in sterile vials containing 2 ml of antibiotic solution. The segments in the sealed vials were then incubated for 24, 48, 72, or 168 hours at 37ºC. After incubation, the preparations were contracted with 60 mM KCl twice followed by the cumulative concentrations of KCl (6 mM - 60 mM). The segments were washed twice and allowed to reach the baseline and then contracted with the cumulative concentrations of U46619 (1 nM - 200 nM).

3.2.2.5. The effect of SNP on the contractile responses of porcine isolated splenic arteries:

These experiments were designed to demonstrate the effect of SNP on phenylephrine and dexmedetomidine in porcine splenic arteries. In the first set of experiments, the segments were pre-contracted with U46619 to achieve 60% of the maximum response of 60 mM KCl followed by cumulative concentrations of SNP (1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 µM). After approximately 20 minutes, the segments were contracted with cumulative concentrations of phenylephrine (10 nM, 30 nM, 100 nM, 300 nM, 1 µM, 3 µM, 10 µM, and 30 µM). In the second set of experiments, SNP (1 µM or 10 µM) were added, followed by cumulative concentrations of phenylephrine (1 µM - 100 µM), dexmedetomidine (1 nM - 1 µM), or mivazerol (1 nM - 1 µM).

3.2.2.6. The contractility study of α_2-adrenoceptor agonists:

To demonstrate the vasoconstriction action of α_2-adrenoceptor agonists in different porcine isolated blood vessels, the segments were exposed to cumulative concentrations (1 nM to 3 µM) of these agonists. The time interval between the concentrations was usually 5 minutes if no contractile response was observed after adding the agonist, or longer to allow the contraction to reach the plateau.

3.2.2.7. The effect of prolonged incubation of porcine splenic artery with α_2-adrenoceptors:

To study the prolonged effect of α_2-adrenoceptor agonists on isolated splenic arteries, different concentrations of dexmedetomidine (1 nM, 3 nM, and 10 nM) were used. After finishing the setup of the isolated splenic arteries, a single dose of dexmedetomidine was added, and the tissues were left in the organ baths for approximately 14 - 16 hours. The organ baths were covered with foil to avoid the reduction in the amount of the Krebs-Henseleit solution in the organ bath due to evaporation.

3.2.2.8. The effect of NA and phenylephrine in pre-contracted porcine splenic artery (sparing effects):

In these experiments, the segments of porcine isolated splenic arteries were pre-contracted with vasoactive agents before the addition of NA. The segments were exposed to one of the following agonists: L-NAME (100 µM), U46619 (10 - 45 nM), or α_2-adrenoceptor agonists such as with or without U46619. These agents induced an increase in the vascular tone by approximately 9 -10% of the maximum KCl response.

Subsequently, the cumulative concentrations of NA or phenylephrine (1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM) were added.

3.2.3. Data Analysis and Statistics

The contraction responses produced by KCl and other drugs were calculated as tension in weight (grams) from the traces using LabChart Reader version 8.1.25. Figure 3.9 illustrates the calculation of the response magnitude. The spontaneous spikes that occurred in some species were ignored. The data were then calculated using the Excel Microsoft Office and expressed either as a percentage of the maximum contraction to 60 mM KCl, or as g wt in the experiments that included overnight incubation at 37ºC. The graphs were then plotted using Prism version 10.0.3 (GraphPad, USA), where the equation non-linear regression (curve fit) followed by the equation (**LogAgonist vs. Response (three parameters)**) was selected to plot the curves and measure the maximum response (**Emax**), and the potency which was determined as the negative logarithm of the concentration of agonist that gives 50% of the response (-Log $\mathbf{EC_{50}}$).

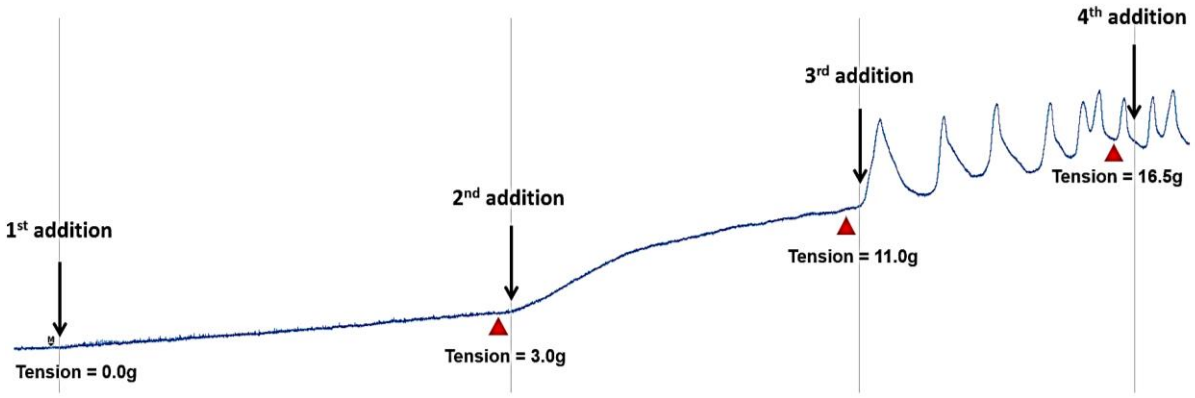


Figure 3.9: Diagram representing the method of calculation of the peak responses induced by cumulative additions of agonists in the porcine splenic artery. The spikes of contractions were excluded from the measurement.

In the experiments that focused on a small range of NA concentrations (1 nM - 200 nM), the responses of cumulative addition were calculated after the peak stabilised (the rapid contraction and spikes were excluded) (Figure 3.10). After plotting the graphs, the potency of NA in these experiments was measured based on the concentration that gives 30% of the maximum response (-Log $EC_{30\ KCl}$). All the data are presented as the standard error mean (±SEM). Student's paired t-test (two-tailed) was used to compare the data between two conditions, and one-way ANOVA followed by Dunnett's test was used to compare two or more groups. The *p-value < 0.05 was considered statistically significant.

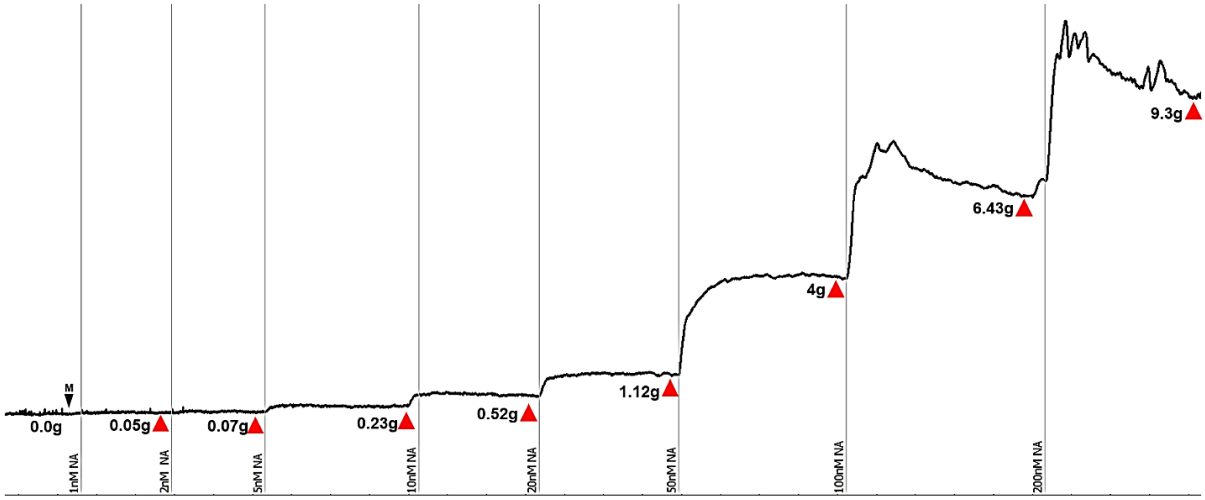


Figure 3.10: Diagram representing the original recordings of a concentration-response curve for isometric contractions of NA induced by cumulative additions of the small range of concentrations (1nM – 200nM) in porcine splenic artery. The tension (g wt) of the peak was measured when the peak reached the plateau excluding the rapid contraction.

3.3. Results

3.3.1. The contraction responses of vasoactive agents in porcine isolated blood vessels:

The contractile responses of NA in the segments of the same porcine isolated splenic arteries were plotted to determine the reproducibility across eight channels. The data were calculated and presented as weight in grams or percentage of the maximum contraction of 60 mM KCl. There was variation in the maximum responses expressed as the weight, which reduced when the data were expressed as the percentage of 60 mM KCl (Appendix 1). The findings of the experiments conducted to demonstrate the variability in the sensitivity of different porcine isolated blood vessels to vasoactive agents showed a considerable variation in the maximum responses achieved by cumulative concentrations of KCl, U46619 and NA in different porcine isolated tissues. The Emax of KCl was higher in the coronary artery followed by the splenic artery, renal artery, and splenic vein.

The Emax of KCl in the splenic artery was nearly 50% higher than the Emax in the renal artery and splenic vein. The Emax of NA was higher in the splenic artery followed by the Emax in the renal artery and splenic vein, meanwhile, the coronary artery was less sensitive to the NA and caused relaxation rather than contraction. The U46619 induced contractions in coronary and splenic arteries were nearly equal and about 50% bigger than the contractions in the renal artery and splenic vein (Table 3.3). Figure 3.11 presented the data as the percentage of 60 mM KCl and showed that the renal artery was more sensitive to all the vasoconstrictors than other blood vessels. The sensitivity of all the blood vessels to NA was bigger than 100% of KCl responses in all blood vessels except in the coronary artery.

Table 3.3: Comparison of the maximum responses (Emax) and -Log EC_{50} (pD2) of vasoconstrictors in the porcine isolated blood vessels. PCA: porcine coronary artery, PSA: porcine splenic artery, PSV: porcine splenic vein, PRA: porcine renal artery, NA: noradrenaline, N: number of animals.

		PCA	**PSA**	**PSV**	**PRA**
KCl	N	**11**	**10**	**7**	**16**
	Emax (g wt)	14.28 ± 1.09	9.67 ± 0.74	4.55 ± 1.48	5.83 ± 1.23
	pD_2	1.89 ± 0.4	1.4 ± 0.08		0.57 ± 0.28
U46619	N	20	10	6	8
	Emax (g wt)	14.2 ± 0.98	10.28 ± 1.03	4.54 ± 1.73	5.22 ± 0.91
	pD_2	7.9 ± 0.07	8.1 ± 0.123	8.15 ± 0.06	8.2 ± 0.06
NA	N	8	11	10	10
	Emax (g wt)	- 0.69 ± 0.11	15.61 ± 1.25	5.8 ± 0.79	10.2 ± 1.28
	pD_2		5.7 ± 0.08	5.3 ± 0.15	5.2 ± 0.09

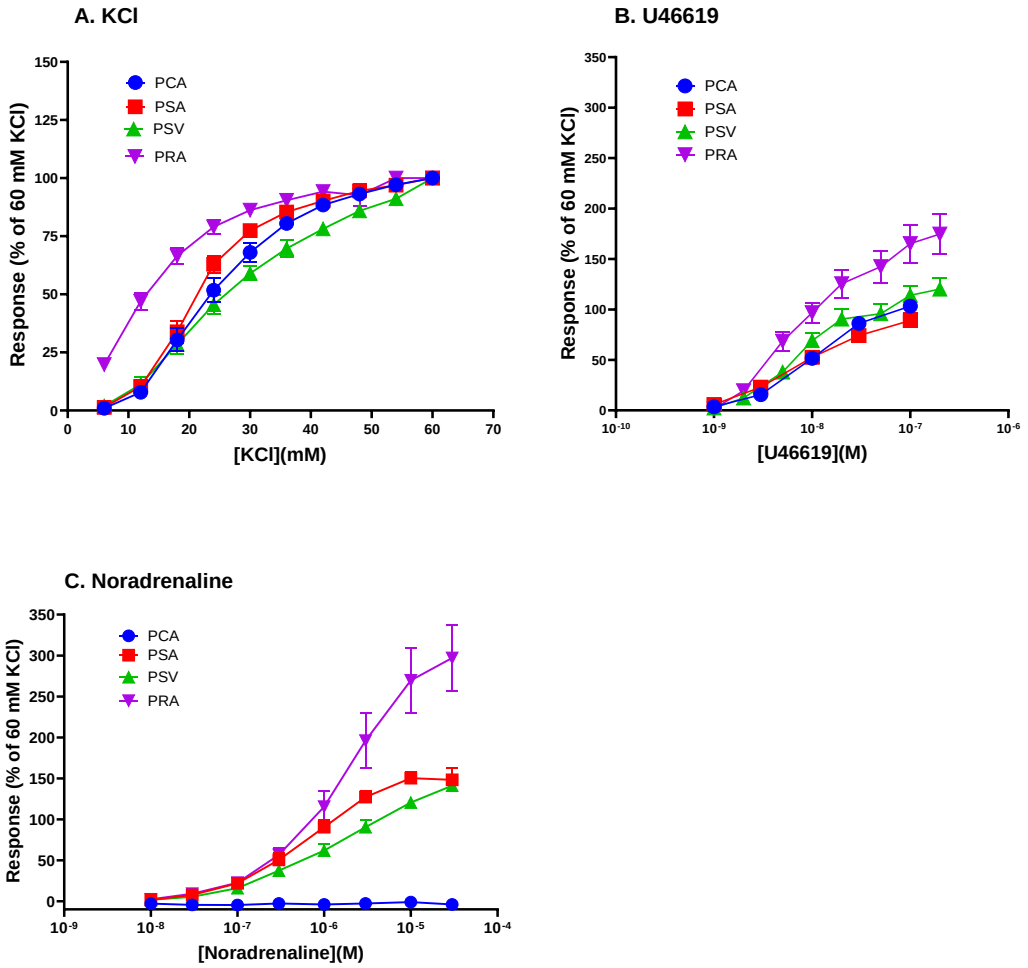


Figure 3.11: Contractile responses of vasoconstrictors (**A**) KCl, (**B**) U46619, (**C**) NA in isolated porcine blood vessels. PCA: porcine coronary arteries, PSA: porcine splenic arteries, PSV: porcine splenic veins, PRA: porcine renal arteries. The contractions are expressed as % of 60 mM KCl and presented as the mean ± SEM, n= 8-10.

3.3.2. The effect of LPS on the contractile responses of vasoconstrictor agents in porcine isolated blood vessels:

The incubation of porcine isolated coronary arteries with 100 µg/ml LPS for 1 or 3 hours in organ bath did not significantly decrease the U46619 induced contractions in both porcine coronary and splenic arteries (Figure 3.12 and 3.13). The SP produced relaxation action of U46619 mediated contractions in both porcine splenic and coronary arteries. LPS significantly affected the SP relaxation responses in porcine splenic artery, where the maximum relaxation of SP in the absence and presence of LPS, were 34.3 ±4.9 and 26.2 ±4.9 % of 60 mM KCl, respectively (p-value = 0.036).

The contraction responses induced by cumulative concentrations of KCl and U46619 were studied in porcine isolated coronary arteries incubated overnight at 37 ºC in Krebs-Henseleit solution alone, or antibiotics solution in the absence and presence of two different concentrations of LPS (Figure 3.14 and 3.15). There was significant reduction in the KCl induced contractions of the segments stored in antibiotics solution compared to those stored in Krebs-Henseleit solution alone (p-value = 0.007) or in the presence of 100 µg/ml LPS (p-value = 0.0043), but no difference was observed in the presence of 1 µg/ml LPS. Also, the results showed some reduction in the contraction responses induced by U46619 but statistically were not significant in all the conditions. There was also no significant change in the relaxation responses of SP in the segments incubated in Krebs-Henseleit solution only or in the presence of LPS compared to the control (Table 3.4).

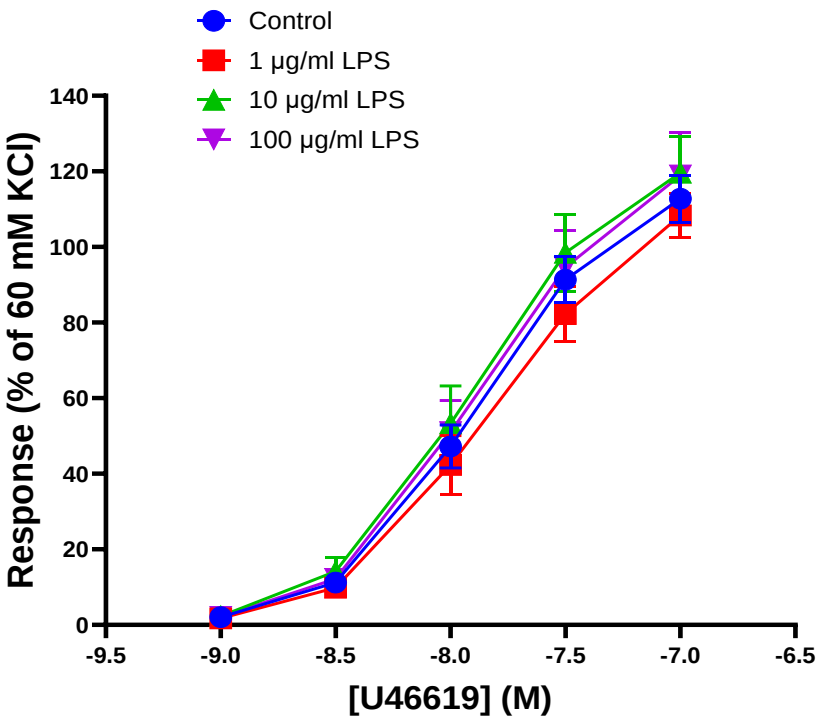


Figure 3.12: The cumulative response curves of U46619 in porcine coronary arteries in the presence of 1-100 µg/ml LPS for 1 hour incubation in organ bath containing Krebs-Henseleit solution. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n=10.

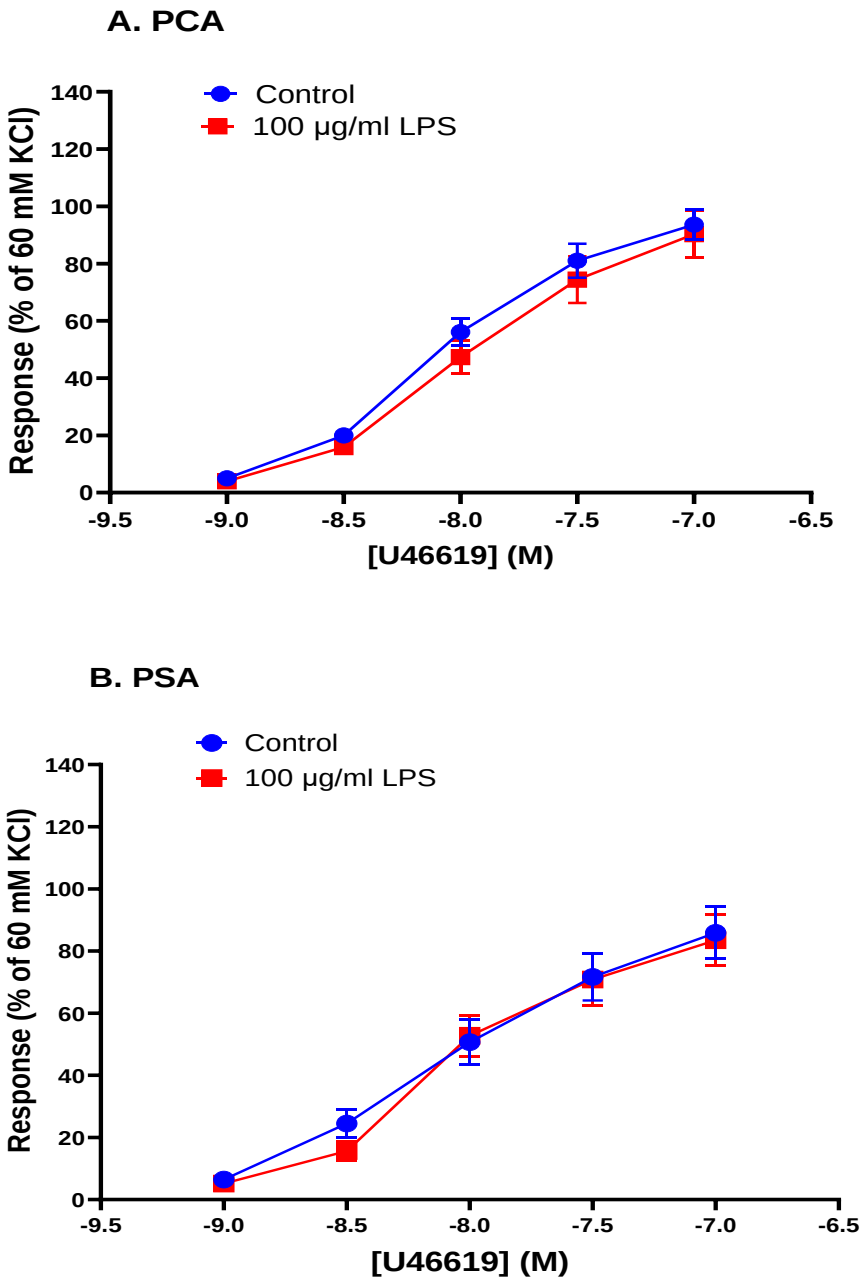


Figure 3.13: The cumulative response curves of U46619 in (**A**) porcine coronary arteries and (**B**) porcine splenic arteries in the presence of 100 µg/ml LPS for 3 hours incubation in organ bath containing Krebs-Henseleit solution. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n=10.

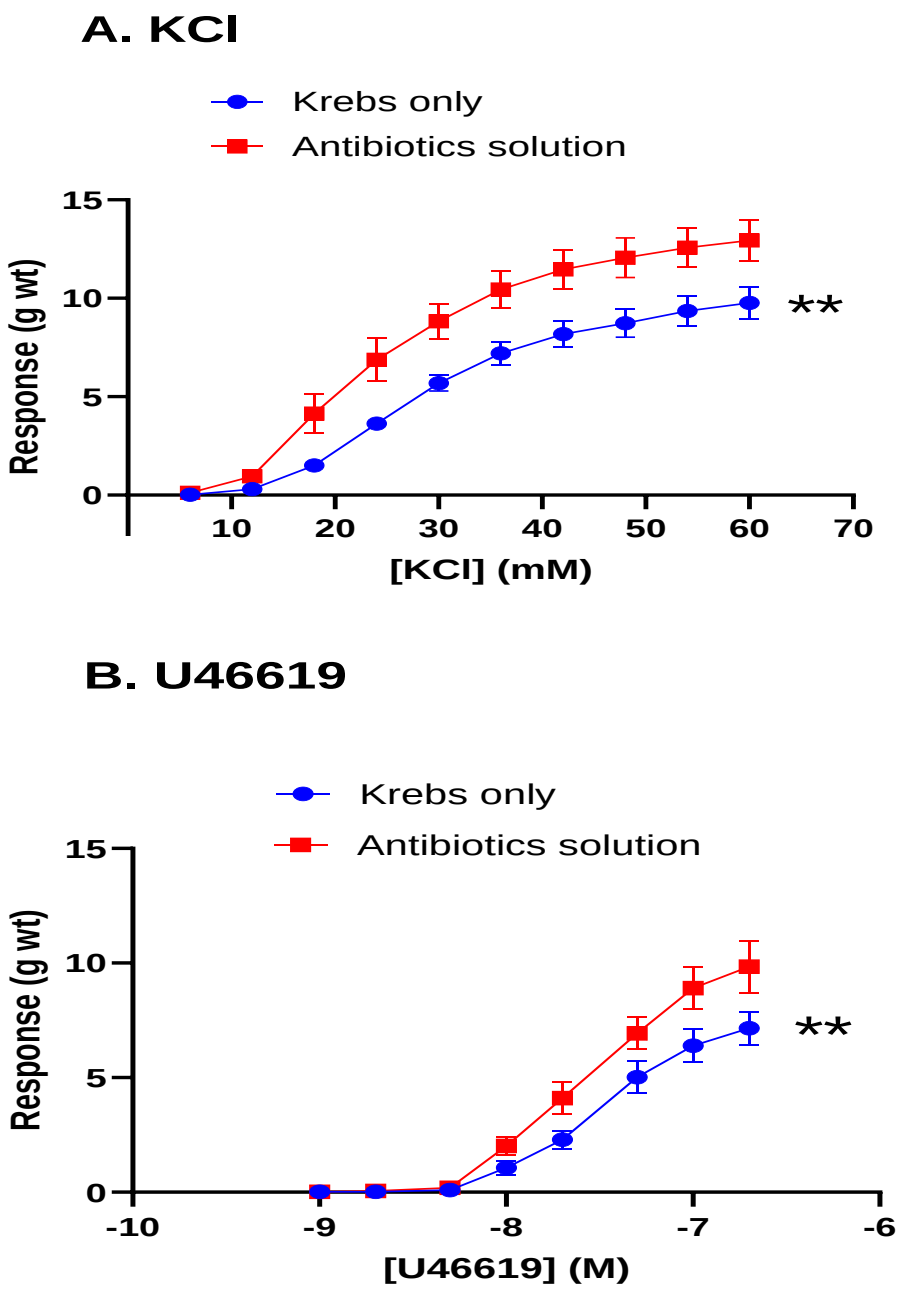


Figure 3.14: The cumulative concentrations-response curves of (**A**) KCl and (**B**) U46619 in porcine coronary artery incubated overnight in Krebs-Henseleit, and antibiotics solution. Data are expressed as weight in grams and presented as mean ± SEM, n=11. *p < 0.01 represented significant differences, paired t-test.

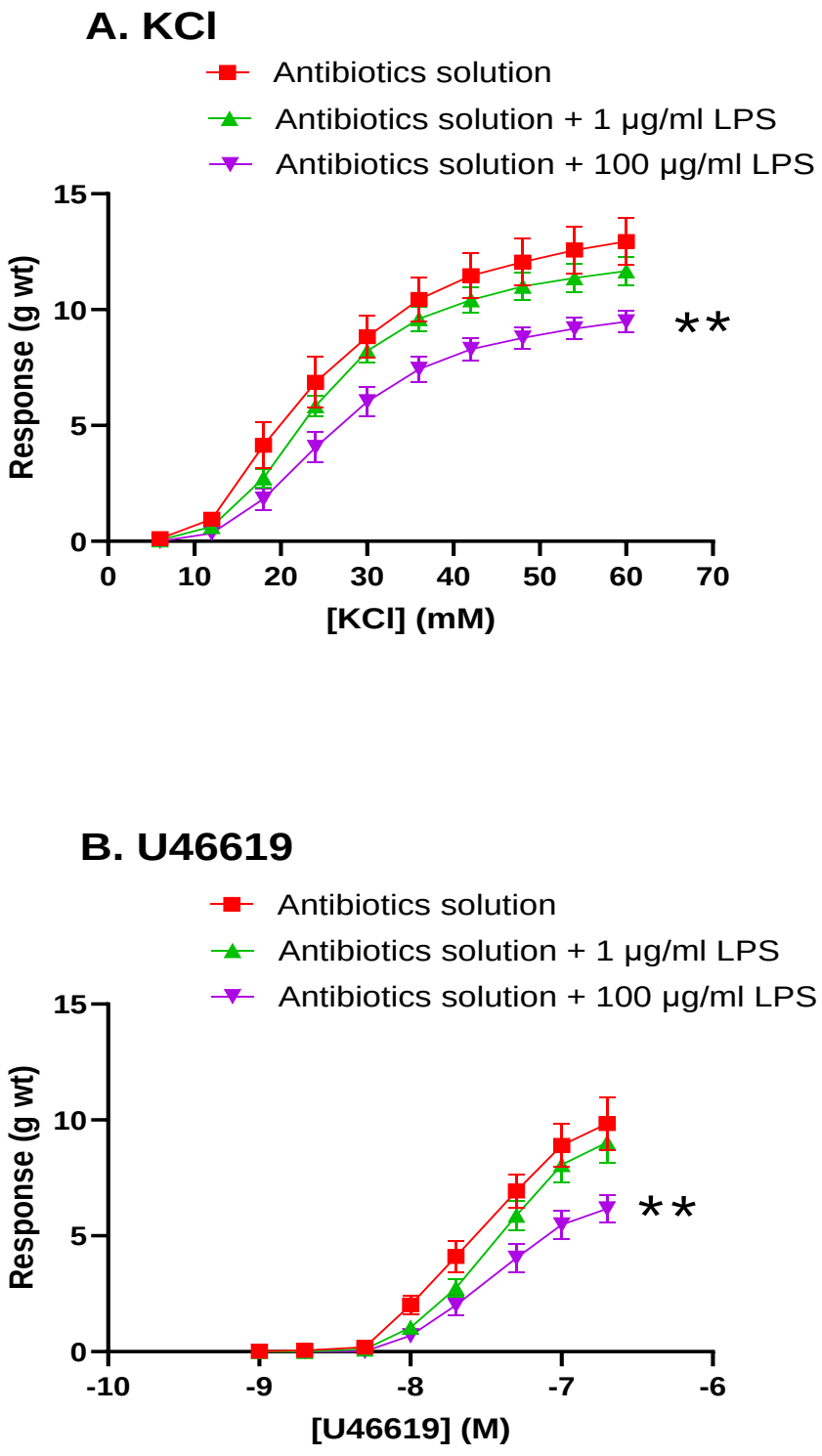


Figure 3.15: The cumulative concentrations-response curves of (**A**) KCl and (**B**) U46619 in porcine coronary artery incubated overnight in antibiotics solution in the presence of 1 µg/ml LPS, or 100 µg/ml LPS. Data are expressed as weight in grams and presented as mean ± SEM, n=11. *p < 0.01 represented significant differences, one-way ANOVA followed by Dunnett's test.

Table 3.4: Comparison of the vascular contractions (Emax) of KCl and U46619, and vascular relaxation of SP in isolated porcine coronary arteries in antibiotics solution (control), Krebs-Henseleit solution, antibiotics solution plus 1 µg/ml, or 100 µg/ml LPS. The data are presented as weight (grams) and expressed as mean ± SEM, n=11. The ******p-values < 0.01 were considered significant differences when compared to the antibiotics solution for each column, one-way ANOVA followed by Dunnett's test.

	Emax		
	KCl	**U46619**	**SP (% of max U46619 responses)**
Antibiotic Solution (Control)	11.6 ± 0.9	12.3 ± 1.5	- 22.8 ± 5.8
Krebs-Henseleit Solution	8.5 ± 0.7******	9.5 ± 1.1	- 33.0 ± 7.6
Antibiotics solution + 1 µg/ml LPS	10.3 ± 0.5	10.4 ± 1.7	- 29.1 ± 7.1
Antibiotics solution + 100 µg/ml LPS	8.4 ± 0.5******	8.7 ± 0.7	- 36.2 ± 8.3

Following the prior issue of the infection, a series of experiments were carried out to investigate how extending the incubation period at two different storage temperatures impacted the contraction responses of KCl and U46619 in isolated porcine splenic arteries. Figure 3.16 shows the number of responsive segments throughout different incubation periods at both 4 and 37ºC. The incubation of splenic artery segments for 24, 48 and 72 hours at 4 ºC did not affect the number of the segments and the magnitude of the contraction responses. The number of responsive segments decreased by over 65% after 168 hours. At 37 ºC, there was a consistent reduction in the number of the responded segments throughout the incubation period, with none of the segments incubated for 168 hours responding to KCl or U46619 (Figure 3.17 and Figure 3.18). In comparing the contraction responses in the responded segments at 4 ºC and 37 ºC, there was no difference in the Emax of KCl in those incubated for 24 hours, but a significant reduction in those incubated for 48 and 72 hours. The significant changes were observed in the contraction responses of U46619 in splenic arteries incubated at either temperature (Table 3.5).

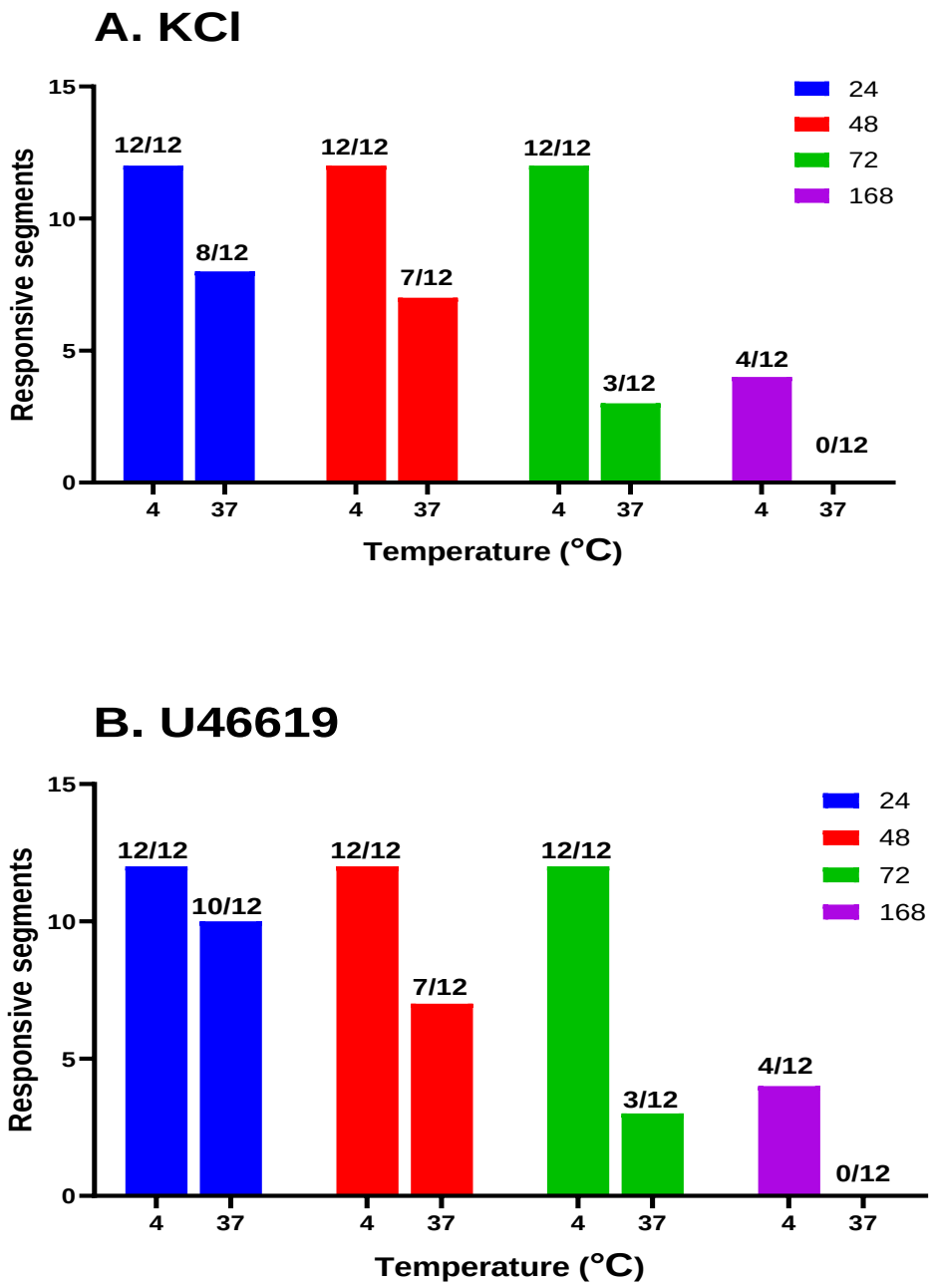


Figure 3.16: Comparison of the number of the segments of porcine splenic arteries that responded to (**A**) KCl, and (**B**) U46619 incubated for up to 168 hours in antibiotics solution at 4 ºC and 37 ºC for 24 h,48 h,72 h, and 168 h. A total number of the tissues examined was 12.

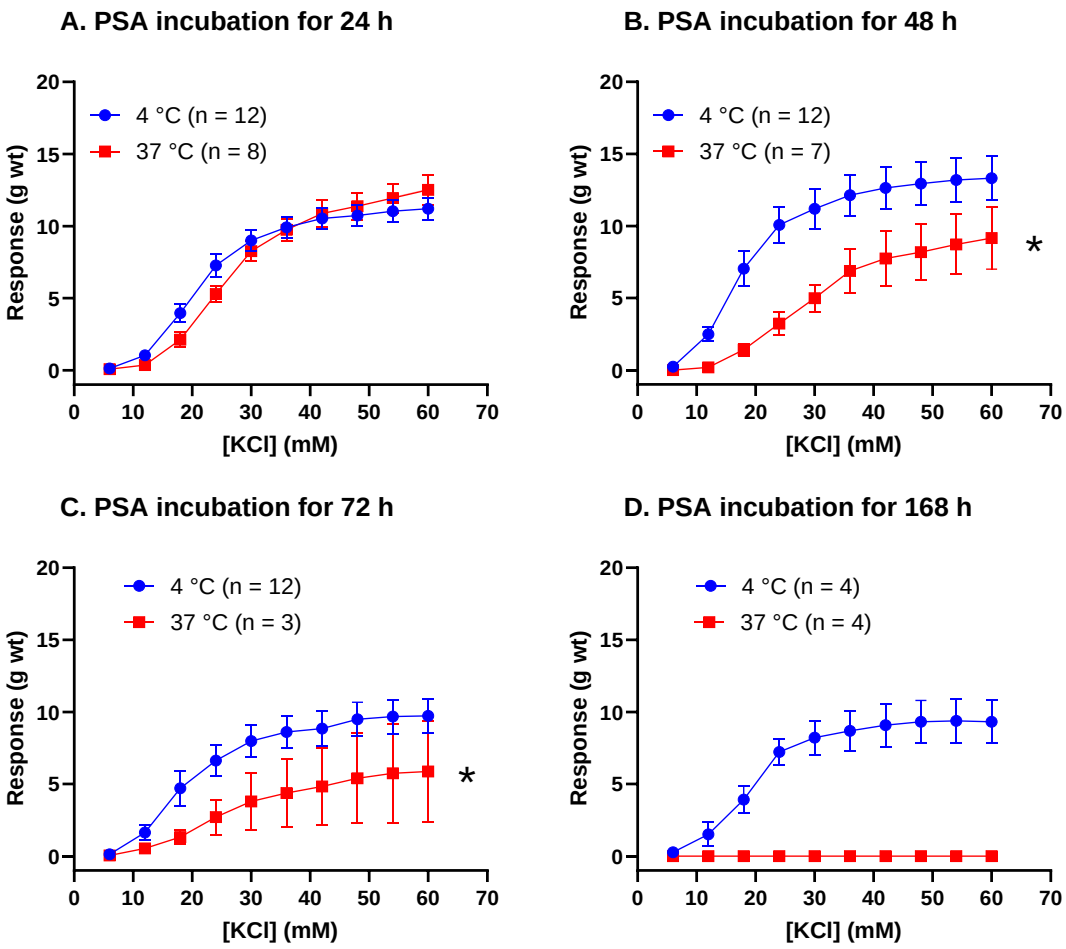


Figure 3.17: The effect of temperature and incubation period on KCl induced contractions in porcine splenic artery. The tissues were incubated for (**A**) 24 h, (**B**) 48 h, (**C**) 72 h, and (**D**) 168 h in antibiotics solutions. Data are expressed as weight (grams) and presented as mean ± SEM. *p-value < 0.05 represented a statistically significant difference (Student's paired t-test).

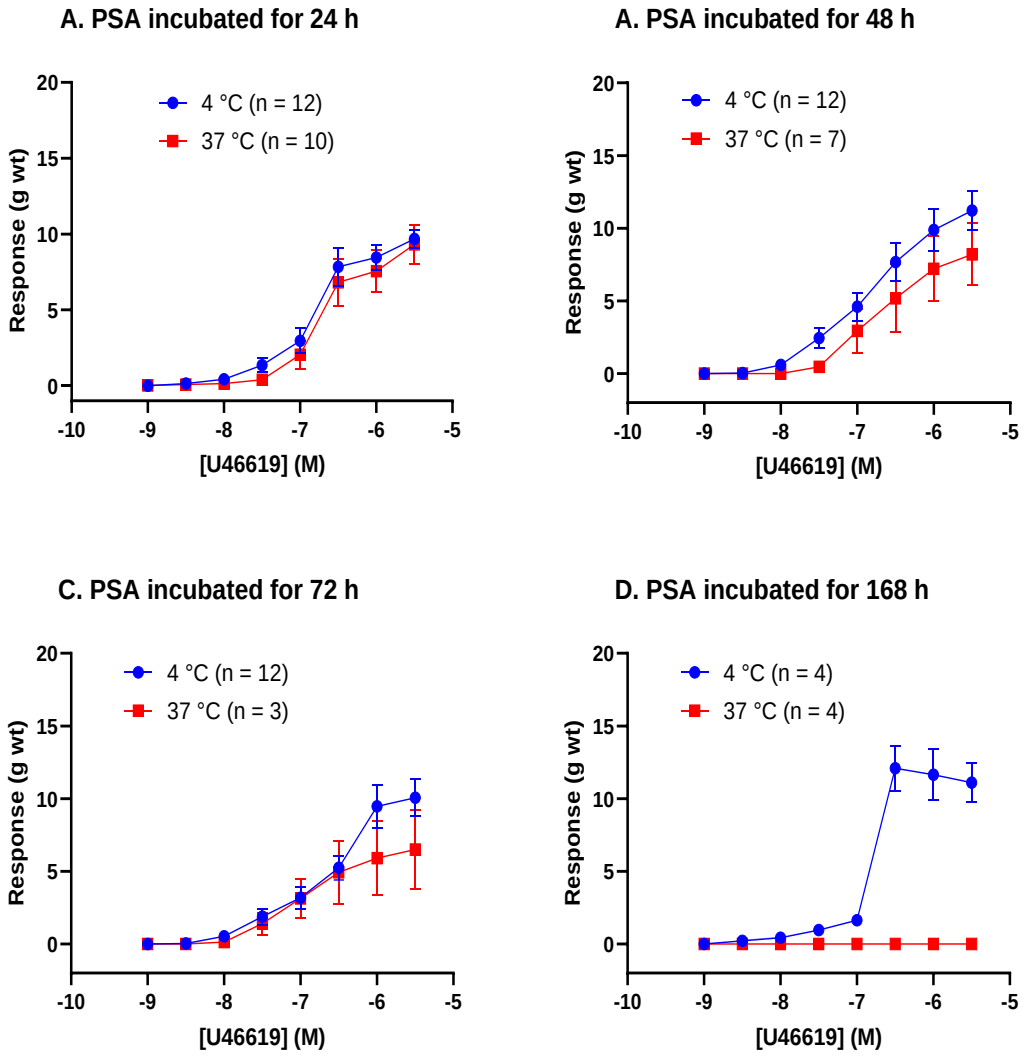


Figure 3.18: The effect of temperature and incubation period on U46619 induced contractions in porcine splenic artery. The tissues were incubated for (**A**) 24 h, (**B**) 48 h, (**C**) 72 h, and (**D**) 168 h in antibiotics solution. Data are expressed as weight (grams) and presented as mean ± SEM.

Table 3.5: The effect of temperature and incubation period on the maximum response (Emax) of KCl and U46619 in porcine splenic artery incubated for 24 h, 48 h, 72 h, and 168 h at 4 ºC or 37 ºC. The Emax values are expressed as weight (grams) and presented as mean ± SEM. The total number of the tissues examined was 12. The *p-value < 0.05 was considered statistically significant (two tailed paired t-test) when comparing group 37 ºC to group 4 ºC. Temp: Temperature, N: number of animals.

Incubation period (h)	Temp	KCl		U46619	
		N	Emax (g wt)	N	Emax (g wt)
24	4 ºC	12	10.33 ± 0.71	12	9.68 ± 0.58
	37 ºC	8	11.66 ± 1.02	10	9.32 ± 1.20
48	4 ºC	12	12.30 ± 1.39	12	11.22 ± 1.40
	37 ºC	7	8.01 ± 2.02 *	7	9.40 ± 1.90
72	4 ºC	12	9.0 ± 1.15	12	10.10 ± 1.22
	37 ºC	3	5.0 ± 3.05 *	3	8.0 ± 2.60
168	4 ºC	4	8.89 ± 1.47	4	11.10 ± 1.15
	37 ºC	0		0	

In a different set of experiments, the antibiotic solution was replaced by the sterile culture media (endothelial cell media) to avoid infection during the incubation of the porcine isolated splenic arteries. The findings showed that there is no difference in the contraction responses of U46619 in the segments incubated for 48 hours with 100 µg/ml LPS (Figure 3.19). Also, LPS did not cause any change in NA mediated contractions of the segments incubated at 37 ºC for either 24 or 48 hours (Figure 3.20).

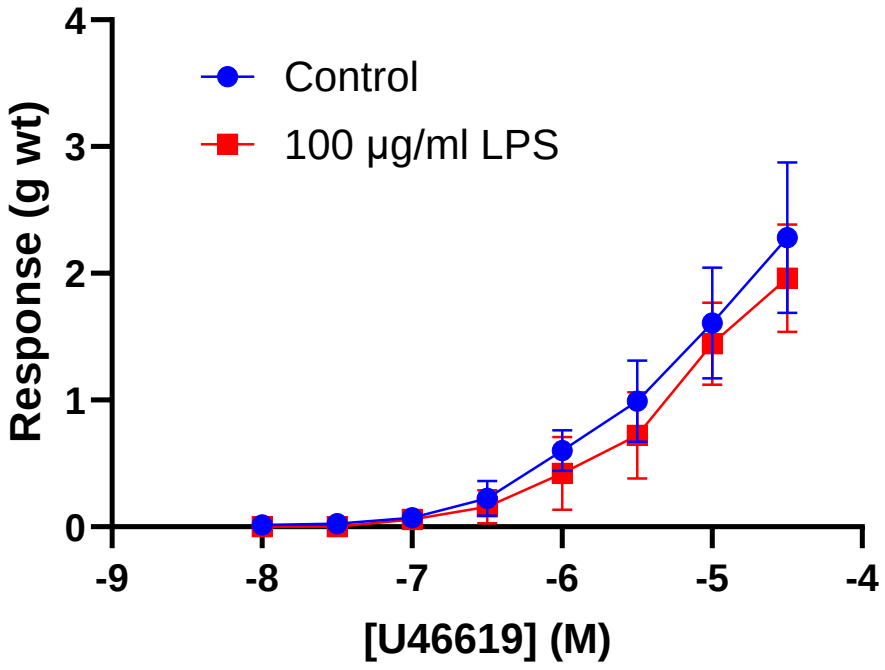


Figure 3.19: The cumulative concentration-response curves of U46619 in porcine splenic artery incubated at 37ºC for 48 hours in culture medium (endothelial cell medium) in the presence of 100 µg/ml LPS. Data are expressed as weight (grams) and presented as mean ± SEM, n = 15.

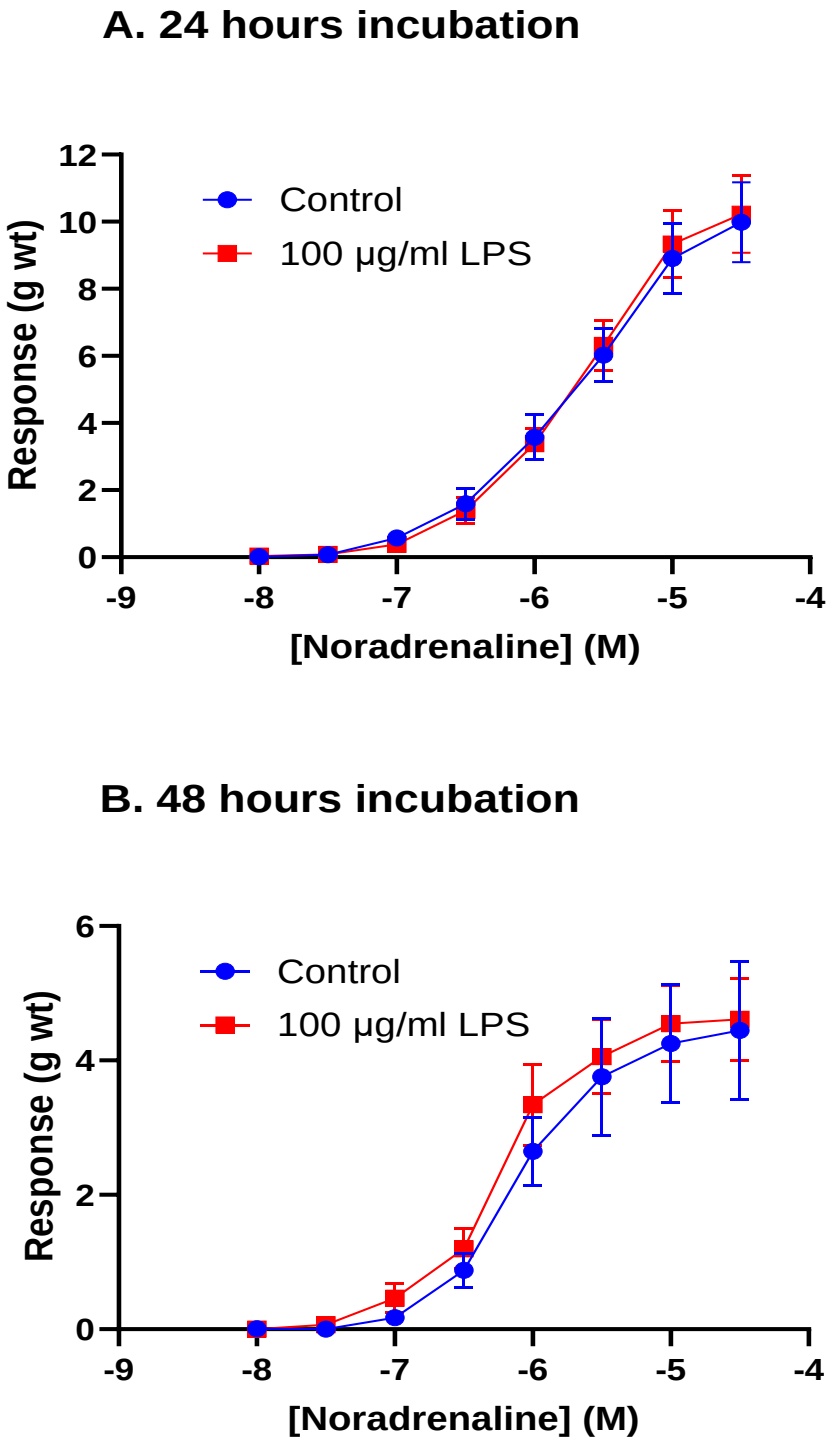


Figure 3.20: The cumulative concentration-response curves of NA in porcine splenic artery incubated at 37ºC for (**A**) 24 hours, n=14 and (**B**) 48 hours n=4 in culture medium (endothelial cell medium) in the presence of 100 µg/ml LPS. Data are expressed as weight in grams and presented as mean ± SEM, n = 4.

3.3.3. The contractile responses of various α_2-adrenoceptor agonists in porcine blood vessels:

The contraction responses of five α_2-adrenoceptor agonists using the cumulative concentrations (1 nM up to 1 μM) were studied in central, and peripheral porcine isolated blood vessels. The maximum contraction responses of 60 mM KCl in porcine splenic artery (16.2 ± 0.70 g wt, n = 75) was higher than the contractile responses in porcine tail artery (PTA) (2.5 ± 0.22 g wt, n = 26), and porcine splenic vein (1.40 ± 0.12 g wt, n = 40). As a percentage of the maximum KCl responses, the splenic vein showed higher sensitivity to α_2-adrenoceptor agonists compared to the splenic and tail arteries (Figure 3.21). In this study, the maximum effect (Emax) of mivazerol was approximately the same as the Emax of dexmedetomidine and higher than the Emax of other agonists in the splenic vein and artery. In the tail artery, the Emax of dexmedetomidine was approximately 50% higher than that of clonidine, and brimonidine, and the pD_2 of brimonidine (6.85 ± 0.15) was lower than pD_2 of clonidine (7.29 ± 0.30), and dexmedetomidine (7.10 ± 0.07). The pD_2 values of mivazerol and guanfacine were non-obtainable in the tail artery as the maximum response could not be achieved at this range of concentrations (Table 3.6).

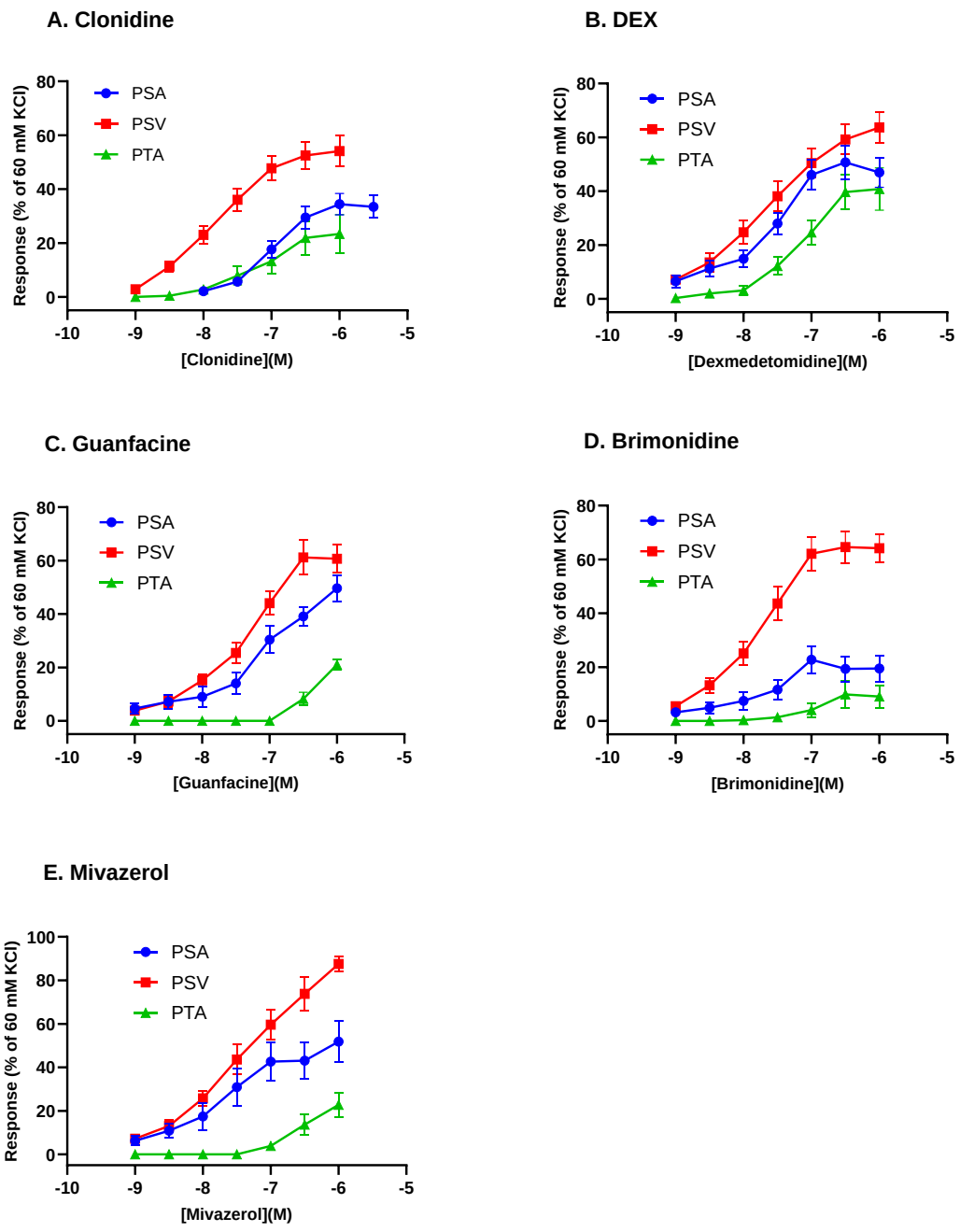


Figure 3.21: Cumulative concentration-response curves of (**A**) clonidine in PSA (n = 12), PSV (n = 13), and PTA (n= 7), (**B**) dexmedetomidine in PSA (n = 15), PSV (n = 10), and PTA (n= 7), (**C**) guanfacine in PSA (n = 15), PSV (n = 10), and PTA (n= 6), (**D**) brimonidine in PSA (n = 14), PSV (n = 10), and PTA (n= 6), and (**E**) mivazerol in PSA (n = 15), PSV (n = 10), and PTA (n= 6). Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM.

Table 3.6: Comparison of maximum response (Emax) and EC50 (pD_2) of α_2-adrenoceptor agonists in different porcine blood vessels. The Emax values are expressed as the percentage of 60 mM KCl responses, and the pD_2 values are obtained from (-Log10 EC50). All the data are presented as mean ± SEM. € indicates the Emax achieved at a higher concentration. PSA: porcine splenic artery, PSV: porcine splenic vein, PTA: porcine tail artery. N: number of animals.

Tissues	**Parameters**	Clonidine	Dexmedetomidine	Guanfacine	Brimonidine	Mivazerol
PSA	**N**	12	14	11-14	11	14
	Emax	36.5 ± 4.28	60.9 ± 7.25	49.6 ± 4.60 €	37.7 ± 6.07	60.2 ± 9.40
	pD_2	8.0 ± 0.10	7.3 ± 0.30	Not obtainable	7.3 ± 0.17	7.3 ± 0.17
PSV	**N**	13	10	10	10	10
	Emax	55.1 ± 5.43	84.0 ± 6.61	66.4 ± 4.24	70.2 ± 6.03	87.5 ± 3.12 €
	pD_2	7.4 ± 0.12	7.6 ± 0.15	7.0 ± 0.12	7.7 ± 0.06	Not obtainable
PTA	**N**	6	6	6	4	4
	Emax	24.1 ± 8.41	46.7 ± 8.25	19.8 ± 1.80 €	20.6 ± 6.27	22.7 ± 5.10 €
	pD_2	7.3 ± 0.30	7.1 ± 0.07	Not obtainable	6.9 ± 0.15	Not obtainable

3.3.4. The effect of SNP on α_2-adrenoceptor agonists induced contraction in porcine splenic artery:

The cumulative addition of SNP produced a concentration dependant relaxation by approximately 50% (n=19) in U46619 induced contractions in porcine splenic artery (Figure 3.22). The presence of SNP (1 µM and 10 µM) significantly reduced the maximum response induced by cumulative concentrations of phenylephrine compared to the control. The addition of 1 µM SNP reduced the Emax of phenylephrine by 42% (Figure 3.23). Accordingly, the concentration of 1 µM of SNP was chosen to study its effect on dexmedetomidine and mivazerol induced vasoconstrictions. The results have shown that SNP reduced the contraction responses of dexmedetomidine and mivazerol (Figure 3.24 and 3.25). The Emax of dexmedetomidine was reduced from 84.4 ± 13.4 to 46.8 ± 9.1 % of 60 mM KCl (p-value < 0.05), and the pD_2 was shifted from 7.62 ± 0.11 to 7.20 ± 0.08 in the presence of SNP. Also, the Emax of mivazerol reduced from 76.4 ± 13.5 to 45.6 ± 9.0 % of 60 mM KCl (p-value < 0.05) (Figure 3.26).

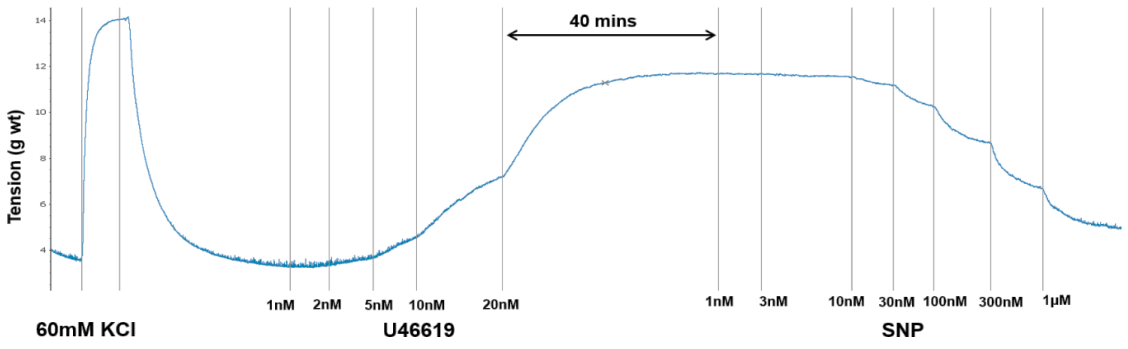


Figure 3.22: Representative traces show the U46619 induced contractile responses followed by SNP relaxation responses in porcine splenic artery.

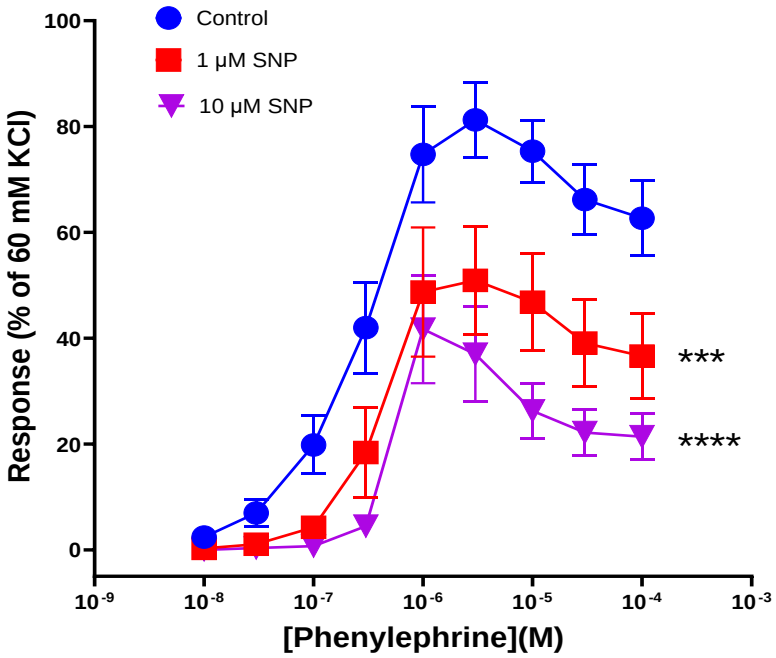


Figure 3.23: Cumulative concentration-response curves of phenylephrine in the presence of SNP (1 μM and 10 μM) in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n = 10. ***p-value < 0.005 and ****p-value < 0.001 were represented statistically significant difference, one-way ANOVA followed by Dunnett's test.

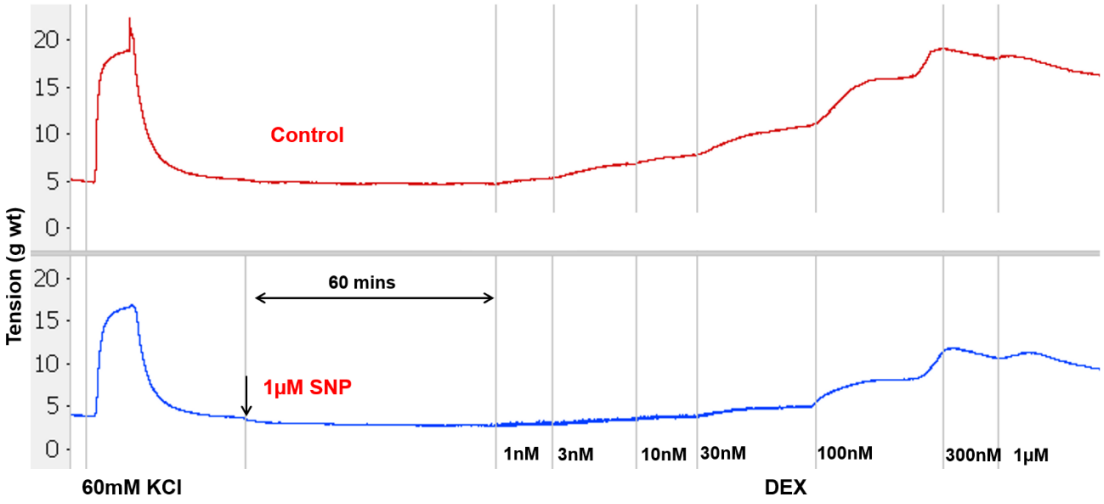


Figure 3.24: Representative traces showing the cumulative responses of dexmedetomidine in the presence of SNP (1 µM) in porcine splenic artery.

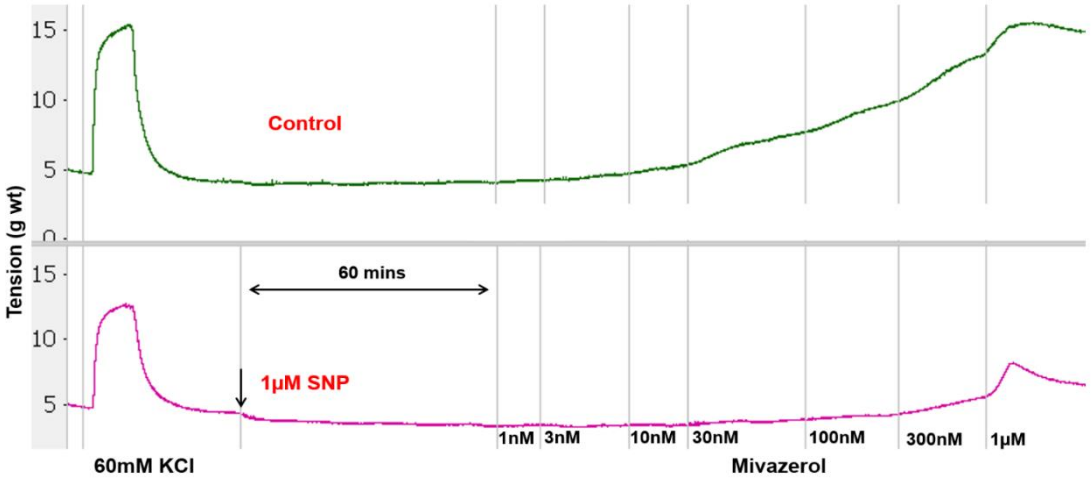


Figure 3.25: Representative traces showing the cumulative responses of mivazerol in the presence of SNP (1 µM) in porcine splenic artery.

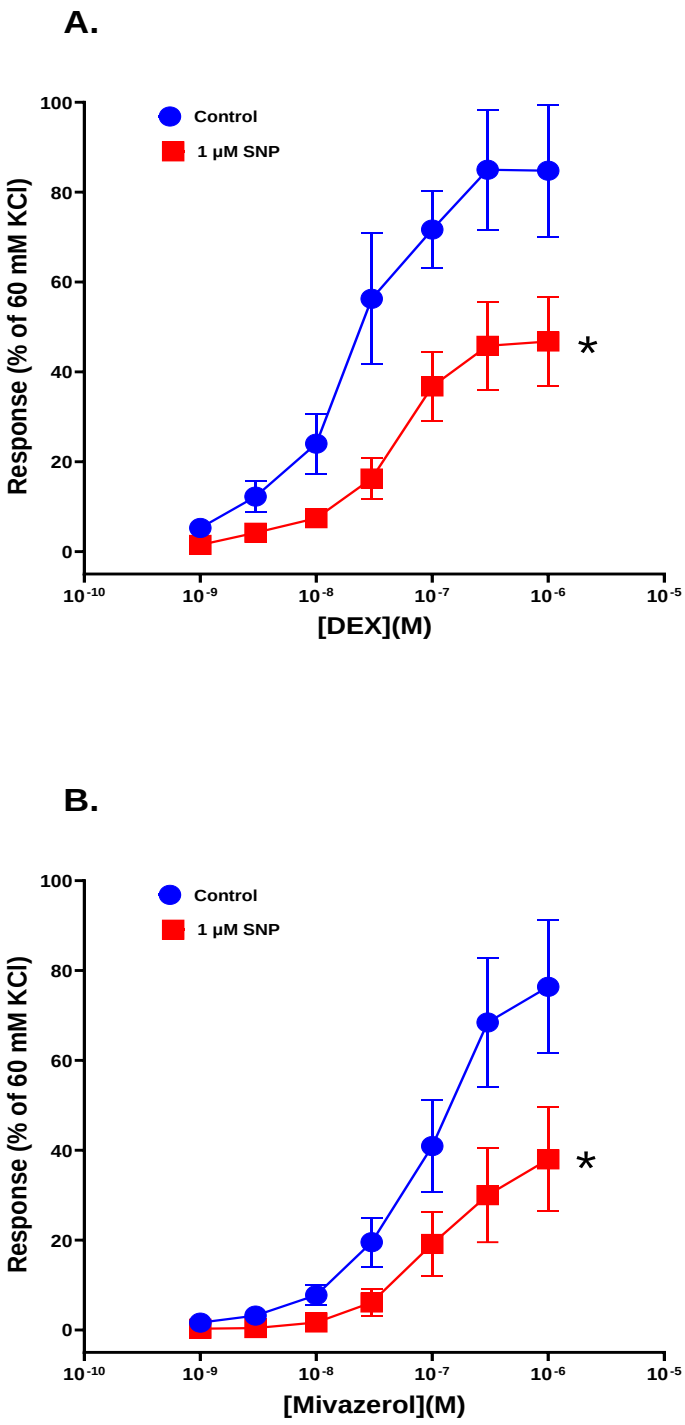


Figure 3.26: Cumulative concentration-response curves of (**A**) dexmedetomidine and (**B**) mivazerol in the presence of 1 μM SNP in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n = 6. *p-value < 0.05 represented a statistically significant difference (Student's paired t-test).

3.3.5. The prolonged effect of α_2-adrenoceptor agonists in porcine isolated splenic artery:

To demonstrate the effects of α_2-adrenoceptor agonists over a long time, the segments of porcine splenic arteries were set and left incubated overnight with α_2-adrenoceptor agonists in the organ bath. There was a consistent increase in the spontaneous contraction of porcine splenic arteries over time up to 14 -15 hours (Table 3.7). The first set of experiments was conducted to demonstrate the reproducibility and consistency of the responses of porcine splenic arteries in different channels (Figure 3.27). The contraction responses of control segments increased by 28.4% of 60 mM KCl over 15 hours, while 10 nM dexmedetomidine group (n=12-13) increased by 32.3% of 60 mM KCl (p-value < 0.01). No significant difference was observed in the presence of 3 nM dexmedetomidine (Figure 3.28).

Table 3.7: The consistent increase in the spontaneous contractions of porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM.

Time (h)	Control (n=13-14)
1	2.7 ± 1.3
2	5.5 ± 3.5
3	9.6 ± 5.9
4	14.4 ± 7.4
5	21.3 ± 8.4
6	21.7 ± 8.9
7	25.6 ± 8.9
8	27.6 ± 7.9
9	27.5 ± 7.4
10	28.2 ± 7.5
11	29.4 ± 7.4
12	27.8 ± 7.4
13	29.6 ± 7.6
14	31.0 ± 6.8
15	26.9 ± 5.9

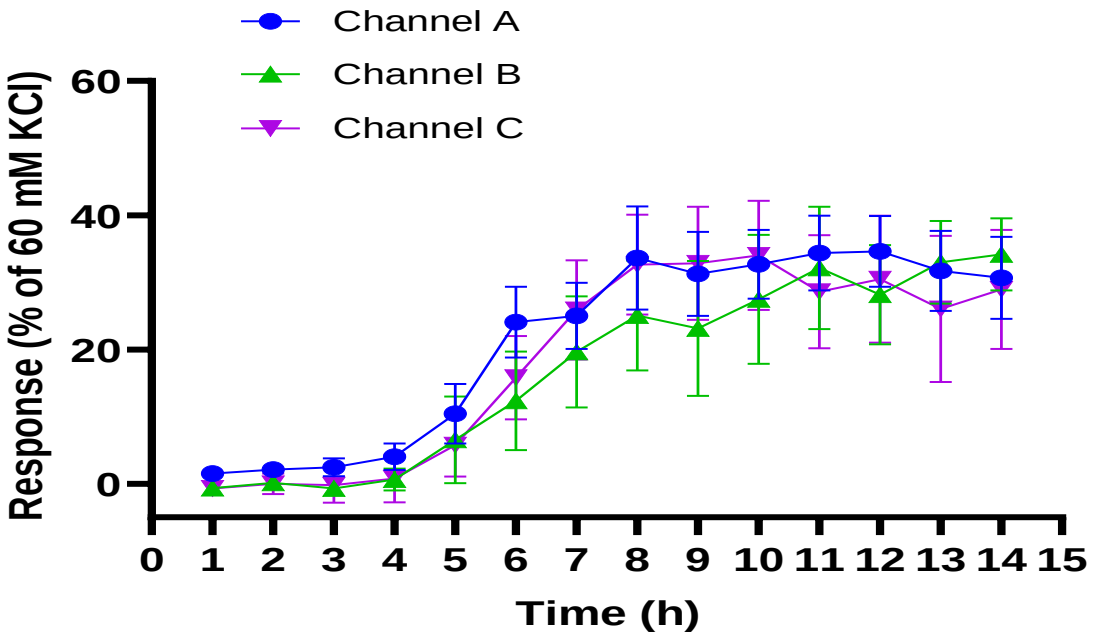


Figure 3.27: The contractile responses of porcine splenic arteries incubated overnight in an organ bath. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n= 7.

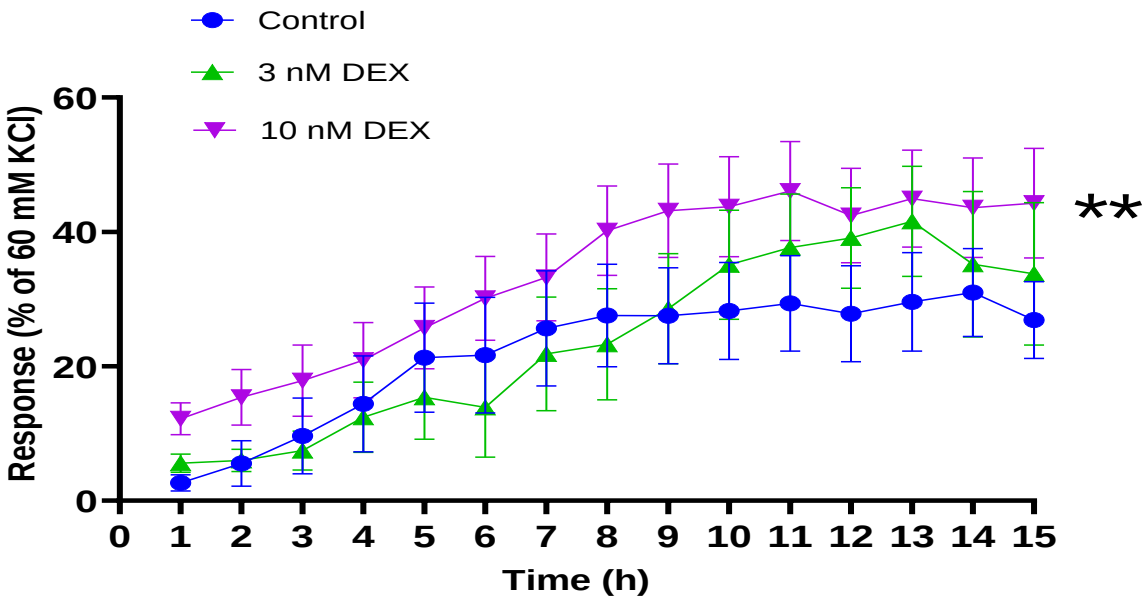


Figure 3.28: The effect of dexmedetomidine on contractile responses of porcine splenic arteries incubated overnight in an organ bath containing Krebs-Henseleit solution. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n= 12 -14. **p < 0.01 represented significant differences, one-way ANOVA followed by Dunnett's test.

3.3.6. NA sparing effects of α_2-adrenoceptor agonists in porcine splenic artery:

The purpose of these experiments was to examine the additive effects of other vasoconstrictors such as L-NAME, U46619, and α_2-adrenoceptor agonists on the contraction responses induced by small concentrations of NA (1 nM - 200 nM). The findings showed that NA mediated contractions were enhanced in the presence of the tone induced by L-NAME (Figure 3.29). The maximum contractions of NA were enhanced in the porcine splenic artery precontracted with L-NAME and U46619 (Figure 3.30). Dexmedetomidine also enhanced NA mediated contraction in the porcine splenic artery (Figure 3.31). Dexmedetomidine (10 nM) alone or with U46619 increased the NA induced contractions (Figure 3.32). NA mediated contractions were not affected by a low concentration of guanfacine 30 nM but enhanced in the presence of a higher concentration of guanfacine 100 nM (Figure 3.33). The maximum contraction of NA was shifted to the left only in the presence of guanfacine 100 nM (Figure 3.34). In addition, the tone induced by U46619 and dexmedetomidine (3 and 10 nM) increased the contractions of phenylephrine in porcine splenic artery (Figure 3.35). Table 3.8 showed a significant change in the potency (-Log $EC_{30\ KCl}$) values of NA and phenylephrine in normal conditions and pre-contracted porcine splenic arteries

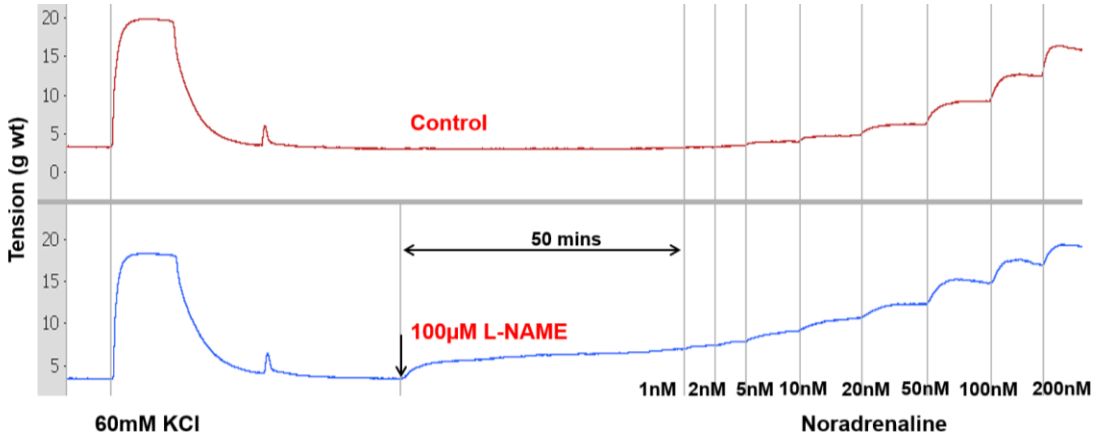


Figure 3.29: Representative traces showing the cumulative responses of NA in the presence of L-NAME (100 μM) in porcine splenic artery.

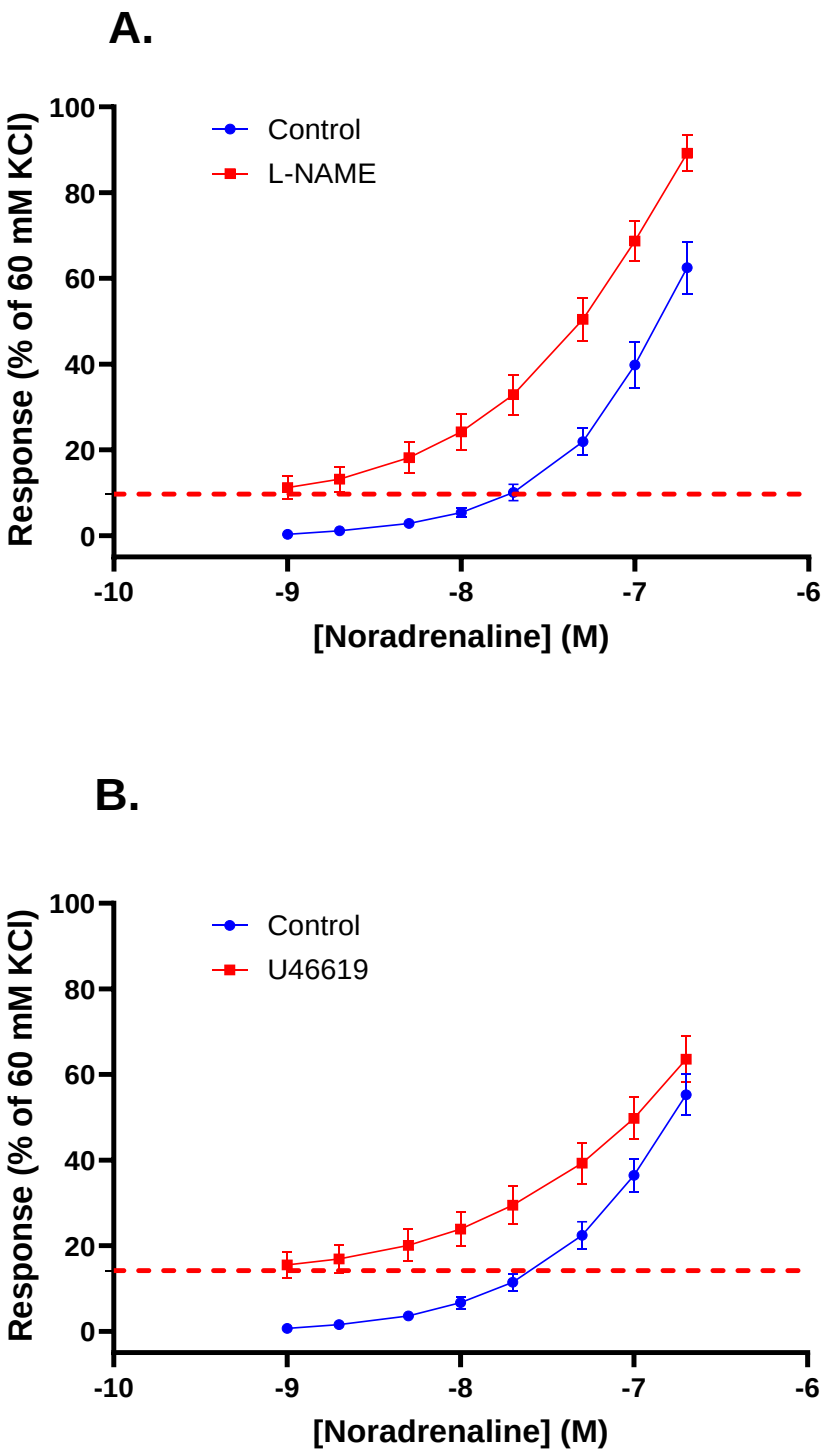


Figure 3.30. Cumulative concentration-response curves of noradrenaline (NA) in the presence of (**A**) 100 µM L-NAME (n=9), and (**B**) 15 -35 nM U46619 (n=16) in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM. Grid lines (---) indicate the baseline tones of drugs.

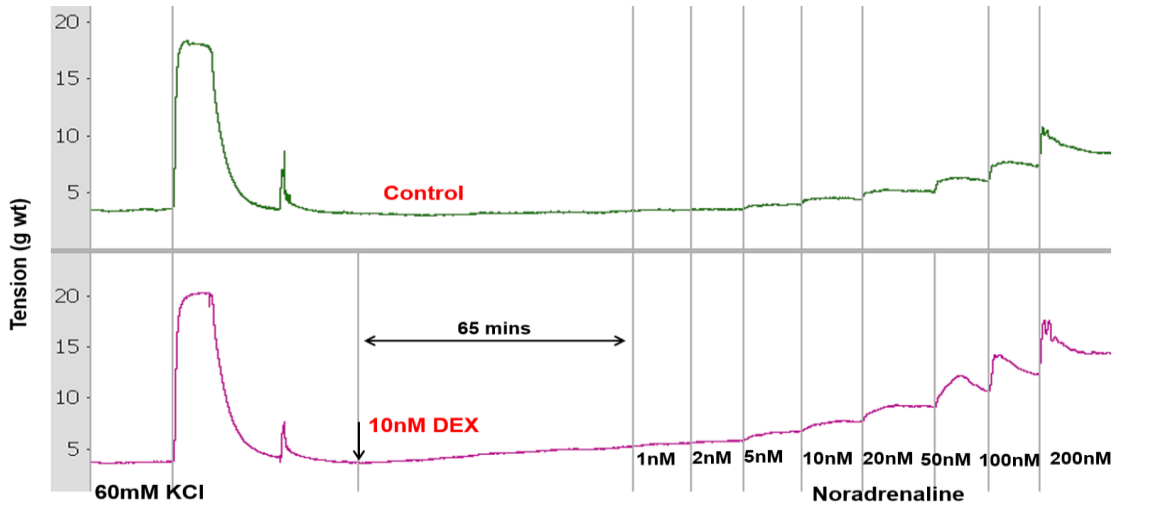


Figure 3.31: Representative traces recording the cumulative responses of NA in the presence of dexmedetomidine (10 nM) in porcine splenic artery.

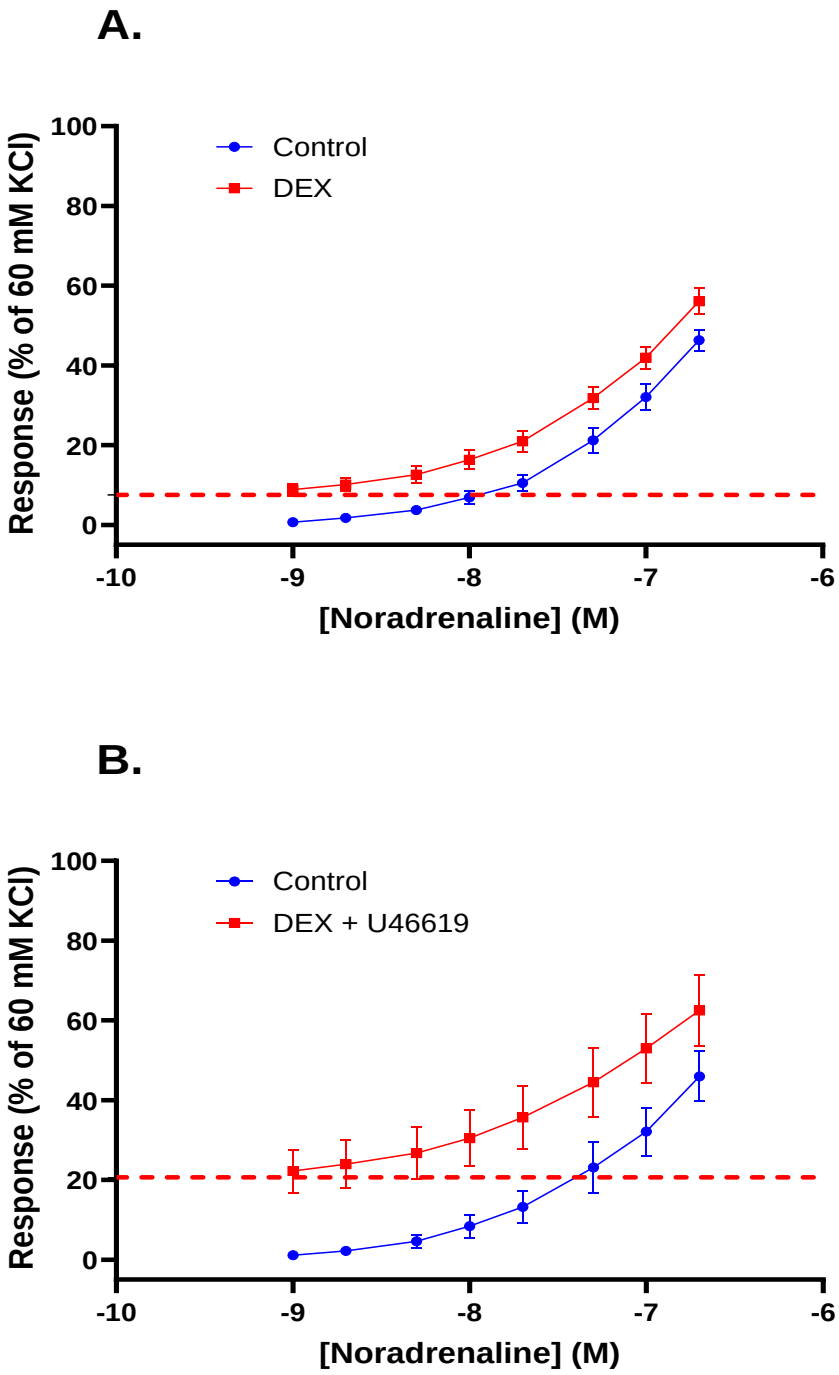


Figure 3.32. Cumulative concentration-response curves of noradrenaline (NA) in the presence of (**A**) 10 nM dexmedetomidine (n=21), and (**B**) 10 nM dexmedetomidine + 15 - 35 nM U46619 (n=7) in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM. Grid lines (---) indicate the baseline tones of drugs.

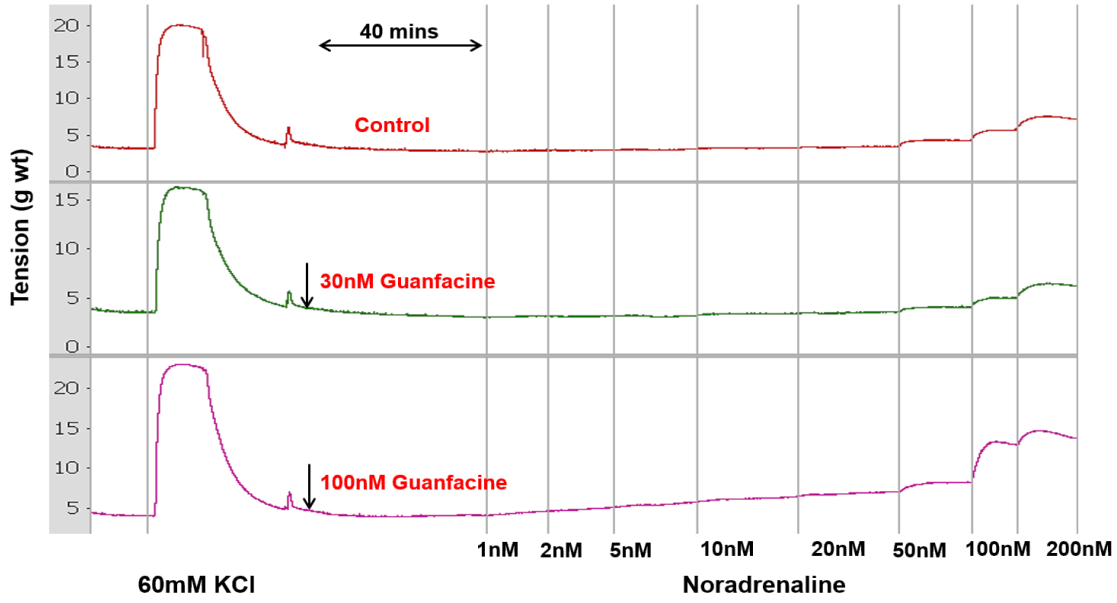


Figure 3.33: Representative traces recording the cumulative responses of NA in the presence of Guanfacine (30, and 100 nM) in porcine splenic artery.

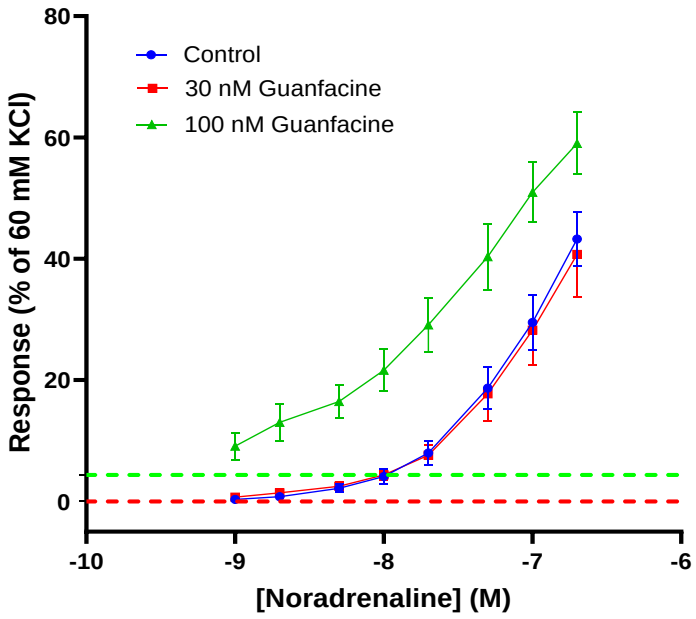


Figure 3.34: Cumulative concentration-response curves of noradrenaline (NA) in the presence of 30 nM and 100 nM Guanfacine in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n=7-8. Grid lines (---) indicate the baseline tones of drugs.

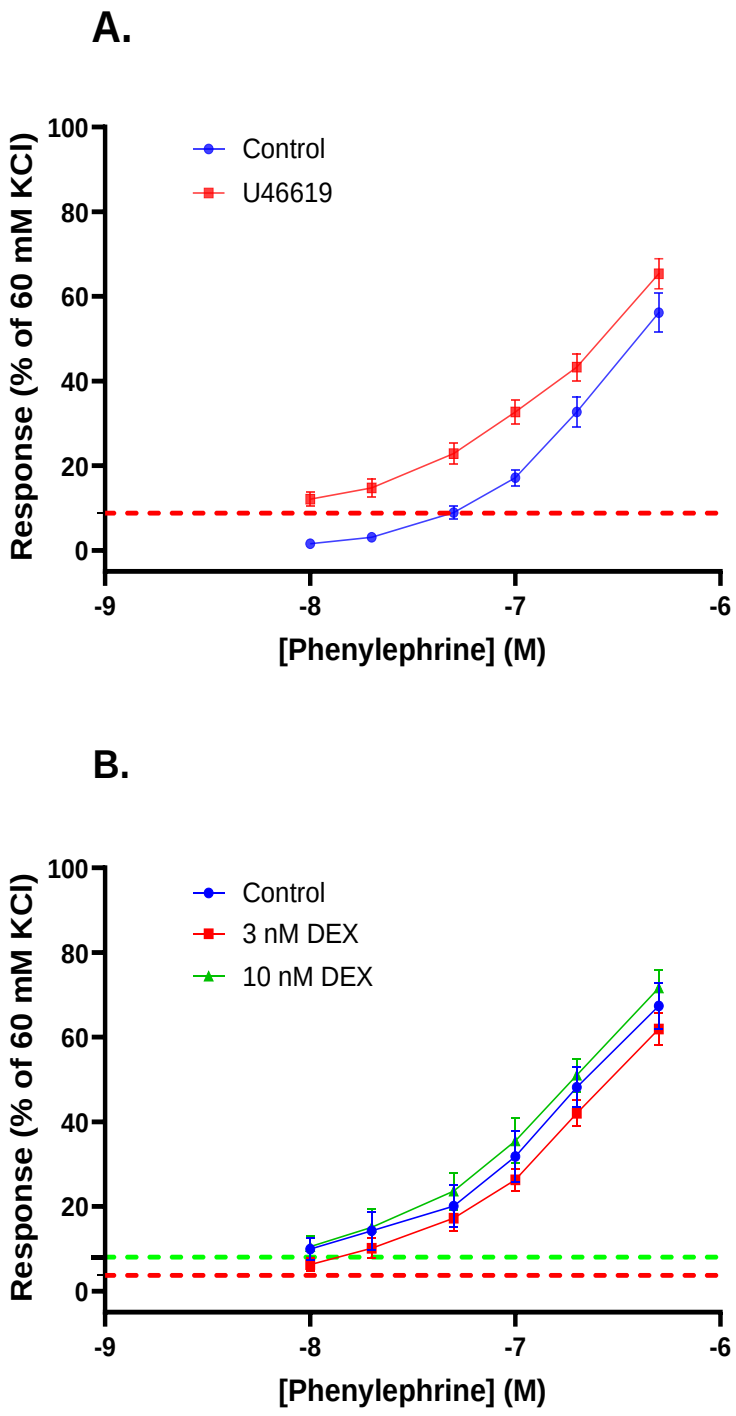


Figure 3.35: Cumulative concentration-response curves of phenylephrine in the presence of (**A**) U46619, (**B**) 3 nM dexmedetomidine, and 10 nM dexmedetomidine in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n= 9. Grid lines (---) indicate the baseline tones of drugs.

Our Table 3.8: Comparison of the potency (-Log$EC_{30\ KCl}$) of noradrenaline (NA) and phenylephrine in the absence and presence of the vascular tones induced by other vasoconstrictors. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM. *p-value < 0.05, and **p-value < 0.01, ***p-value < 0.005, ****p-value < 0.001 were considered statistically significant compared to the control, two tailed paired t-test or one-way ANOVA followed by Dunnett's test.

	n	-Log $EC_{30\ KCl}$
NA (control)	9	7.15 ± 0.06
NA + L-NAME tone		7.80 ± 0.15****
NA (control)	16	7.12 ± 0.08
NA + U46619 tone		7.62 ± 0.15****
NA (control)	21	7.06 ± 0.10
NA + 10 nM Dexmedetomidine tone		7.40 ± 0.09****
NA (control)	7	7.10 ± 0.16
NA + (Dexmedetomidine + U46619) tone		8.0 ± 0.36*
NA (control)	8	6.92 ± 0.10
NA + 30 nM Guanfacine tone		6.87 ± 0.15
NA + 100 nM Guanfacine tone		7.63 ± 0.14***
Phenylephrine (control)	9	6.71 ± 0.10
Phenylephrine + U46619 tone		7.05 ± 0.10***
Phenylephrine (control)	9	6.71 ± 0.10
Phenylephrine + 3 nM Dexmedetomidine tone		6.94 ± 0.10
Phenylephrine + 10 nM Dexmedetomidine tone		7.13 ± 0.10***

3.4. Discussion

The isolated animal blood vessels used in this study were first contracted with KCl, a highly reproducible vasoconstrictor that activates the smooth muscle by bypassing GPCRs and activating the voltage-sensitive calcium channels leading to vascular contraction (Ratz *et al.,* 2005). Hence, the maximum response to 60 mM KCl was used to standardise the responses induced by all the vasoactive agents. The preliminary experiments using the randomised conditions in different channels have been conducted to examine the reproducibility of the vascular contractions in isolated tissues and to avoid biasing the experiments. Some variability in the maximum contractions and EC_{50} were observed in the responses of the same animals when the data were expressed as weight (g) (Appendix 1). This variability is possibly due to the differences in the size of the dissected segments, technical errors, or calibrations of the channels. The variability in the contraction responses of the tissues was reduced by plotting the data as the percentage of maximum contractions of the KCl.

The sensitivity of different isolated blood vessels to vasoconstrictors was studied, and the findings demonstrated that porcine coronary arteries resulted in relaxation rather than contraction in response to NA. It is well known that catecholamines produce contraction of vascular smooth muscles, which is mediated through α-adrenoceptors. It has been reported previously that NA caused relaxation of the pig coronary arteries through the phosphorylation of L-type Ca^{++} channel mediated via β-adrenoceptors, and that cAMP could be involved in the mechanism (Fukumitsu *et al.,* 1990). The goal of the present study mainly included vasoconstriction mediated through

catecholamines, particularly NA. Therefore, the splenic artery was chosen to be used in this study because of its great contractile responses induced by NA and phenylephrine, in addition to several benefits over other blood vessels. It is accessible and long enough with regular internal diameter, and it has been previously used in the same laboratory and under the same conditions. The splenic veins and renal arteries were also used due to their potential importance in the context of raising vascular resistance and subsequently blood pressure, in addition to the fact that both α_1- and pre/post- junctional α_2-adrenoceptors are present in the venous circulation, which contribute to the vascular contractions (Gornemann *et al.,* 2007). On the other hand, the results of the contractility study also showed that renal arteries are highly sensitive to NA, showing that the contraction responses were higher in response to NA when the data were compared to the maximum KCl contractions. Accordingly, the renal artery was also chosen to be used in some experiments. It has been reported that kidneys play a substantial role in maintaining the body's homeostasis which is highly distracted during early hours of sepsis leading to hypotension (Daniels *et al.,* 2011).

3.4.1. The impairment of vascular smooth muscle contraction induced by LPS or infection in porcine isolated splenic artery:

Previous studies described vascular hypo-reactivity induced by LPS through prolonged incubation of the isolated tissues with LPS, which is largely mediated through the cGMP pathway. After incubation of rat mesenteric arteries with 1 µg/ml LPS, vascular contractions to phenylephrine were impaired after 6 hours and completely abolished after 20 hours of incubation with LPS (O'Brien *et al.,* 2005). The present study aimed to design a model of sepsis and study the effect of LPS on peripheral and central vasculatures. Subsequently, to investigate the effect of α_2-

adrenoceptor agonists on the vascular contraction of the isolated porcine splenic artery under septic conditions. The isolated porcine coronary artery (PCA) was used as a standard experiment for the protocol of the sepsis model since these tissues were previously used under the same conditions. The results showed that short-term incubation of coronary and splenic arteries with LPS in an organ bath did not produce any significant changes in the contraction activity induced by vasoconstrictors. These findings disagreed with the previous in vitro study on rat isolated thoracic aorta, where one-hour exposure to 10 µg/ml of LPS lowered the phenylephrine mediated contractions, which were further reduced with a longer LPS exposure (Beasley *et al.,* 1990). The overnight incubation of the isolated segments of coronary arteries with 1 µg/ml LPS at 37ºC didn't have any obvious effect on the contraction responses of KCl or U46619, while the higher concentration (100 µg/ml LPS) significantly reduced the KCl mediated contractions only. However, in previous studies from this lab on porcine isolated coronary arteries, the overnight exposure to 1 and 100 µg/ml LPS lowered the KCl and U46619 vascular contractions and impaired the endothelium-mediated relaxation induced by substance P (Wei *et al.,* 2008, Al-Shalmani *et al.,* 2011).

The KCl responses were significantly greater after overnight storage at 37 ^{o}C in Krebs-Henseleit with antibiotics compared to Krebs-Henseleit alone, where the incubated media turned to cloudy media because of the infection which in turn affected the vascular contractile responses (Figure 3.36). Nevertheless, the antibiotics solution used for incubating the porcine isolated splenic arteries was observed to become cloudy with a foul smell at 37°C, and this condition worsened over time (Appendix 2). Subsequently, this affected the contractility activity of these segments. Our results identified that the bacterial infection impaired the vascular smooth muscle contraction

and the integrity of the vascular endothelium. The antibiotics (penicillin-streptomycin) used to prepare the antibiotics solution were not enough to control the infection raised by isolated porcine splenic arteries. Several ways were used to minimise infection, such as washing the spleens with a mixture of warm water and chlorous solution (0.1%) and dissecting the tissues with previously sterilised tools. The spleens arrived mostly contaminated with animal faeces due to their location in the abdominal section of the animal, making it difficult to isolate the spleens without contaminating them. This could explain the higher susceptibility of splenic arteries to the infection than coronary arteries during incubation. Therefore, we should have focussed on using vessels from encapsulated organs such as kidneys and lungs as our model of central blood vessels to examine the effect of LPS, where the encapsulated organs could be less disposed to infection following dissection.

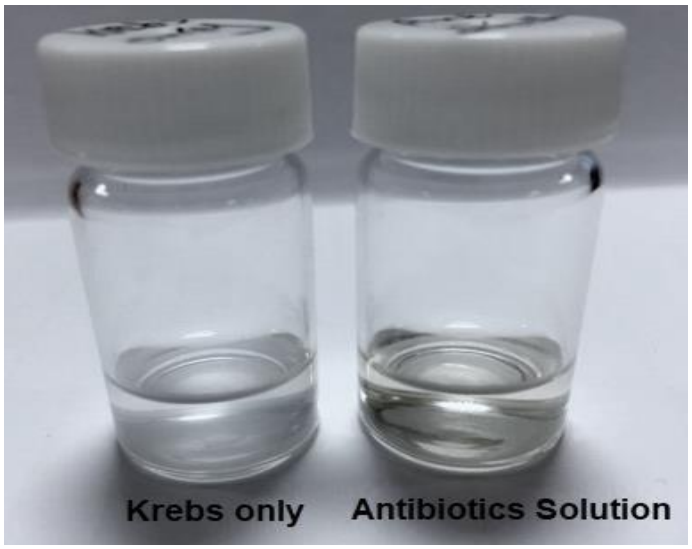


Figure 3.36: The comparison of the incubation media of porcine isolated coronary arteries incubated in either Krebs-Henseleit solution or antibiotics solution.

The purpose of the subsequent experiments was to investigate the effect of the incubation period on the number of infected segments and their contractility characteristics. It has been found that the number of the infected segments increased with the prolonged incubation at 37ºC, where the number of the responsive segments to KCl or U46619 decreased by approximately 25%, 50% and 75% after 24,48, and 72 hours respectively. On the other hand, all the segments were completely responsive to KCl and U46619 when the segments were stored at 4 ºC for up to 72 hours and showed a 66% decrease in responsive segments after 1 week of incubation. These results demonstrated that the bacterial infection could reduce or abolish the vascular reactivity. The complete ECM was then used to design LPS mediated sepsis model in porcine splenic arteries, and the media was found to be clear after incubation at 37ºC, suggesting that no infection developed (Appendix 3).

A variety of animal tissues have been used to study LPS-induced vascular hyporeactivity. The contractile responses to NA were significantly reduced by LPS in rat aorta (McKenna, 1990, Beasley *et al.,* 1990). Surprisingly, and despite of using high concentration of LPS, the maximum contractile responses of U46619 and NA were unaffected by the presence of LPS for 24 and 48 hours in porcine splenic arteries. Therefore, further studies are needed to discover a proper sepsis condition using different blood vessels and to gain vascular hyporesponsiveness at lower concentrations of LPS.

3.4.2. The vasoconstriction action of α_2-adrenoceptor agonists and its potential additive effect on NA and phenylephrine induced vasoconstriction:

The present study aimed to address the earlier question regarding the better α_2-adrenoceptor agonists concerning their peripheral vasoconstriction and potential efficacy in treating hypotension in sepsis. Thus, this study demonstrated the vasoconstriction activity of different α_2-adrenoceptor agonists in peripheral blood vessels (tail artery) and central blood vessels (splenic artery vein, and renal artery). Investigating the vasoconstrictive actions of these agonists in a septic model was crucial. However, as mentioned, this was impossible due to the failure to develop a proper septic model in porcine splenic arteries. In the present study, the vasoconstriction action of dexmedetomidine and mivazerol was demonstrated in the presence of SNP which significantly reduced the maximum contractions, indicating that NO mediated vasorelaxation affected the contractile responses of α_2-adrenoceptor agonists in porcine splenic artery. SNP induced vasodilatation by increasing cyclic GMP through the release of NO, causing the relaxation of the vascular smooth muscle in both peripheral arteries and veins (Ranadive *et al.,* 2017). The recent results emphasised that the vasoconstriction action of α_2-adrenoceptor agonists could be considerably impaired but not abolished in sepsis.

In previous studies, porcine splenic arteries showed that α_2-adrenoceptors mediated the vasoconstriction but it is less predominant than that mediated by α_1-adrenoceptors, where the contraction induced by phenylephrine and clonidine were about 93% and 31% of the NA maximum contraction, respectively (Barbieri *et al.,* 1998). dexmedetomidine causes vasoconstriction in various species of animals such as dog

and porcine coronary arteries, as well as human gastroepiploic and brachial arteries (Seyrek *et al.,* 2011). There is a variation in the contribution of cell-derived Ca^{++} to contractile responses between anatomically distinct vessels. It has been found that the initial transient contractions were dominant in the isolated abdominal aorta, distal saphenous artery, and renal vein, and were mainly associated with α_1-adrenoceptors. However, the post-junctional α_2-adrenoceptors can partly mediate the initial transient contractions to NA in the ear vein and plantaris vein (Daly *et al.,* 1990). The current findings showed that α_2-adrenoceptor agonists exhibit varying degrees of potency in different vascular beds with higher potency in the splenic vein and artery compared to the tail artery. It was reported that there is a heterogeneous population of α-adrenoceptors in dog vasculature having characteristics of both α_1 and α_2-adrenoceptor subtypes. However, the splenic artery was more sensitive to α_2-adrenoceptor blocker (Rauwolscine) than the splenic vein. Meanwhile, the selective α_1-adrenoceptor antagonist (prazosin) was equipotent in dog and rabbit tissues (Hieble and Woodward, 1984).

In our study, the vasoconstriction activity of α_2-adrenoceptor agonists initiated at the lower range of the concentrations, typically within 1 nM - 100 nM. These concentrations fall within the range of plasma concentrations of dexmedetomidine and guanfacine. The plasma concentration of dexmedetomidine used as a sedative agent in ICU was reported to be 1 to 70 nM (Dyck *et al.,* 1993, Ebert *et al.,* 2000). Moreover, the plasma concentration of guanfacine exhibits sedation was nearly 34 nM (Dollery and Davies, 1980). The vasoconstriction actions of guanfacine and its sedative activity increase the potentiality of its usage as an alternative to dexmedetomidine, suggesting its efficacy in raising vascular resistance when administered with other

vasoconstrictors in the ICU. However, based on the lipophilic property of guanfacine, the dose of guanfacine might need to be increased to achieve the sedation action equivalent to that of dexmedetomidine. In addition, the present study established the prolonged vasoconstriction action of dexmedetomidine in porcine splenic artery as shown in Figure 3.28. It was reported that dexmedetomidine has biphasic action on MAP, where the blood pressure decreased in the beginning followed by a gradual increase with continuous dexmedetomidine infusion, indicating that the distribution of dexmedetomidine in peripheral vasculature could overcome the central action of dexmedetomidine and potentially maintain the blood pressure (Ebert *et al*., 2000).

Patients with vasodilatory shock who require high doses of vasopressors may have an increased susceptibility to adverse effects induced by catecholamine, and this led clinicians to consider the usage of adjunctive therapeutic options. The high dosage of catecholamines in septic shock might cause impaired adrenergic receptor functionality and receptor desensitisation. The prolonged exposure to the catecholamines might cause overstimulation of the sympathetic nervous system resulting in dysrhythmia, tachyarrhythmia (Buckley *et al.,* 2019), irreversible heart remodelling leading to heart failure (Bode *et al.,* 2024), in addition to immune modulation (Stolk *et al.,* 2020). Vasopressin and angiotensin II were demonstrated to have adrenaline sparing properties and could potentially alleviate the adverse effects linked to the excessive vasopressor dosing requirements (Annane *et al.,* 2018). Methylene blue a guanylate cyclase blocker, was reported to have positive effects in the cases of nitric oxide up-regulation. It is not a vasoconstrictor but can facilitate the NA vasoconstrictor effect through the inhibition of the cGMP system (Evora *et al.,* 2009). Moreover, the titration of vasopressin in septic patients could lead to more than a 50% reduction in the

required dose of NA, where the infusion rate of NA was decreased from 25.0 μg/min to 5.3 μg/min at 4 hours while maintaining the MAP and improvement in the renal functions (Patel *et al.,* 2002). In the recent randomised study, NA dose requirement decreased by more than 50% in patients receiving methylene blue during the first 4 days. It has been found that the administration of methylene blue within the first 24 hours reduced time to vasopressor discontinuation and increased NA-free days at 28 days. It also reduced the adverse effects, and the length of ICU stay (Ibarra-Estrada *et al.,* 2023).

This study demonstrated the additive vasoconstrictive effects of various vasopressors when combined with the catecholamines, targeting a 50% reduction in catecholamine dosage. The potency was calculated using the EC_{30} as a percentage of the maximum KCl contraction ($EC_{30\ KCl}$) since the small range of NA concentrations was used to fall in the range of the plasma concentrations of NA, where the maximum effect of NA was not achieved. The $EC_{30\ KCl}$ of NA was significantly reduced when combined with U46619 or with L-NAME suggesting their sparing activity to NA. In a clinical study, the infusion of the L-NAME to patients with severe sepsis increased the MAP, vascular resistance, and pulmonary arterial pressure, in addition to the reduction in the dosage of catecholamines. However, these beneficial effects were predominant at the early stage of L-NAME infusion only with limited effect on the mortality rate (Avontuur *et al.,* 1998). The present findings support the hypothesis that dexmedetomidine could exhibit some peripheral vasoconstriction action when administered as a sedative agent in ICU patients. In addition, the results showed some potential sparing activity of α_2-adrenoceptor agonists such as dexmedetomidine and guanfacine to NA and phenylephrine. These results suggest that the co-administration of α_2-adrenoceptor agonists with NA might enhance the outcomes of septic patients by minimising the

dosage of NA required to raise the blood pressure. However, further investigations are needed to confirm that guanfacine can be used as an alternative sedative agent to dexmedetomidine and reduce the dosage of NA by 50% in animal models of sepsis.

4. Chapter Four: The effect of dexmedetomidine on LPS induced endothelial cells hyperpermeability.

4.1. Introduction

4.1.1. Endothelial cell permeability

The endothelium is a semi-permeable barrier that covers the inner surface of blood vessels. It regulates a wide range of functions, such as vascular smooth muscle tone, fluid tissue hemostasis, angiogenesis, and modulation of platelet aggregation. The vascular endothelial cells control the passage of macromolecules, cells, and fluid through the endothelial barrier to the interstitial tissue (Mehta and Malik, 2006). Depending on the type of the tissues, the endothelial barrier divided into fenestrated or non-fenestrated endothelial barriers (Aird, 2007). The non-fenestrated continuous endothelium is found in arteries, veins, brain, placenta, skin, heart, and lung, while the fenestrated continuous endothelium lines the exocrine and endocrine glands, gastric and intestinal mucosa, and renal glomeruli (Wisse, 1970).

The endothelial lining of the vasculatures in different organs varies in morphology and permeability. For example, the pulmonary endothelium has high expression of adhesion molecules that promote the margination of the large intravascular pool of leucocytes within the lung. The endothelial cells of BBB exhibit an extensive tight junction and sparse pinocytotic vesicular transport which allows strict control of the exchange of the solutes and circulating molecules between the plasma and the interstitial fluid (Pries and Kuebler, 2006). The fetal placental endothelial barrier is continuous non-fenestrated endothelium, mainly maintained through adherens and tight junctions, and the molecules present within these cell-cell junctions could influence the stability and the permeability of these junctions (Desforges and Sibley, 2010).

4.1.2. Permeability pathways

The regulation of endothelial permeability is a complex process which involves two pathways transcellular and paracellular. Transcellular pathways can mediate the transportation of macromolecules (large sized) proteins such as albumin and insulin across both the apical and basolateral membranes through passive diffusion through ion channels, receptor-mediated or fluid phase manners. The paracellular permeability is determined by three main types of junctions: adherens junctions, tight junctions, and gap junctions. Tight junctions are composed of the occludin, claudins, and junctional adhesion molecules. The tight junctions regulate the movement of ions and solutes such as urea, glucose, and dextran between the cells (Mehta and Malik, 2006). Vascular endothelial cadherin (VE-cadherin) forms adherens junctions. The VE-cadherin linked with an F-actin acts as a basic cytoskeleton in maintaining cell-cell contacts and mediating the maintenance of the endothelial monolayer. The adherens junctions adjust the permeability of endothelium via tyrosine phosphorylation, endocytosis, and dissociation of VE-cadherin. In this study, the dextran with small molecular weight was used to assess the endothelial permeability and it is believed that dextran passes through the paracellular pathway (Figure 4.1) (Leach *et al*., 2009, Frost *et al*., 2019).

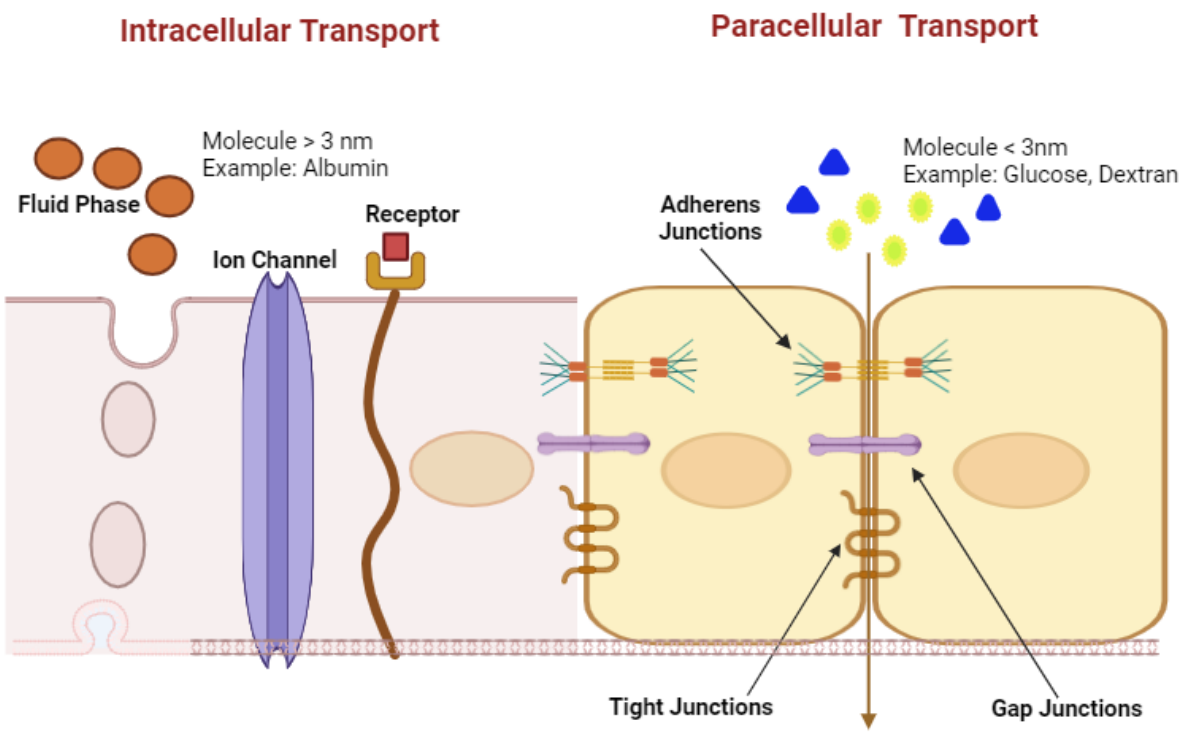


Figure 4.1: The diagram displays the permeability pathways of endothelial cell membrane including the intracellular and paracellular transports (created **in Biorender.com**).

4.1.3. The effect of LPS on the vascular endothelium

The endothelium synthesises and expresses molecules that are critical in regulating haemostatic functions. In normal hemostasis, the endothelium regulates the fibrinolysis and coagulation so that the blood can flow freely without systemic bleeding or clotting. However, severe sepsis is associated with the dysfunction of endothelial cell functions leading to dysregulation of haemostasis, vascular reactivity, tissue edema, and endothelial hyperpermeability (Ince *et al.,* 2016). This dysregulation increases the endothelial permeability and contributes to the development of septic shock, and multiple organ failure. It has been shown that LPS causes endothelial barrier disruption in different types of endothelial cells. Table 4.1 summarises the LPS-related dysfunctions of various endothelial cells in in vitro studies. The signalling pathways by which the LPS induces endothelial apoptosis are not fully characterised. It has been reported that LPS induced endothelial apoptosis through the induction of tumour suppressor gene p53 and pro-apoptotic Bax as well as the activation of caspase-3 and caspase-1 in HUVECs (Munshi *et al.,* 2002).

One of the important pathological processes of endothelial hyperpermeability in sepsis is via the attachment of hyaline leukocytes to vascular endothelial cells, which influence the expression and distribution of VE-cadherin. It has been found that the LPS increased the endothelium-hyaline leukocyte conglutination and VE-cadherin, and reduced the vascular cell adhesion protein 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin (Yu *et al.,* 2015). The mitogen-activated protein kinase (MAPK) family with p38 MAPK is one of the major members that plays a key

role in modulating the actin–myosin cytoskeleton and regulating vascular permeability. The LPS is a well-known activator of MAPKs, where this interaction is critical in developing sepsis. It caused the abnormal distribution of F-actin and the formation of stress fibers and subsequently, a disturbance of intercellular junctions through the activation of p38 MAPK resulting in increased endothelial permeability (Xia *et al.,* 2014). The other mechanisms involved in the LPS-induced barrier disruption of the HUVECs is suggested to be via the activation of nuclear factor-κB (NF-κB) or/and SIRT3 (NAD+-dependent protein deacetylase) leading to the damage of adherens junctions and endothelial hyperpermeability (Wu *et al.,* 2019, Wang *et al.,* 2017).

Table 4.1: The effect of LPS on different endothelial cells in *in vitro* studies. ↑: increase, ↓ decrease, VEGF: vascular endothelial growth factor, HSPA12B: heat shock protein A12B, RAGE: receptor of advanced glycation end products, VCAM-1: vascular cell adhesion protein 1, ICAM-1: intercellular adhesion molecule 1, HUVECs: human umbilical vein endothelial cells, HPMVEC: human pulmonary microvascular endothelial cell, HBMVECs: human brain microvascular endothelial cells, MIMVEC: primary mouse intestinal microvascular endothelial cells. TEER: transepithelial electrical resistance.

Type of endothelial cells	Study design	Outcomes	Reference
HUVECs	The cells were treated with *Escherichia coli* 0111: B4 LPS 10-1000 ng/ml for 24 h	↑ Endothelial apoptosis which was blocked by VEGF	(Munshi *et al.,* 2002)
HUVECs	The cells were treated with *Escherichia coli* LPS 1 µg/ml for 12 h TEER was measured	↑ Endothelial permeability which was reduced by HSPA12B	(Kang *et al.,* 2016)
HUVECs	The cells were treated with LPS 1 µg/ml for 12 h or 24 h FITC dextran leakage was measured.	↑ Endothelial permeability which was reduced by polydatin	(Wu *et al.,* 2019)
HUVECs	The cells were treated with *Escherichia coli* 055: B5 LPS (0.01, 0.1, 1,10 µg/ml) for 24 h	↑ Protein expression of RAGE.	(Wang *et al.,* 2017)
HUVECs	The cells were treated with 1 µg/ml LPS for 24 h. TEER was measured to check the cells monolayer and permeability	↑ VCAM-1 ↑ ICAM-1 ↓ VE cadherin ↑ Endothelial permeability	(Yu *et al.,* 2015)
HUVECs	The cells were treated with *Escherichia coli* 055: B5 LPS (200ng/ml). TEER was measured after 8 h.	↑ Endothelial permeability which was reduced by doxycycline Hyclate	(Xia *et al.,* 2014)
HPMVECs	The cells were treated with LPS 0.5 endotoxin units EU/ml. TEER was measured up to 48 h.	↑ Endothelial permeability which was reduced by hydrocortisone + Vitamin C	(Barabutis *et al.,* 2017)
HPMVECs	The cells were treated with *Escherichia coli* O127: B8 LPS 10 mg/ml for 48 h	↑ Endothelial apoptosis	(Huang *et al.,* 2019)
HBMVECs	The cells were treated with LPS 1 µg/ml for 24 h	↓ p120 catenin expression and disrupted the BBB integrity	(Liu *et al.,* 2015)
HBMVECs	The cells were treated with LPS 100 ng/ml for 24 h	↓ Endogenous ET-1 secretion ↑ ET(B) receptor expression and activated necroptosis	(Abdul *et al.,* 2018)
HBMVECs	The cells were treated with E. coli 0127: B8 LPS 50 ng/ml. TEER was measured after 24 h	↓ TEER over time	(Ramirez *et al.,* 2012)
MIMVEC	The cells were treated with *Escherichia coli* 055: B5 LPS 1 µg/ml for 24 h	↓ TEER	(Bian *et al.,* 2020)

4.1.4. The effect of α_2-adrenoceptor agonists on endothelial permeability:

It has been demonstrated that dexmedetomidine has a protective action on the vascular endothelial barrier dysfunction in animals of sepsis. Dexmedetomidine can inhibit mitochondrial fission by restoring the mitochondrial morphology of endothelial cells, where it reduces the sepsis induced ER-MITO by regulating the polymerization of actin via α_2-adrenoceptors (She *et al.,* 2021). It can also decrease the LPS-induced leukocyte-endothelial interactions (Miranda *et al.,* 2015). Moreover, clinical study on healthy volunteers have shown that local administration of clonidine to the ischemic brachial artery improved its flow mediated dilatation, suggesting its protective effect on endothelial function (Gourdin *et al.,* 2012). Dexmedetomidine might improve the mitochondrial function via activation of the mitochondrial ATP-sensitive potassium channel and enhance the mitochondrial membrane function (Yuan *et al.,* 2017).

4.1.5. Aims of the study

1. To produce a monolayer endothelial membrane by culturing HUVECs on transwell inserts.
2. To study the effect of LPS on endothelial cell permeability of HUVECs.
3. To investigate the role of dexmedetomidine in reducing the vascular endothelial permeability of the LPS treated HUVECs.

4.2. Methods

4.2.1. Materials and Chemicals

The details of the cell lines, chemicals, materials, and devices used in the cell culture have been listed in the Tables 4.2 and 4.3.

Table 4.2: Type of the cell lines and other chemical reagents used in cell culture.

Compounds/Reagents	Resource/Manufacture
Human Umbilical Vein Endothelial Cells (HUVEC)	PromoCell Heidelberg, Germany (Cat. No. C-12203)
Endothelial Cell Medium (ECM)	ScienCell Research Laboratories California, USA (Cat. No. 1001)
Human EC Serum-Free Defined Medium without Phenol Red	Cell Applications, Inc Sandiago, USA (Cat. No. 113PR-500)
Fetal Bovine Serum (FBS)	ScienCell Research Laboratories California, USA (Cat. No. 0025)
Endothelial cell growth supplement (ECGS)	ScienCell Research Laboratories California, USA (Cat. No. 1052)
Antibiotic (Penicillin/Streptomycin P/S) solution	ScienCell Research Laboratories California, USA (Cat. No. 0503)
Trypsin-EDTA solution x10	Sigma-Aldrich Merck, UK
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich Merck, UK
Fluorescein isothiocyanate-dextran (FITC-dextran) (Mwt 4kD)	Sigma-Aldrich Merck, UK (CAS. No. 60842-46-8)
Lipopolysaccharides (LPS) from *Escherichia coli* O111:B4	Sigma-Aldrich Merck, UK (L2630)
Trypan blue solution	Sigma-Aldrich Merck, UK
Calcein, AM, cell-permeant dye	ThermoFisher Scientific, UK Cat. No. C3100MP
Dimethylsulfoxide (DMSO)	Sigma-Aldrich Merck, UK

Table 4.3: List of the materials and devices used in cell culture.

Materials/ Devices	Resource/Manufacture
Cell Culture Flasks T25 (25 cm^2) and T75 (75 cm^2)	Thermo Fisher Scientific, UK or Corning, USA
Cell Culture Plates (12 wells, 96-well Plates)	Corning, USA
Glass Pipettes 5 ml, 10 ml, and 25 ml	Greiner bio-one, Germany
Centrifuge Tubes (15 ml and 50 ml)	SARSTEDT, UK
Falcon® Permeable Support for 12-well Plate with 0.4 µm Transparent PET Membrane (Inserts)	Corning, USA Product number: 353180
Pipette Tips 20, 200, and 1000 µl	Sigma-Aldrich Merck, UK
Eppendorf 1.5 ml	Sigma-Aldrich Merck, UK
TC Counting Slides	Bio-Rad, UK
TC20 Automated Cell Counter	Bio-Rad, UK
The SpectraMax M2e Multimode, Microplate Readers	Molecular Devices, UK

4.2.2. Thawing and Culturing the Cryopreserved HUVECs:

The ECM was supplemented with 5% vol/vol FBS, 1% endothelial cell growth supplement (ECGS), and 1% antibiotic P/S solution to prepare a complete growth medium and was warmed in an incubator (37°C, 5% CO_2). A cryovial of HUVECs, passage one (P1) was removed from the liquid nitrogen and thawed by immersing the vial in a 37°C water bath for 1-2 minutes. The laminar flow hood and materials were rinsed with the industrial methylated spirit (IMS). The cryovial was thoroughly rinsed with IMS under a laminar flow hood. The cells were removed from the vial and immediately transferred to a 1% gelatin-coated T25 flask (surface area 25 cm^2) containing 8-10 ml of pre-warmed complete ECM (seeding density 5,000–10,000 cells per cm^2). The flask was labelled with the name of the cells, the number of the passage and the culturing date, then placed in a humidified incubator (37°C) with 5% CO_2 for cell attachment. The medium was changed after 24 hours and every other day until they reached approximately 70-90% confluency (Figure 4.2).

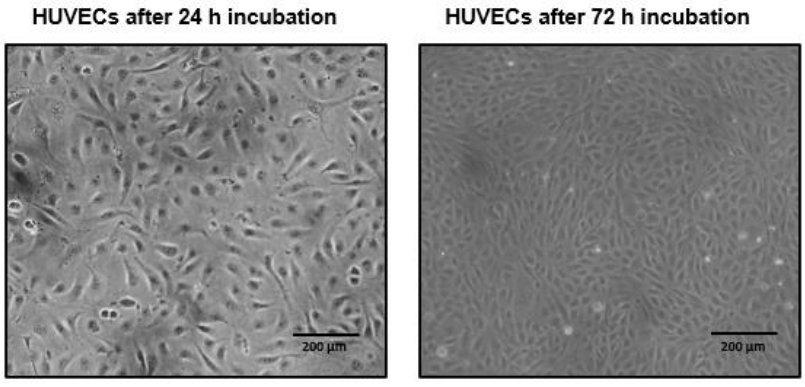


Figure 4.2: Representative phase contrast microscopic images of HUVECs P2 cultured in ECM in culture flask T25 after 24 and 72 hours.

4.2.3. Subculturing the HUVECs

The reagents (ECM, Trypsin/EDTA x1 and DPBS) were placed at room temperature or warming box for at least 30 minutes before use. The medium was carefully aspirated from the culture flask and the cells were rinsed with (1-2 ml) DPBS solution to wash the cells; this was carefully aspirated after 15 seconds. The cells were then covered with 2-3 ml Trypsin/EDTA and placed in the 37°C incubator for 3 minutes. Contrast phase microscopy was used to check that the cells were fully detached and then the Trypsin/EDTA was neutralised with 3 ml pre-warmed complete ECM, the cell suspension was aspirated, transferred into a centrifugation tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 3 ml pre-warmed ECM by triturating before transferring into a 1% gelatin-coated T75 flask containing 18 ml of complete media. The flask was then left in the incubator (37°C, 5% CO2) and the medium was changed every two or three days.

4.2.4. HUVECs Passaging, Counting and Freezing

The cells in the T75 flask were passaged once they reached 80-90% confluence. The expected number of the HUVECs at confluency in T75 is approximately 5 - 8 x 10^9 cells. Therefore, they were passaged from one into three T75 flasks. They were first washed with DPBS and detached with Trypsin/EDTA as outlined in the previous section. For passaging the cells, three pre-coated T75 flasks, each containing 19 ml complete ECM were prepared and the HUVECs pellet in centrifuged tube was suspended into 3 ml complete ECM and transferred to pre-coated T75 flasks (1 ml each) to be incubated and used later for the experiments.

To freeze the HUVECs, the cell pellet was resuspended in a freezing solution (10% DMSO + 90% FBS). 10 µl of cell suspension was mixed with the 10 µl of Trypan blue solution, and then 10 µl of the mixture was loaded on a counting slide and placed in the TC20 Automated Cell Counter which is used to test the viability of the cells by counting the living cells. After counting the cells, 1 million cells were resuspended in 1 ml of the freezing solution and transferred immediately to the cryovial (1 ml/vial) labelled with the name of the cell line, passage, and date. The cryovials were then placed in the Mr Frosty container to be stored in a - 80 ºC freezer overnight so that the cells freeze slowly (1°C/min) and then transferred into the liquid nitrogen container on the next day for long-term storage. The stored cells were removed when needed thawed as prescribed before and cultured in the T75 flask to be used for the experiments (Figure 4.3).

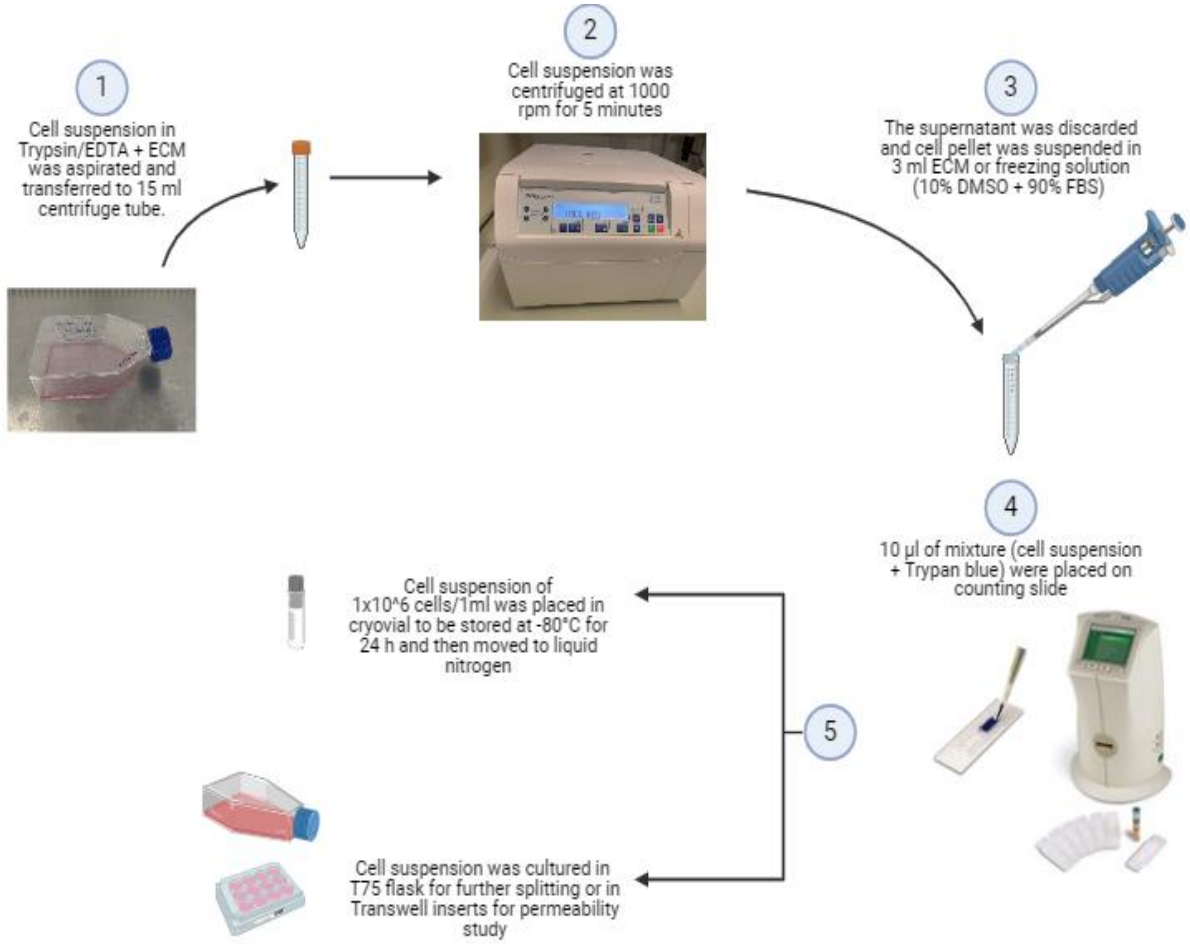


Figure 4.3: Diagram to display the cell counting and freezing steps in cell culture (created **in Biorender.com**).

4.2.5. Experimental protocol of the permeability study:

The HUVECs (P4-5) were seeded on 12 transwell inserts with 0.4 µm pores (1.12 cm^2 culture surface area). The HUVECs were seeded on the gelatin-coated apical (upper) chamber containing 500 µl complete ECM, which was placed in a basolateral (lower) chamber containing 1500 µl complete ECM. The seeding density of the HUVECs in the upper chamber was 50000 cells/ insert 1.12 cm^2. The phase contrast microscope showed that the cells obtained were 80-90% confluence after 24 hours (Figure 4.4). The cells were then treated by replacing the media in the upper chamber with new

media that contained either LPS 0.1 µg/ml, 1 µg/ml, or 10 µg/ml, or dexmedetomidine (50 µM). The cells were then incubated for another 24 hours.

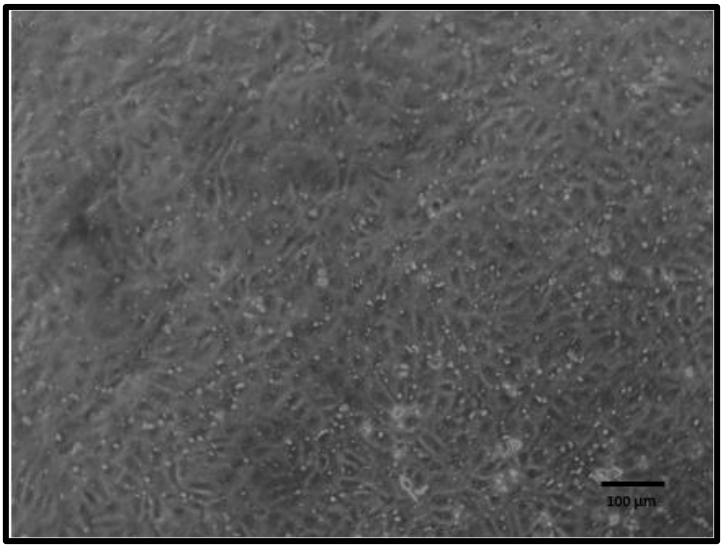


Figure 4.4: Representative phase contrast microscopic images of HUVECs P 4-5 cultured in ECM on insert membrane after 24 hours. The HUVECs were ready to be treated and incubated for another 24 hours.

The FITC-dextran solution (0.5 mg/ml) was prepared by dissolving 5 mg of FITC-dextran in a 10 ml human endothelial cell medium (free of the serum and phenol red). The media from the upper and basolateral chambers were removed and replaced with 500 µl FITC-dextran mixture in the upper chamber and 1500 µl Human EC serum free medium without phenol red in the basolateral chamber. All the solutions that contain FITC-dextran were covered with foil and the experiments were performed under low light conditions. The samples (200 µl) were collected from the basal chamber at the following time intervals (0.5, 1, 1.5, 2, 3, and 5 hours), and pipetted in the 96 well plates (100 µl in each well). The samples in the lower chamber were replaced with 200 µl fresh phenol red free ECM to maintain the volume in the basal chamber to 1500 µl.

The fluorescence intensity was then measured using a SpectraMAX plate reader with 485 nm excitation and 535 nm emission (Figure 4.5).

The concentration of FITC‑dextran was calculated from the equation obtained from the standard curve in Prism software. The standard curve was created by plotting the known concentrations (500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.6 µg/ml, 7.8 µg/ml) against the fluorescence intensity (optical density) in Prism software creating non-linear fit regression curve (Figure 4.6), which was analysed by one site-specific binding to get the equation:

$$Y = Bmax * X/ (Kd + X)$$

Bmax: the maximum specific binding in the same units as Y.

Kd is the equilibrium dissociation constant, in the same units as X.

The unknown concentrations (x) of FITC-dextran were interpolated from the standard curve, which was converted to the amount (µg) by multiplying it with the volume of the sample (200 µl) and then summing the amount with the amount of FITC-dextran in sample discarded at a previous time point, and then with final volume (1500 µl) to get the final concentrations of FITC-dextran. For example:

If the **optical capacity (fluorescence) = 618.8**

Concentration obtained from standard curve = 5.62 µg/ml

Final Concentration = 5.62 x 1.5 + (amount in 200 µl that is discarded in the previous sample)/ final volume

= (5.62 x 1.5 + 0.83)/ 1.5

= 6.17 µg/ml

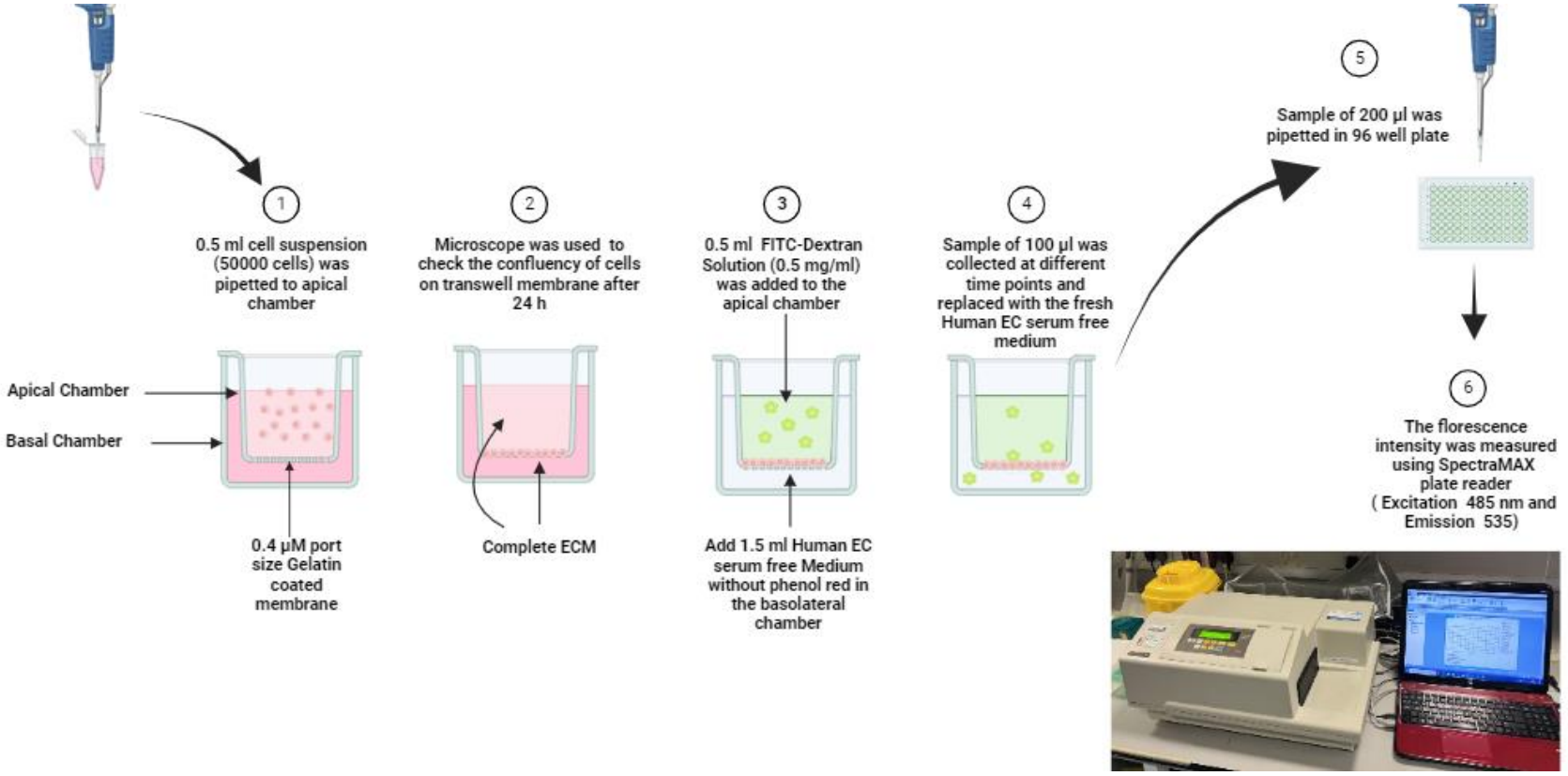


Figure 4.5: A diagram shows the main steps of the FITC-dextran permeability study (created **in Biorender.com**).

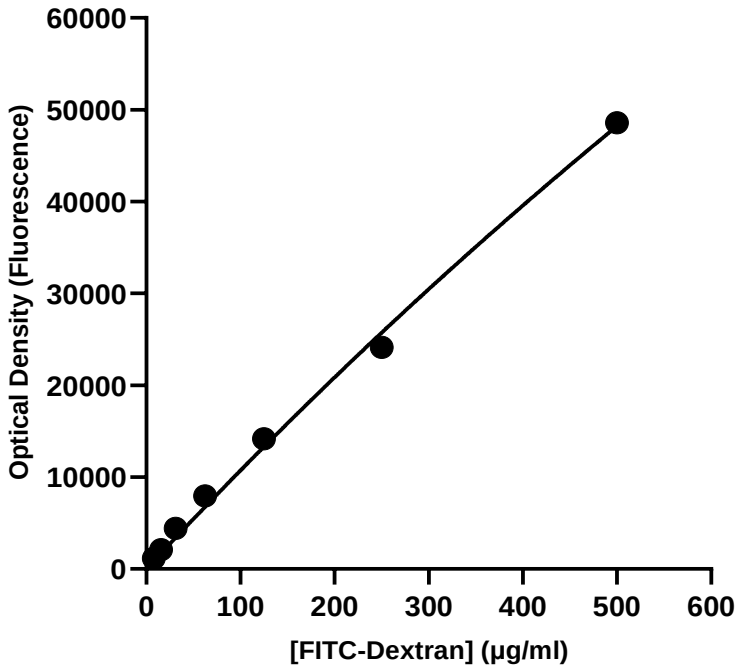


Figure 4.6: Representative calibration line for fluorescence intensities of known concentrations of FITC-dextran. The calibration curve was used to interpolate the unknown concentrations of FITC-dextran.

Determination of the permeability rate constant was performed by Dr Paul Smith as follows: if it is assumed that the flux of FITC-dextran (Jf) across the insert monolayer is a passive first order process, then it can be described by a simple two compartment model described by:

Jf = kf (Cu,t -Cl,t)

Where kf is the rate constant

Cu,t and Cl,t the concentration of FITC-dextran in the upper and lower chambers respectively at time t.

Since measurements of Cl are made at 5 different time points with that at time = 0 assumed to be zero, then Cu,t and Cl,t can be calculated as above for the standard form of measurement. These data values can then be used to fit a two-compartment

model and derive kf. For this, the flux of FITC-dextran was simulated via a multicompartment model (Modelmaker Ver 4.0, AP Benson). The effect of incremental sampling of Cl,t was simulated and compared to values measured (Figure 4.7). The optimal value for kf was obtained by Marquardt Optimization with ordinary least squares comparison of the raw and simulated data for each experimental condition.

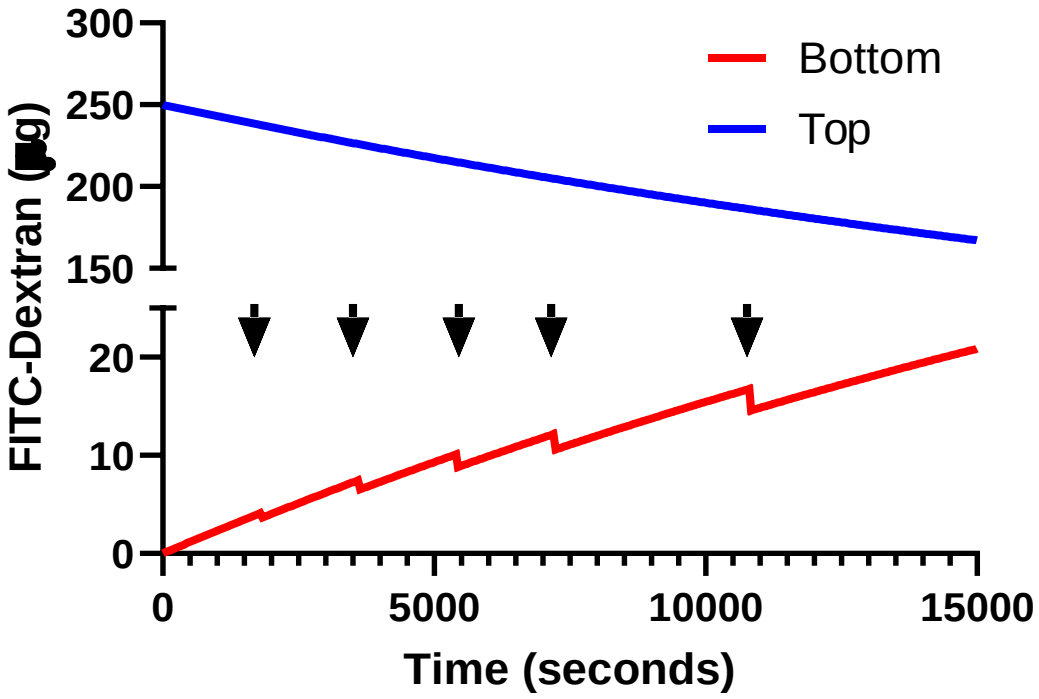


Figure 4.7: Representative simulation of FITC-dextran concentration in the upper (top) chamber and lower (bottom) chamber of insert under control condition. Arrows indicate times at which 200 µl of the solution was removed from the lower chamber for colorimetric measurement (performed by Dr Paul Smith).

4.2.6. Experimental protocol for imaging the cells:

HUVECs (P4-5) were grown on the gelatin coated 12-well plates (seeding density 10 000 cells per 3.5 cm^2). When the cells reached about 80% confluency, they were treated with 0.1, 1, and 10 µg/ml LPS, and 100 µM and 1 mM azide as a positive control for 24 hours. The calcein, AM, and cell-permeant dye were used to identify the cells. Calcien loading solution was prepared by adding 5 µl of calcein green-AM dissolved in DMSO (1 mM) into 6 ml of Hanks solution with 5 mM glucose to make the final concentration of 0.8 µM which was kept in the dark. After 24 hours of incubation, the media were replaced with the calcein loading solution and incubated for 30 minutes in a dark place at room temperature. Images were captured with a Coolsnap HQ2 camera (Photometrics, Tucson, AZ, USA) with a Zeiss XBO 75 xenon short-arc lamp (Zeiss, Oberkochen, Germany). Images were viewed with a 20x objective 1.3 NA and filter sets as indicated above. Recordings were recorded as TIFF files with Imaging Workbench version 6.0 software (INDEC Biosystems, Los Altos, CA, USA) and then transferred as TIFF images.

HUVECs (P4-5) were grown on the gelatin-coated inserts (0.4 µm pore size) placed in the 12-transwell plate as described previously. At about 80% confluency, the cells were treated with 1 mM azide, 10 µg/ml LPS, and 50 µM dexmedetomidine for 24 hours. After which the media of the upper chambers was replaced with calcein green-AM and incubated for 30 minutes in a dark place at room temperature. The Digimizer version 6.3.0 (MedCalc Software Ltd) was used to analyse the images and calculate the cell coverage area of the HUVECs through binarization. The binarization is a technique used to convert a grayscale image or a colour image into a binary image, where the dark empty areas are covered by red colour which represents the area

without cells. The final area of the cell coverage was measured using the equation: **Cell coverage = 100 x (1- sum /total area).**

4.2.7. Data Analysis and Statistics:

The number of samples was considered by the number of repeats (plates) of each experiment. The data obtained were calculated using Microsoft Excel and analysed with Prism software version 10.0.3 (GraphPad, USA). The data was tested for normality distribution by the Shapiro-Wilk test and subsequently analysed by Student's paired t-test (two-tailed) to compare the data between two conditions, and one-way ANOVA followed by Dunnett's test was used to compare two or more groups. The *p-value < 0.05 was considered statistically significant. Data are expressed as the mean ± standard deviation (SD) of observations from repeated experiments.

4.3. Results

4.3.1. Effect of LPS on endothelial cell permeability:

Gradual leakage of FITC-dextran across the inserts with or without cells occurred with time (Figure 4.8). There was no significant difference in the concentrations of FITC-dextran of HUVECs treated with LPS (0.1 and 1 µg/ml) at any time points. Whereas cells treated with the highest concentration of LPS (10 µg/ml) had a significant increase in the concentration of FITC-dextran in the samples collected after 1 hour and 1.5 hours from adding FITC-dextran (Figure 4.9). The first order constant rate for each of the cell groups showed that there was a significant leakage of FITC-dextran in blank (no cells) inserts (4.7 µg/ml ±0.6) compared to control (3.18 µg/ml ±0.2) (p-value = 0.012). However, no significant difference was observed between the LPS treated groups and the control group (Figure 4.10).

To further verify that stimulation of the HUVECs with LPS 10 µg/ml for 24 hours can increase the FITC-dextran leakage, another set of the experiments were conducted. The samples collected after 1 hour from adding the FITC-dextran showed that the concentration of FITC-dextran in the control insert (cells only) was lower (1.56 µg/ml ±0.26) when compared to blank insert (no cells) (3.205 µg/ml ±0.144, p-value = 0.014) or insert with LPS treated cells (3.33 µg/ml ±0.51, p-value = 0.001). These results suggested that the cells on inserts block some of the leakage of FITC-dextran through the membrane pores, while the LPS treated cells increased the endothelial cell permeability (Figure 4.11).

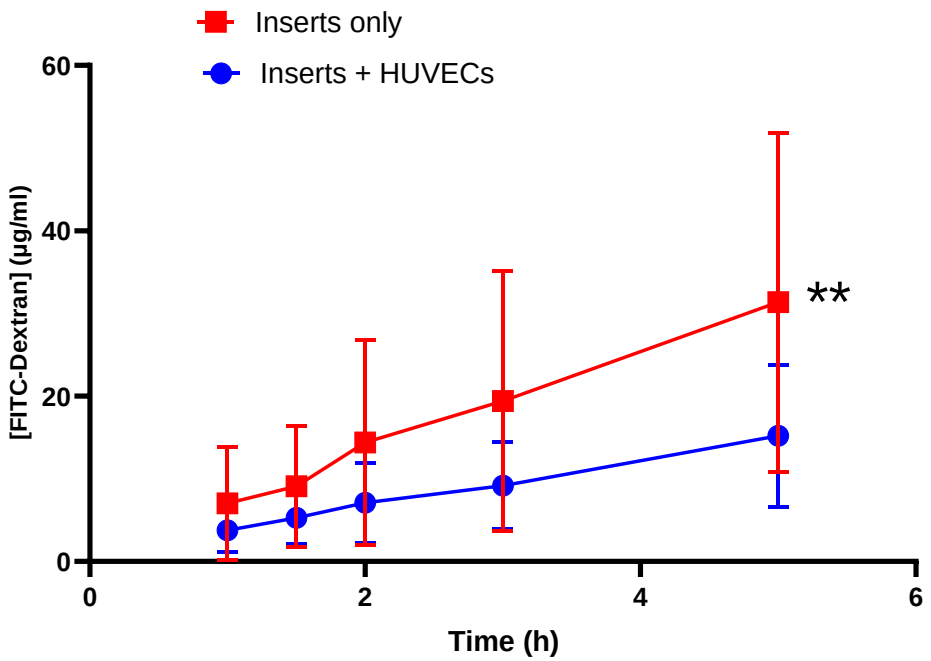


Figure 4.8: The concentration of FITC-dextran (µg/ml) over time unit obtained from inserts with or without cells. The data are presented as the mean ±SD, n=9. ** p <0.01 considered significant difference, two tailed paired t-test.

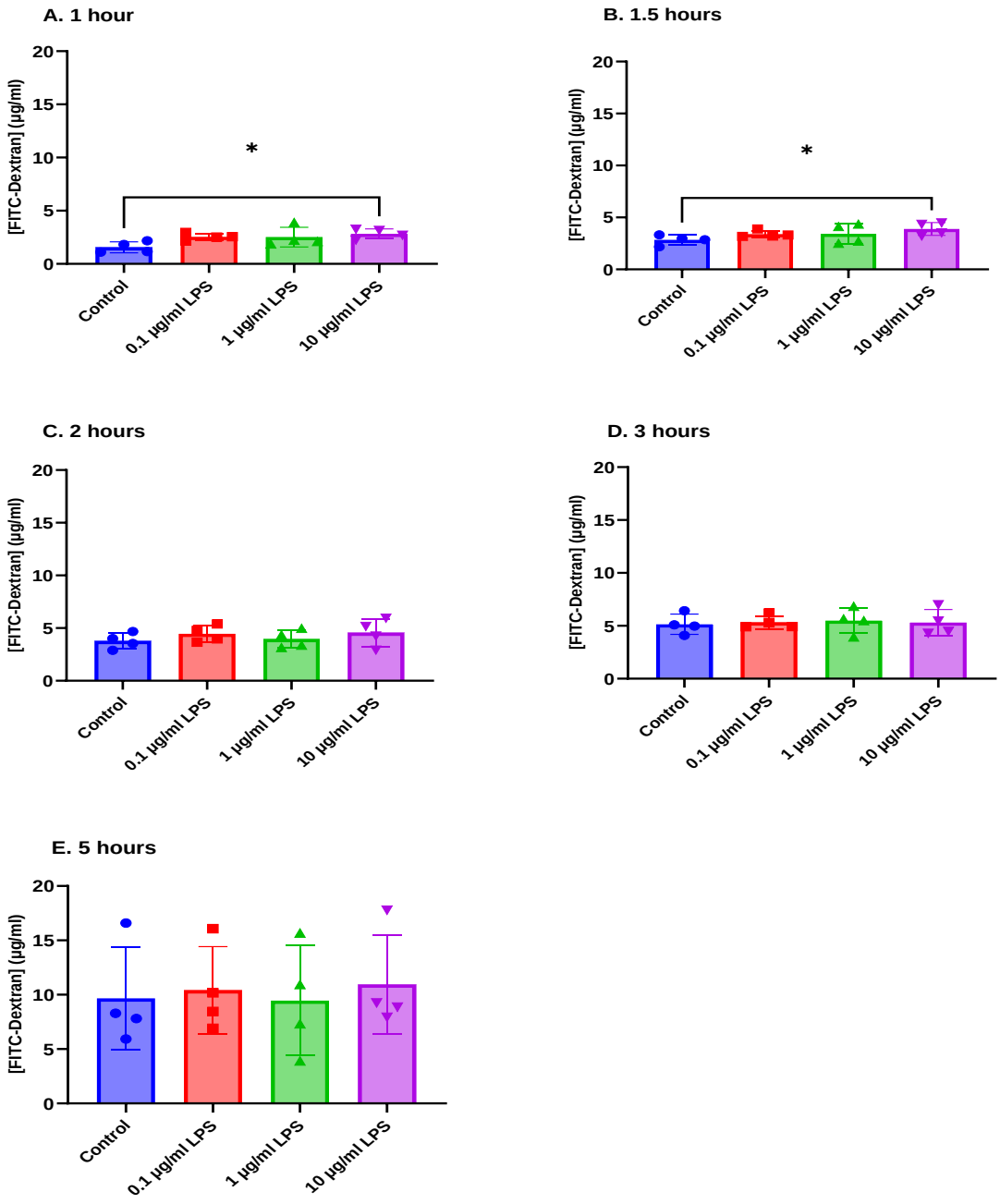


Figure 4.9: The effect of LPS (0.1, 1 and 10 µg/ml) on HUVECs permeability in transwell inserts by measuring the concentrations of FITC-dextran at different time points (**A**) 1 hour, (**B**) 1.5 hours, (**C**) 2 hours, (**D**) 3 hours and (**E**) 5 hours. The data are presented as the mean ±SD, n=4. *p< 0.05 considered a significant difference, one-way ANOVA followed by Dunnett's test.

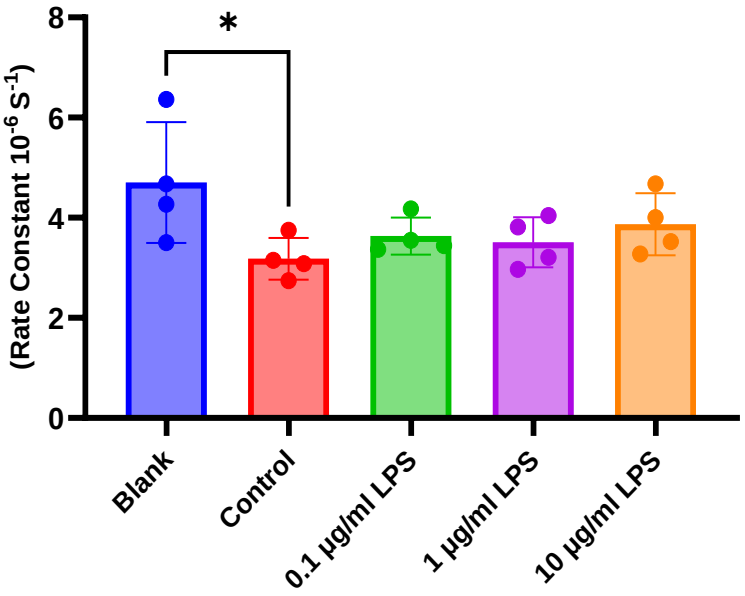


Figure 4.10: Effect of LPS (0.1, 1 and 10 µg/ml) on HUVECs permeability by measuring the first order constant rate. The data are presented as the mean ±SD for n=4. *p-value < 0.05 considered a significant difference, one-way ANOVA followed by Dunnett's test.

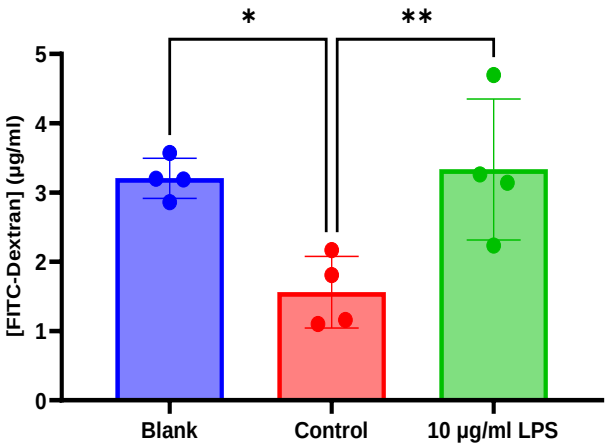


Figure 4.11: The concentrations of FITC-dextran in blank inserts (no cells) and control inserts (untreated HUVECS) and inserts with LPS treated HUVECs. The samples were collected after 1 hour of adding the FITC-dextran solution. The data are presented as the mean ±SD for n=4. *p-value < 0.05, **p < 0.01 considered a significant difference, one-way ANOVA followed by Dunnett's test.

The images of the cells stained with the calcein AM displayed that different concentrations of LPS can affect the morphology as well as decrease the covered area (Figure 4.12). The cell coverage area (PX^2) of the HUVECs cultured on the insert membrane was measured by binarization of the images in Digimizer software as illustrated in Figure 4.13. There was a significant reduction in the area of cell coverage for both the azide group (p-value =0.106), and the LPS group compared to control group. These results suggested that LSP 10 µg/ml affects the viability of the vascular endothelial cells (Figure 4.14).

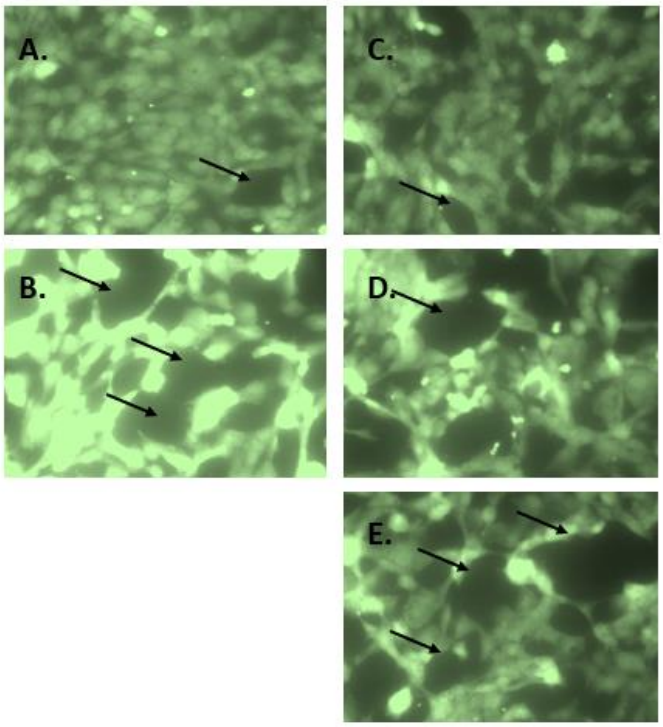


Figure 4.12: Fluorescent microscopic images of HUVECs P 4-5 cultured in ECM in a 12-well plate for 24 hours without treatment (**A**), or treated with 100 µM Azide (**B**), 0.1 µg/ml LPS (**C**), 1 µg/ml LPS (**D**), and 10 µg/ml LPS (**E**). The width of the images is 407. The arrows indicate the uncovered area (no cells).

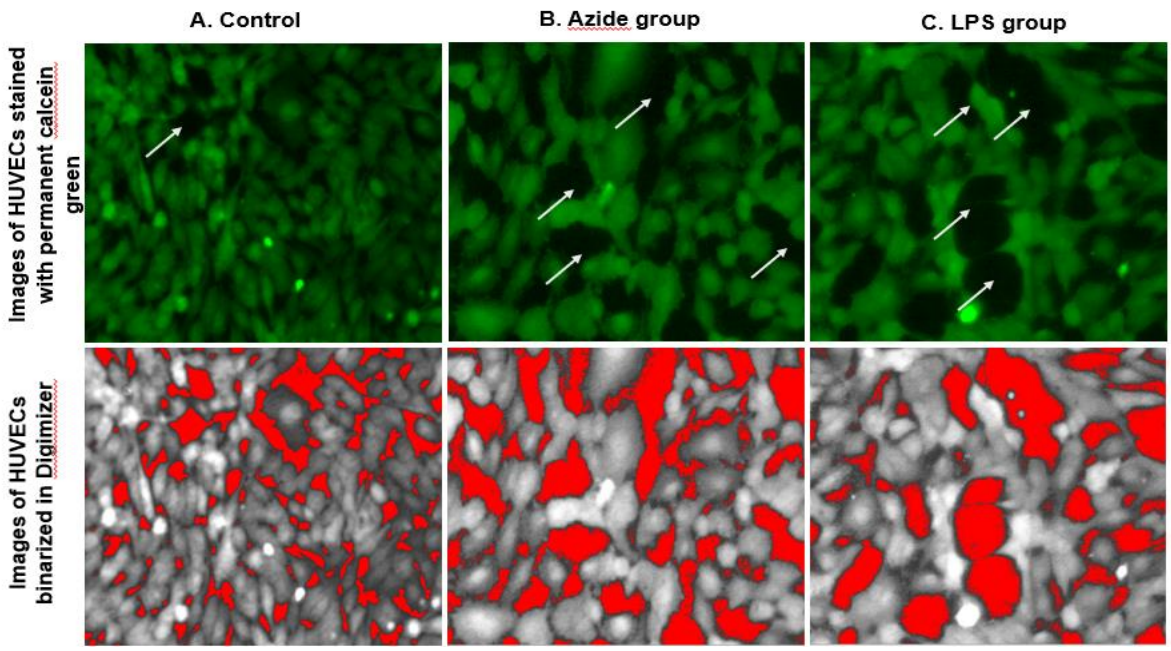


Figure 4.13: Fluorescent microscopic images of HUVECs P 4-5 cultured in ECM on insert membrane as (**A**) control, (**B**) azide, and (**C**) LPS. The images display the covered (grey) and uncovered (red colour) areas after the binarization of the cells in Digimizer software. The width of the images is 407 µm. The arrows indicate the uncovered area (no cells).

4.3.2. Effect of dexmedetomidine on endothelial cell permeability:

To investigate the effect of dexmedetomidine on LPS treated cells, the experiment was divided into 4 groups: control (cells only), LPS group, dexmedetomidine group, and LPS + dexmedetomidine group. The results showed that 10 µg/ml LPS significantly increased the endothelial permeability (p-value = 0.35), which was abolished in the presence of 50 µM dexmedetomidine (Figure 4.15).

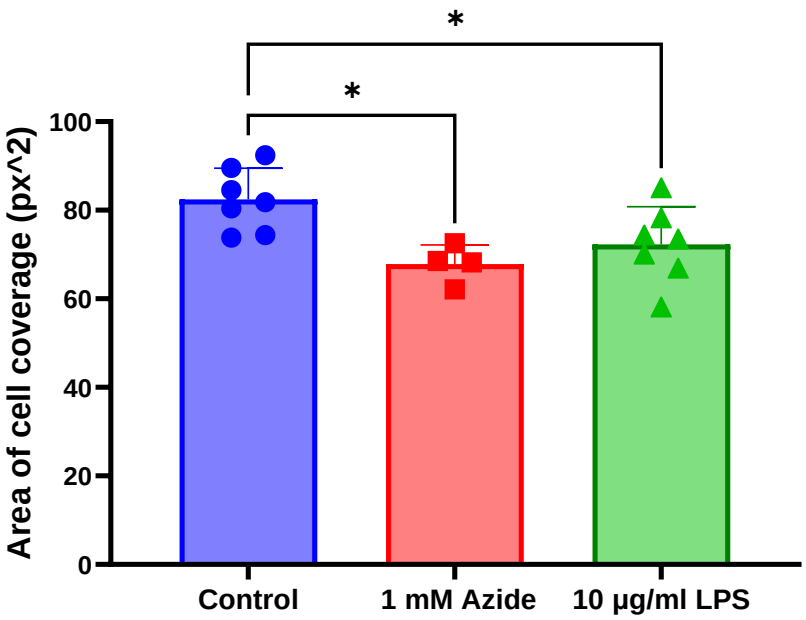


Figure 4.14: Area of cell coverage on inserts seeded with HUVECs control (n=7) treated with 1 mM azide (n=4) as a positive control and 10 µg/ml LPS (n=7). The data are presented as the mean ±SD, *p-value < 0.05 considered a significant difference, one-way ANOVA followed by Dunnett's test.

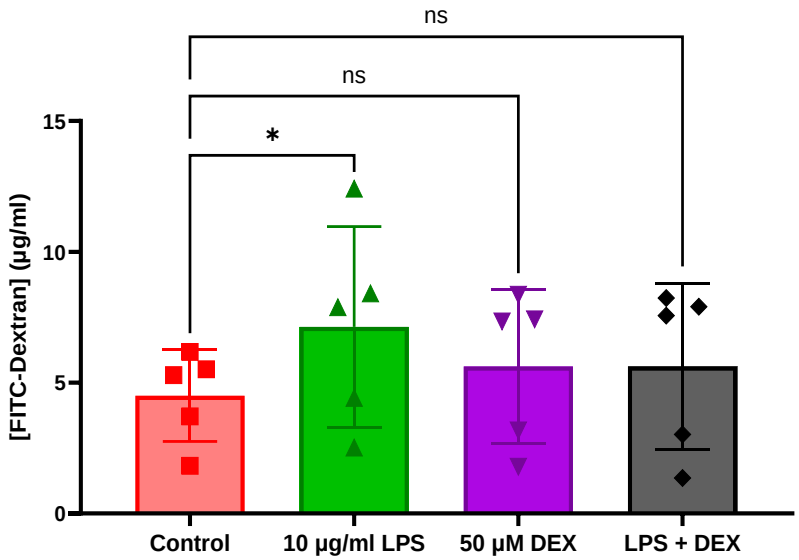


Figure 4.15: The effect of dexmedetomidine (50 µM) on LPS treated HUVECs. The samples were collected after 1 hour of adding the FITC-dextran solution. The data are presented as the mean ±SD for n=5. *p-value < 0.05 considered a significant difference, one-way ANOVA followed by Dunnett's test.

4.4. Discussion

The dysfunction of the endothelial barrier plays an important role in the induction of multiple organ failure in sepsis. Several in vitro studies have shown that LPS resulted in endothelial barrier hyperpermeability or barrier dysfunction (Table 4.1). The FITC-dextran leakage through the HUVECs seeded on the insert membrane was measured to establish an in vitro sepsis model and study the protective effect of dexmedetomidine on vascular endothelial permeability. This procedure along with the protocol used to measure the Trans Epithelial Electrical Resistance (TEER) by the EVOM and STX2 electrodes were previously reported to study the endothelial monolayer permeability (Wu *et al.,* 2019). Unfortunately, the TEER measurements taken in this study were excluded due to the variability of the device.

Several adjustments were needed to improve the results obtained from the permeability assay. It was difficult to get the monolayer of HUVECs on the inert membrane. This was partly solved by coating the surface of the inserts with 1% gelatin. However, gaps between cells were still observed even when the cells reached 90% confluence (Figure 4.12 A and Figure 4.13 A). This could affect the accuracy of the results because of the unregulated passage of the FITC-dextran expected through these gaps. Most previous studies reported that 1 µg/ml LPS results in endothelial apoptosis and hyperpermeability. In the present study, despite of that LPS affected the cell viability at all concentrations tested 0.1, 1, and 10 µg/ml (Figure 4.11C, D, and E), the barrier leakage of FITC-dextran was only significant at the highest concentration of LPS 10 µg/ml.

These results agreed with the study by (Sarmiento *et al*., 2014) which showed that LPS causes dose-dependent endothelial dysfunction in HUVECs through cell migration with 10 µg/ml LPS via TLR-4 receptors and the NF-κB pathway leading to significant dysfunction (Sarmiento *et al.,* 2014). A study on primary human pulmonary microvascular endothelial cells (HPMVECs) showed that LPS induced endothelial cell migration at concentrations lower than 1 µg/ml, which could help the barrier to repair itself through the cell division cycle protein 42 without affecting the endothelial permeability. These results suggest that HPMVECs were not damaged by low doses of LPS. Whereas the concentrations of LPS higher than 1 µg/ml reduced cell viability through the suppression of cell migrations, downregulation of VE-cadherin and claudin-5, and reduction in Akt phosphorylation (Lv *et al.,* 2017). Additionally, a study on HPMVECs showed that LPS has a biphasic concentration-dependent effect on HPMEC barrier function via the PI3K/Akt pathway, where the low doses of LPS (0.01 and 0.1 µg/ml) reduced the permeability by enhancing the cells migration and increasing VE-Cadherin and Claudin-5 expression. Conversely, high doses of LPS (10 and 100 µg/ml) increased the monolayer permeability (Zheng *et al.,* 2018), and these results agreed with the present findings.

The recent results showed that exposure to 10 µg/ml LPS significantly increased the barrier permeability of endothelial cells, and thus this concentration of LPS was chosen to study the effect of dexmedetomidine on endothelial permeability of HUVECs. The results showed that LPS at 10 µg/ml reduced the area covered by the cells and increased the permeability leakage after 24 hours of incubation, suggesting that the LPS induces endothelial apoptosis and hyperpermeability. The studies reported that dexmedetomidine plays a vital role in protecting organ functions in sepsis by improving

vascular leakage. Dexmedetomidine protects the vascular leakage following sepsis by inhibiting the ferroptosis (cell death) in septic cells obtained from humans and rats. Dexmedetomidine could effectively alleviate sepsis-induced vascular endothelial barrier dysfunction in septic rats by inhibiting the mitochondrial division and polymerization of the actin in the vascular endothelial cells via α_2-adrenoceptors (She *et al.,* 2021). Similarly, dexmedetomidine reduced cerebral vascular leakage and improved brain function in mice by elevating the expression level of occludin. It reduced the cytotoxicity, hyperpermeability, abnormal expression of occludin, and inflammatory factors in hypoxia/reoxygenation-induced endothelial dysfunction in HBMVECs (Zhao *et al.,* 2021).

The present study showed some protective role of dexmedetomidine on endothelial permeability, where dexmedetomidine abolished the LPS induced hyperpermeability suggesting its protection action of HUVECs endothelium barrier in sepsis (Figure 4.15). The concentration of 50 µM dexmedetomidine used in this study was previously reported to have the maximum protective effect against the LPS induced neuronal loss and apoptosis (Chen *et al.,* 2021). It is necessary to determine the effect of therapeutically relevant concentration of dexmedetomidine on endothelial permeability, but this was not possible in this study due to the limited access to the transwell inserts. Further investigations are also needed to study the effect of other α_2-adrenoceptor agonists such as guanfacine and mivazerol on endothelial permeability of different cell lines such as HBMVECs and HPMVECs.

5. Chapter Five: NA uptake1 inhibitors enhance the duration of noradrenergic vasoconstriction in porcine isolated blood vessels.

5.1. Introduction

NA uptake1 inhibitors are mainly used for the treatment of ADHD and major depression. These medical conditions are associated with the deficit in the central NA and this group of drugs is known to elevate the NA in the CNS (Zhou, 2004). Atomoxetine, a potent and selective NA uptake1 inhibitor could increase blood pressure by elevating the concentration of NA in peripheral sympathetic neurons. A randomised clinical study on patients with impaired central and peripheral autonomic nervous systems showed that atomoxetine caused a significant increase in the blood pressure of seated patients with central autonomic impairment (Shibao *et al.,* 2007). In addition, systematic searches of eighteen clinical trials on ADHD children and adolescents reported that atomoxetine significantly increased the SBP and HR (Hennissen *et al.,* 2017). This supports the hypothesis that co-administration of NA uptake1 inhibitors with NA infusion in ICU could enhance the vascular actions of NA and lower the required doses of NA. Subsequently, this reduces the risk of lethal complications associated with high doses of NA in septic shock (Stolk *et al*., 2020, Bode *et al*., 2024). Moreover, a recent study on septic murine models showed that amitriptyline, a NA reuptake inhibitor, has anti-inflammatory and protective actions in sepsis by decreasing macrophage TNF-α. It also improved the survival rate, increased IL-10 levels, and alleviated the pro-inflammatory responses in LPS treated mice, suggesting amitriptyline as a promising approach for treating inflammation in sepsis and septic shock (Xia *et al*., 2019a, Xia *et al*., 2019b).

5.1.1. Pharmacology of neuronal uptake transporters:

Catecholamines are translocated across plasma membranes by neuronal (uptake1) and non-neuronal (uptake2) transporters. The neuronal (uptake1) transporter involves dopamine transporters at dopaminergic neurons and noradrenaline transporters (NAT) at noradrenergic neurons. The action of neuronal uptake1 transporters on the extracellular space is the primary mechanism for the inactivation of endogenously released catecholamines and the rapid termination of their actions.

Catecholamines are polar chemicals that cannot readily penetrate the cell membrane to be metabolised by intracellular enzymes. The neuronal transporters remove the catecholamines at the synaptic across the cell membranes (Eisenhofer, 2001). The estimated lifetime of NA released is about 1.5 seconds, and the blockade of neuronal uptake will not only considerably increase the lifetime of the released NA, but also increase the amplitude of the response and diffusion of the transmitter away from synaptic junctions. Generally, neuronal uptake of catecholamine is more important than non-neuronal in terms of terminating catecholamine; however, this may vary between tissues due to different patterns and distribution of NAT (Stjarne *et al.,* 1994). There is a higher density of sympathetic innervation and NATs in the heart than in the kidneys and skeletal muscle resulting in more efficient neuronal uptake of NA in the cardiac cells than in renal cells and skeletal muscles (Kopin *et al.,* 1998). The existence of neuronal transporters near the site of catecholamine release suggests that the neurotransmitters removed by neuronal uptake mechanisms have a shorter duration and smaller action than the neurotransmitters removed by non-neuronal reuptake. Non-neuronal uptake may be particularly important to remove the catecholamines in tissues where high concentrations of adrenergic receptors exist at

extra-junctional locations. NA is metabolised via two major metabolic pathways: deamination by monoamine oxidase (MAO) and O-methylation by catechol-*O*-methyltransferase (COMT) (Axelrod and Kopin, 1969). However, most NA released by sympathetic nerves is removed by neuronal uptake1, while small amounts are removed by non-neuronal mechanisms or enter into the circulation (Eisenhofer, 2001). Figure 5.1 illustrates the release of NA and its degradation processes at noradrenergic neurons.

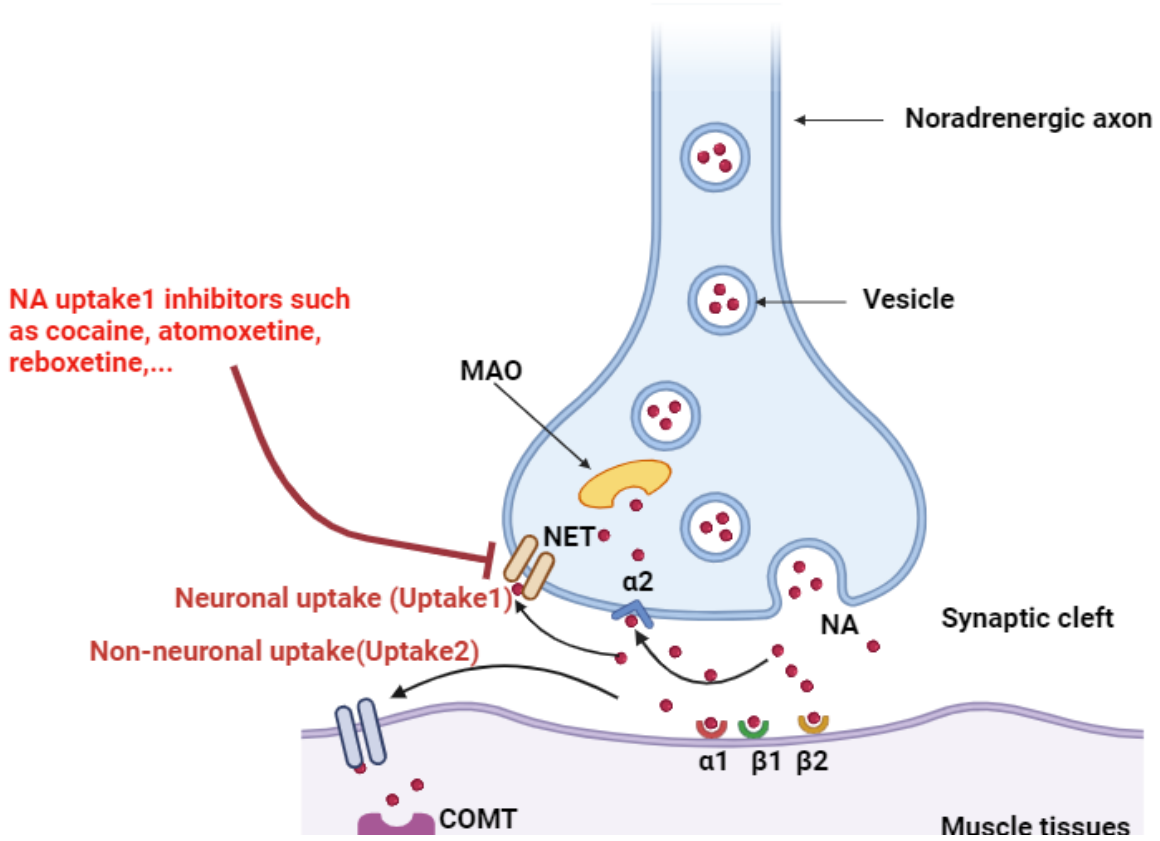


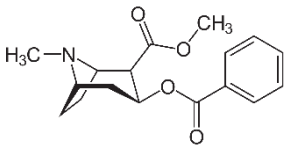
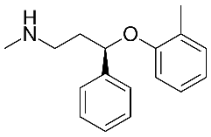
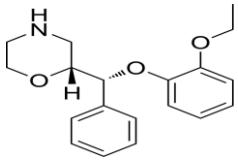
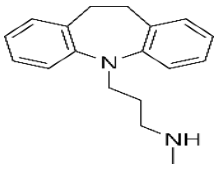
Figure 5.1: Representative diagram illustrates the release, neuronal (uptake1) of NA and uptake1 inhibitors at the noradrenergic neurons. NAT: NA transporters, MAO: monoamine oxidase, COMT: Catechol-O-methyltransferase (created **in Biorender.com**).

5.1.2. NA uptake1 inhibitors and their effect on sympathetic activity:

Inhibition of the NAT located in the plasma membrane of noradrenergic neurons is a mechanism for increasing the concentration of NA in the neuroeffector junction. Neuronal reuptake inhibitors block the presynaptic neuronal uptake of NA and serotonin (5-HT) and prolong the persistence of these monoamines in the synaptic cleft within the central nervous system (CNS). There are multiple clinical indications for serotonin-NA reuptake inhibitors, including treatments for depression, anxiety, and chronic pain (Fanelli *et al.,* 2021). Several clinical studies reported that the dysregulation of neuronal uptake of NA by sympathetic nerves contributes to essential hypertension, in addition to an increase in the vascular reactivity to exogenously administered NA and endogenously released NA. This dysregulation might be also the reason for the elevation in the level of NA in circulation in the elderly more than in young individuals (Goldstein, 1981, Goldstein *et al.,* 1983).

NA uptake1 inhibitors have a high affinity to NAT and potentiate the activation of postsynaptic α- and β- adrenoceptors. The high neuroeffector concentration of NA may lead to increased sympathetic activity and hence increase peripheral blood pressure and prolong the effects of NA in the body. A vasoconstrictor effect can be therefore achieved by using neuronal reuptake inhibitors (Eisenhofer *et al.,* 1991). Table 5.1 shows the chemical structure, indications, and plasma concentrations of NA uptake1 inhibitors such as cocaine, atomoxetine, reboxetine, and desipramine, which were used in this study.

Table 5.1: The chemical structures, therapeutic indications, and plasma concentrations of NA unptake1 inhibitors. Extensive and poor metabolisers refer to people with active and poor metabolic capabilities, respectively.

NA uptake1 inhibitors	Chemical structure (Kim *et al*., 2023)	Main therapeutic indications (NICE, 2023, Kim *et al*., 2023)	Plasma concentrations (Reference)
Cocaine		Used as a local anaesthetic agent in UK but it is not approved by the FDA for clinical uses.	100-250 ng/ml (330 nM – 820 nM) in cocaine used participants (Coe *et al.,* 2018)
Atomoxetine		ADHD in children and adults	159.70 (51.9) ng/ml in extensive metabolisers (~500nM) 914.72 (30.5) ng/ml in poor metaboliser (~3.5µM) (Sauer *et al.,* 2005)
Reboxetine		Major depression	111 ± 28 ng/ml (350 nM) in young healthy volunteers 271 ± 86 ng/ml (700 nM) in elderly volunteers (Bergmann *et al.,* 2000)
Desipramine		Depression, ADHD	21.8 ± 6.5 ng/ml (78.8 nM) in healthy volunteers (Harris *et al.,* 2007)

5.1.2.1. Cocaine:

Cocaine and its derivatives are not FDA-approved but are only allowed to be used as topical anaesthetic agents in oral, laryngeal, and nasal operations with severe restrictions. Cocaine had been widely used by ENT physicians since its discovery in ophthalmology as a local anaesthetic and vasoconstrictor in 1884. It is categorized as

a Schedule II substance under the UK Controlled Substances Act, where its administration causes serious CNS and CVS side effects such as restlessness, bradycardia, or tachycardia and may lead to severe psychological or physical dependence. It has a high potential for misuse and is rarely accepted to be medically used in the USA (Saif *et al.,* 2016). Its pharmacodynamic actions result from several mechanisms, including the blockage of sodium/potassium channels, in addition to the blockage of neuronal reuptake transporters causing an enhanced and prolonged sympathetic nervous system activity (Richards *et al.,* 2017).

It is also believed that cocaine might increase the affinity of post-junctional receptors to the agonist and block the pre-synaptic α-adrenoceptor causing an increase in the amount of released NA. It also modifies the properties of the post-junctional muscle membrane by suppressing the membrane K-conductance, with little effect on the Ca^{+2} receptors and the contractile proteins in the cell. Therefore, the super-sensitivity to NA may not only be related to the reduction in the uptake of NA but rather due to an increase in the NA sensitivity of the extra-junctional adrenoceptors. It has been found that there is a difference in the contractile action of cocaine on the vascular smooth muscle of various organs depending on the average distance between the adrenergic nerves and muscular junction. In guinea pig mesenteric arteries, cocaine did not mainly block the intra-junctional adrenoceptors but increased the sensitivity of the post-junctional adrenoceptors by increasing the overflow of NA (Kuriyama and Suyama, 1983).

Furthermore, the effect of cocaine on coronary circulation and peripheral vascular system might involve α and β adrenoceptors. In an animal study on dogs, cocaine

produced dose-dependent increases in MAP. The high doses resulted in significant vasoconstriction and increased the coronary resistance while no effect was observed at low doses. Also, cocaine reduced the systemic vascular resistance after treating the animal with phentolamine (α-blocker) and increased it after propranolol (β-blocker) suggesting that the pharmacological mechanisms of cocaine on the peripheral vascular system might involve both α and β adrenoceptors (Kuhn *et al*., 1990). Figure 5.2 shows the real time of NA release (NA current) in parallel to its contraction induced by EFS in the rat tail artery. The electrochemical techniques were proved to be used in monitoring NA release by combining the electrochemically treated carbon fiber electrodes with differential pulse voltammetry (DNPV) or with differential pulse amperometry (DPA). The inhibition of NA reuptake by cocaine increased the amplitude and duration of the released NA induced by EFS (Gonon *et al.,* 1993).

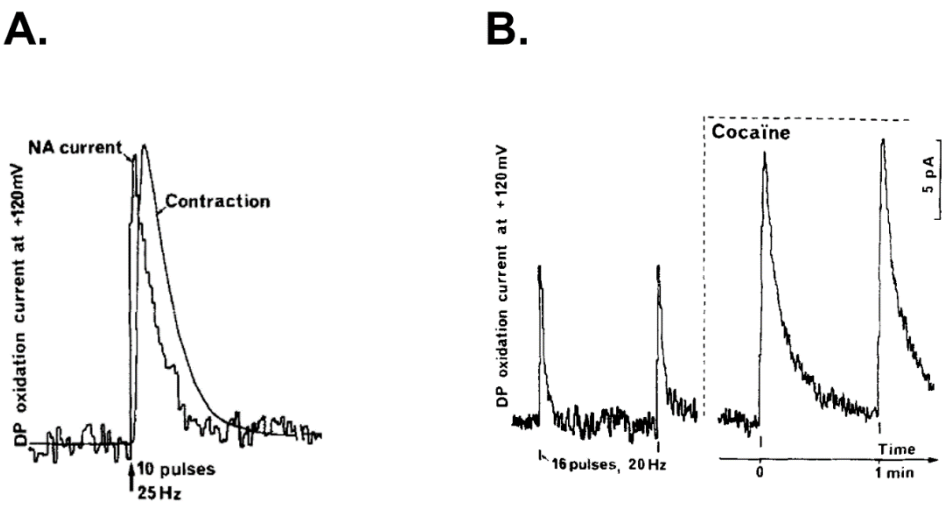


Figure 5.2: Traces showing the comparison between the local NA release and contraction induced by EFS (10 pulses at 25 Hz) (**A**), and the effect of cocaine (10 µM) on local NA concentration (**B**) induced by EFS (16 pulses at 20 Hz) in isolated rat tail artery (adopted from Gonon *et al.,* 1993).

5.1.2.2. Atomoxetine:

Atomoxetine is a potent and selective presynaptic NA uptake1 with an inhibitor constant of 3.3 nM, and a relatively weak ligand for α- and β-adrenoceptors, muscarinic, histaminergic H1, GABA and benzodiazepine receptors (Wong *et al.,* 1982). In healthy volunteers, administering atomoxetine at doses of 20 up to 90 mg produced a slight and transient increase in the BP without affecting the HR. It has been reported that there was a marked increase in the pressor sensitivity to the NA infusion by 261 ± 69% of control, which was enhanced by increasing the dose of atomoxetine (Zerbe *et al.,* 1985). Atomoxetine is a non-stimulant FDA approved drug that is used as a first or second choice for the treatment of ADHD with no risk of abuse or addiction. It is known to be metabolised by cytochrome P450 (CYP) 2D6, which is the reason for its non-linear kinetics between the doses and plasma concentrations. There are also some population differences in the metabolism of atomoxetine based on the polymorphisms of CYP enzyme. Accordingly, two groups of populations were identified in metabolising the atomoxetine. Those having rapid metabolic capabilities (extensive metabolisers) and it is the majority group with more than 90% of the population and those with poor metabolic capabilities (poor metabolisers) with up to 7% of the population. After a single oral dose (20 mg), atomoxetine achieved the maximum plasma concentration in 1 to 2 hours. However, the average steady state in poor metabolisers was approximately 10-fold higher than in rapid metabolisers. This is due to the difference in the half-life of atomoxetine which were 5.2 and 21.6 hours in rapid and poor metabolisers, respectively (Sauer *et al.,* 2005).

A randomised clinical trial on atomoxetine showed that it causes a small but significant rise in SBP in adults and DBP in adolescents and children, in addition to a significant

increase in the pulse rate in all groups. The increases in BP and pulse rate occurred in the early stages of treatment before reaching a plateau (Wernicke *et al.,* 2003). Moreover, the results from a randomised trial on children with impaired central and peripheral autonomic nervous systems revealed that atomoxetine (18 mg) acutely increased seated and standing SBP suggesting that atomoxetine enhanced vascular actions of NA by elevating the concentration of endogenous NA in peripheral sympathetic neurons (Shibao *et al.,* 2007).

5.1.2.3. Reboxetine:

Reboxetine is a highly selective NAT inhibitor, with low affinity to α-adrenoceptors and muscarinic receptors. It is FDA approved drug which belongs to the tricyclic class of antidepressants. It has been used to treat other psychiatric conditions including narcolepsy, ADHD, panic attacks, and depression in patients with Parkinson's disease and cocaine dependence disorder (Sepede *et al.,* 2012). Reboxetine has linear pharmacokinetics in young and healthy male individuals receiving single doses of 1-5 mg, and up to 4 mg twice a day in female and elderly individuals. It is rapidly absorbed within 1-2 hours after administration and has a half-life of termination equal to 13 hours (Dostert *et al.,* 1997).

Taylor 2008 has reported that reboxetine is considered cardiotoxic and might increase blood pressure when taken in overdose (Taylor, 2008). However, a recent review on anti-depressants has reported that reboxetine is a safe option and well tolerated in comparison to other TCA because of its neutral effect on BP in long-term treatment. Animal and human studies have shown that reboxetine has no significant effect on BP

and orthostatic hypotension with a very low effect on pulse rate. It might result in the reduction of BP in acute administration due to inhibiting presynaptic α_2-adrenoceptors. The desensitisation of the α_2-adrenoceptors in long-term treatment will initially enhance the release of NA followed by a later reduction in the sympathetic outflow, resulting in neutral effects on BP (Calvi *et al.,* 2021).

5.1.2.4. Desipramine:

It is a principal active metabolite of imipramine and belongs to the group of tricyclic antidepressants commonly used for treating depression. It is believed that its pharmacological action is due to inhibition of NA uptake1 in the central and peripheral nervous system, causing an increase in sympathetic activity. It also has anticholinergic and antireserpine-like actions (Wali and Greenidge, 1990). In an experimental study of anti-depressants on healthy volunteers, desipramine 100 mg potentiated the NA vasoconstriction during local infusion into the superficial dorsal hand vein. In addition, desipramine significantly increased the heart rate and reduced salivation suggesting that these results are secondary to NA uptake inhibition (Abdelmawla *et al.,* 1999). It could be safely used for treating the symptoms of ADHD in children and adults (Ghanizadeh, 2013). It is metabolised into 2-hydroxy desipramine by CYP2D6 enzyme and has a half-life of 21 hours (Harris *et al.,* 2007). The results obtained from the rat isolated seminal vesicle showed that desipramine enhanced the contractions induced by endogenous and exogenous contraction due to their NA uptake inhibitor activity. On the other hand, desipramine reduced the clonidine vasocontraction suggesting its blockade effect of adrenoceptors in the rat seminal vesicle (Wali and Greenidge, 1990).

Due to the potential role of atomoxetine in raising the BP by increasing NA concentrations in peripheral sympathetic neurons (Shibao *et al*., 2007, Hennissen *et al*., 2017). The present study hypothesised that the co-administering of NA uptake1 inhibitors with NA infusion in critically ill patients could enhance the vascular effects of NA. This suggests the potential benefit of NA uptake1 inhibitors in lowering the doses of NA infusion, causing a reduction in the risk of lethal complications in septic shock (Stolk *et al*., 2020, Bode *et al*., 2024).

5.1.3. Aims of the study:

1. To demonstrate the effect of selective NA uptake1 inhibitors on peak contractions induced by EFS in porcine isolated splenic and renal arteries.

2. To demonstrate the effect of selective NA uptake1 inhibitors on the duration of 50% relaxation of the contractions induced by EFS in porcine isolated splenic and renal arteries.

3. To demonstrate the NA sparing effects of cocaine and atomoxetine in porcine isolated splenic arteries.

5.2. Methods

5.2.1. Materials and Chemicals:

The salts and chemicals used for preparing the Krebs-Henseleit solution are mentioned in Chapter 3 (Table 3.2). The chemicals and drugs used in the current study are listed in Table 5.2.

Table 5.2: List of the chemicals and drugs used in this contractility study.

Chemicals and drugs	Solvents	Source/Manufacture
Atomoxetine Hydrochloride	DW	Sigma Aldrich, Germany
Cocaine Hydrochloride	DW	Sigma Aldrich, Germany
Desipramine Hydrochloride	DW	Sigma Aldrich, USA
Reboxetine Mesylate	DW	MedChem Express, USA
Prazosin Hydrochloride	DW	TOCRIS Bioscience, Bristol, UK

5.2.2. Experimental Protocols using EFS:

In the present study, two protocols of electrical field stimulation (EFS): trains of frequency (2 - 64 Hz) and single repeated frequency (16 Hz) were used to study the effect of neuronal uptake1 inhibitors on endogenous NA. Table 5.3 summarises the main experimental protocols of this study.

Table 5.3: Experimental protocols of EFS and isolated blood vessels used in this study.

Tissues and protocols	EFS (10 min interval)	NA uptake1 inhibitors
(A) Splenic Artery	2 - 64Hz, 0.3ms 200mA 10s	10 nM Atomoxetine 3 µM Cocaine 100 nM Desipramine 300 nM Reboxetine 100 nM Reboxetine
(B) Renal Artery	2 - 64Hz, 0.3ms 200mA 10s	10 nM Atomoxetine 3 µM Cocaine
(C) Splenic Artery	16Hz, 0.3ms 200mA 10s	10 nM, 100 nM, 1 µM Atomoxetine
(D) Splenic Artery	16Hz, 0.3ms 200mA 30s	10 nM, 100 nM, 1 µM Atomoxetine
(E) Renal Artery	16Hz, 0.3ms 200mA 10s	10 nM, 100 nM, 1 µM Atomoxetine 0.3 µM, 3 µM Cocaine

5.2.2.1. EFS produced by trains of frequencies (2 - 64 Hz):

The collection and dissection of the porcine isolated blood vessels were carried out as mentioned in section 3.2.1. The segments mounted in the organ bath were stimulated using two stimulator electrodes linked to the Digitimer Multistim System D330 (Figure 5.3). The tissues were then contracted with 60 mM KCl (as described previously in section 3.2.2.1) and allowed to recover and stabilise for about 40 minutes. The tissues

were then exposed to EFS at increasing frequencies: 2 Hz, 4 Hz, 8 Hz, 16 Hz, 32 Hz, and 64 Hz at 0.3 milliseconds (ms) pulse width, 200 milliamperes (mA) for 10 seconds duration at 10 minutes intervals. In the beginning, the segments of isolated splenic and renal arteries were exposed to two sets of EFS (2 - 64 Hz) without adding the drugs to assess the consistency of the responses. To study the effect of NA uptake1 inhibitors on frequency response curve (FRC), the segments from splenic and renal arteries were exposed to either cocaine (3 μM), atomoxetine (10 nM), desipramine (100 nM), or reboxetine (100 nM and 300 nM) after the first set of EFS (2 - 64 Hz). The segments were then left for an incubation period of 40-50 minutes before starting the second set of EFS (2 - 64Hz).

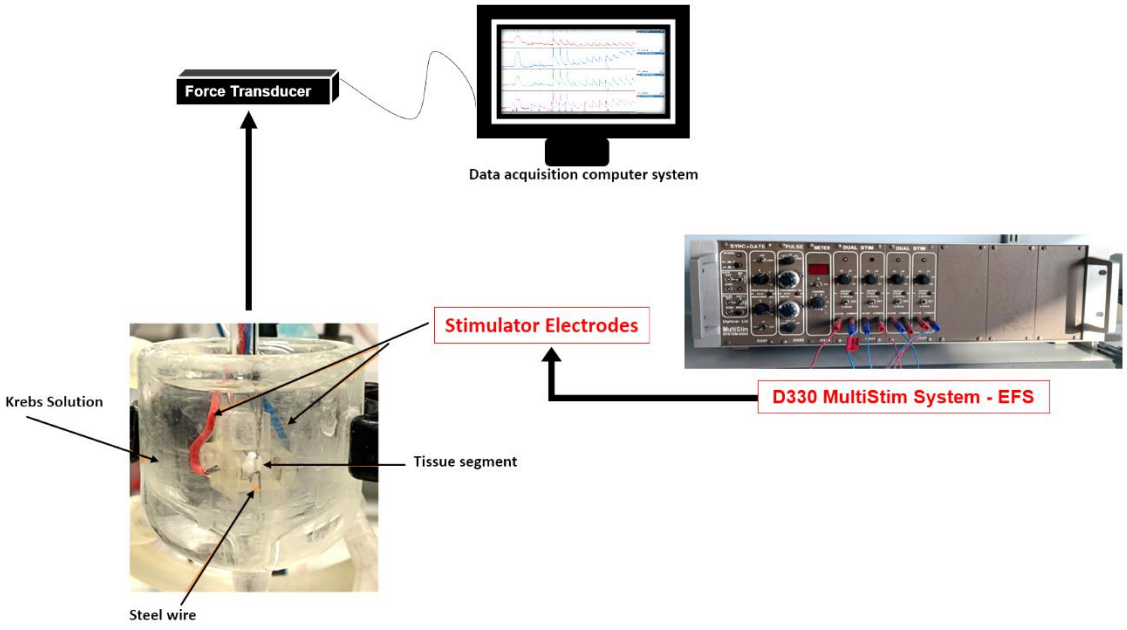


Figure 5.3: Schematic diagram shows EFS equipment linked to the stimulator electrodes in an organ bath containing a segment of porcine splenic artery mounted in Krebs-Henseleit solution.

5.2.2.2. EFS using single frequency (16 Hz):

Following the tissue setup of isolated porcine blood vessels in an organ bath as mentioned in the previous section, the segments were stimulated with a repeated EFS at a single frequency of 16 Hz, 0.3 ms pulse width, and 200 mA for a stimulation duration of either 10 or 30 seconds. The time interval between the stimulations was 10 minutes. To assess the consistency of the responses, the tissues were stimulated for up to 160 minutes without adding the drugs. To study the effect of NA uptake1 inhibitors, the tissues were stimulated for 60 minutes, and then the drug was added at the point following the 6th EFS. The tissues were stimulated for a further 60 minutes to achieve a total of 12 EFSs (Figure 5.4). The magnitude and time to 50% relaxation of the response at 30 minutes (3rd EFS) before exposure to the drug was used as the control and this was compared to 30 minutes (9th EFS) and 60 minutes (12th EFS) after exposure to the drug. Prazosin (100 nM) was added at the end of the experiment to check the effectiveness of NA responses on postsynaptic α_1-adrenoceptors induced via electrical field stimulation in isolated blood vessels.

5.2.3. Experimental protocols for studying the NA sparing effect of NA uptake1 inhibitors in porcine splenic artery:

In these experiments, the segments of porcine isolated splenic arteries were pre-contracted with NA uptake1 inhibitors before the addition of NA. The segments were exposed to one of the following agonists: cocaine (1 and 10 µM), atomoxetine (10 and 100 nM), or dexmedetomidine (10 nM) with or without atomoxetine (10 and 100 nM). Subsequently, the cumulative concentrations of NA (1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM) were added.

5.2.4. Data Analysis and Statistics:

All the data obtained from the traces were calculated using the Excel Microsoft Office, and statistically analysed and plotted as graphs using Prism version 10.0.3 (GraphPad, USA). The magnitude of the electrically stimulated contractions was expressed as a percentage of the 60 mM KCl in experiments using increasing frequencies (2 - 64 Hz), or a percentage of the 6th EFS for the experiment using a single frequency (16 Hz). The time required for the peak response to relaxing by 50% was measured by calculating the time from the beginning of the stimulation (baseline) to the time point where there is a 50% decline in the peak response as prescribed in Figure 5.4, and it was expressed as the % to the first EFS obtained at 64 Hz, or to the 6^{th} EFS when single frequency 16 Hz was used. All values were expressed as the mean ± standard error of the mean (± SEM) of observations from repeated experiments. For statistical analysis, the Student's paired t-test (two-tailed) was used to compare between two groups, and the ordinary one-way ANOVA parametric test followed by Dunnett's test was used to compare the results of more than two groups. The *p-value < 0.05 was considered statistically significant.

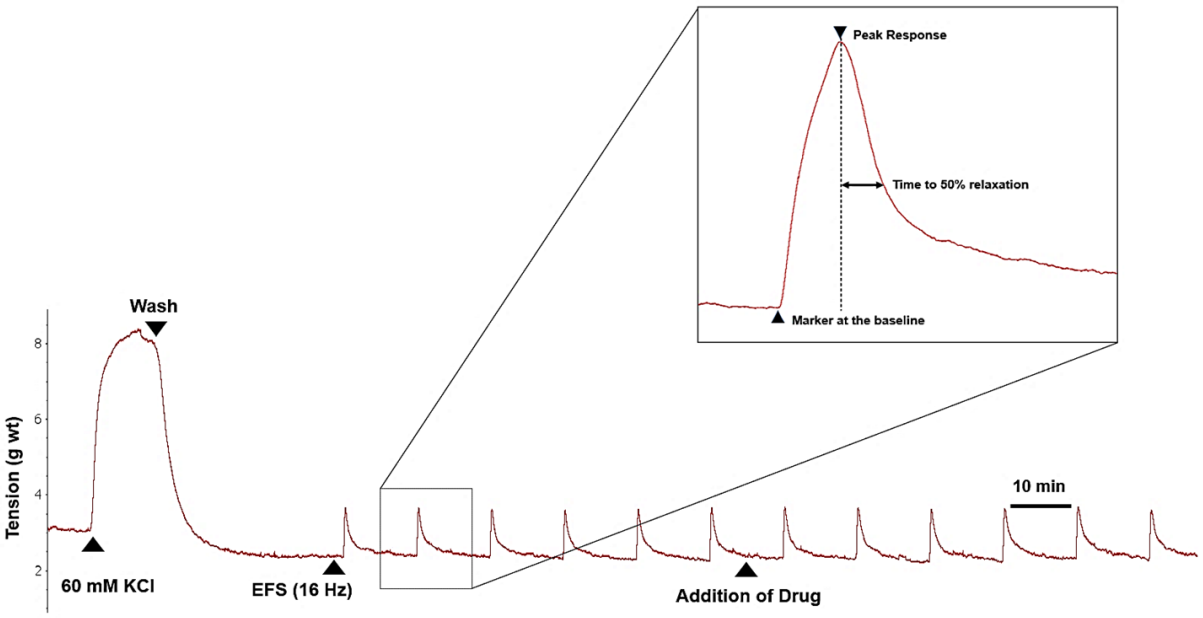


Figure 5.4: Representative diagram showing the recorded traces of EFS induced contractions and the protocol of adding the drug and measuring the peak response and the time to 50% relaxation.

5.3. Results

5.3.1. The responses of EFS (2 - 64 Hz) in isolated porcine blood vessels:

The contractile responses obtained from 60 mM KCl in porcine splenic and renal arteries were equivalent to 7.8 ± 0.47 g wt (n=21), and 6.84 ±1.53 g wt (n=6), respectively. The EFS (2 - 64 Hz) of the porcine splenic and renal arteries resulted in frequency-dependent contractions (Figure 5.5). In the comparison of the absolute values of the splenic and renal arteries, the magnitude of peak contraction of the 1st EFS at frequencies (2 - 64 Hz) in splenic arteries was slightly bigger than that of renal arteries, on the other hand, the time to 50% relaxation in EFS response at 64 Hz was higher in renal than that of splenic arteries. The small responses that couldn't be analysed were excluded from the results such as the responses observed at low frequencies (2 Hz and 4 Hz) in porcine renal artery (Table 5.4). The peak responses of the 2nd EFS were significantly higher than the 1st EFS at frequencies (16, 32, and 64 Hz) in splenic arteries (Figure 5.6). Also, significant differences were observed in relaxation time to 50% between 1st and 2nd EFS at frequencies 4 Hz and 8 Hz in porcine splenic artery and frequencies 16 Hz and 32 Hz in porcine renal artery (Figure 5.7). Therefore, the responses obtained from the 2nd EFS were used to compare the results of treatment groups with the control group.

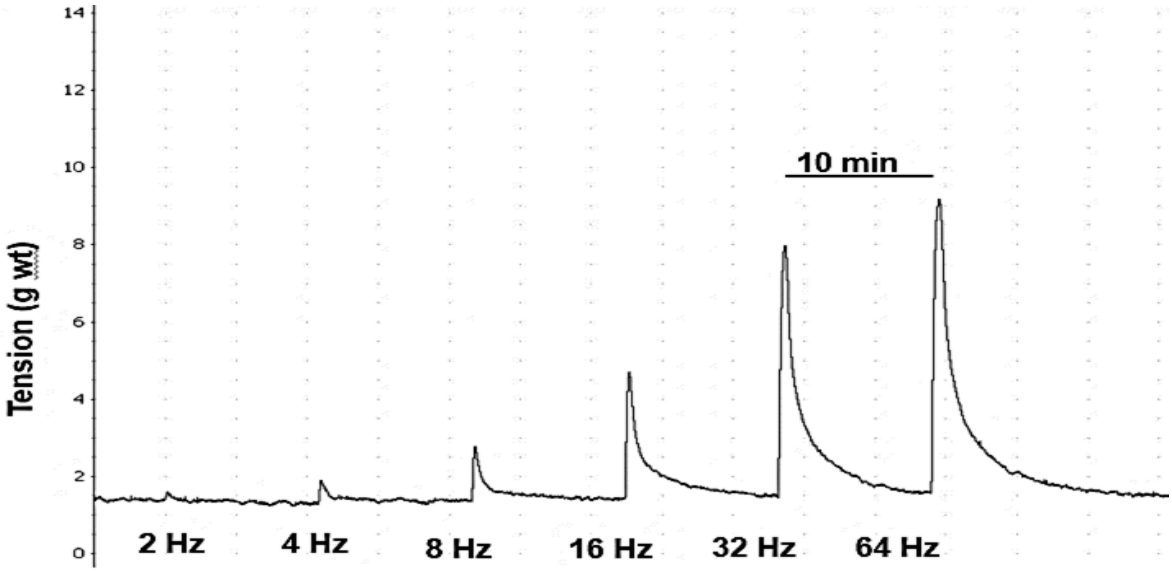


Figure 5.5: Representative traces show the responses of electrically stimulated contractions (EFS, 2 - 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in porcine isolated splenic arteries.

Table 5.4: The absolute values of the peak contractions (g wt) and time to 50% relaxation (seconds) in porcine splenic artery (PSA) and PRA (porcine renal artery) following electrical stimulation at different frequencies. Data are presented as mean ± SEM.

PSA (n=18-21)						
Parameters (unit)	**2 Hz**	**4 Hz**	**8 Hz**	**16 Hz**	**32 Hz**	**64 Hz**
Peak contraction (g wt)	0.17 ± 0.04	0.54 ± 0.08	1.28 ± 0.17	3.41 ± 0.35	7.02 ± 0.49	8.57 ± 0.55
Time duration to 50% relaxation (Sec)	18.9 ± 2.72	18.2 ± 1.79	21.9 ± 1.97	28.9 ± 1.93	40.6 ± 3.33	47.7 ± 4.29
PRA (n=5-6)						
Peak contraction (g wt)			0.61 ± 0.28	1.80 ± 0.61	5.01 ± 1.05	6.89 ± 1.27
Time duration to 50% relaxation (Sec)			12.74 ± 1.02	17.84 ± 2.90	40.48 ± 11.43	79.63 ± 16.40

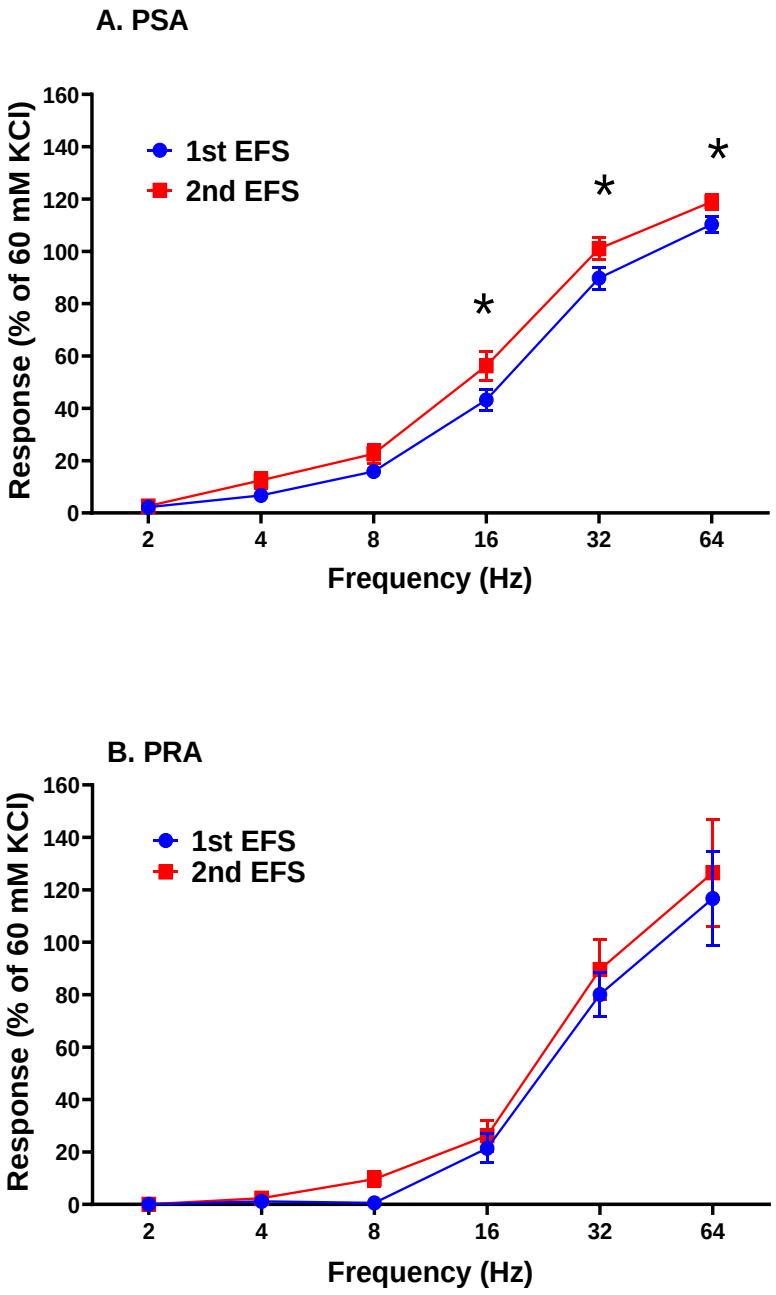


Figure 5.6: Peak responses of the 1st and 2nd electrically stimulated contractions (EFS, 2 to 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in (**A**) porcine splenic arteries (n= 21), and (**B**) porcine renal arteries (n= 6). Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM. *p <0.05 represented significant differences, multiple two tailed t-tests.

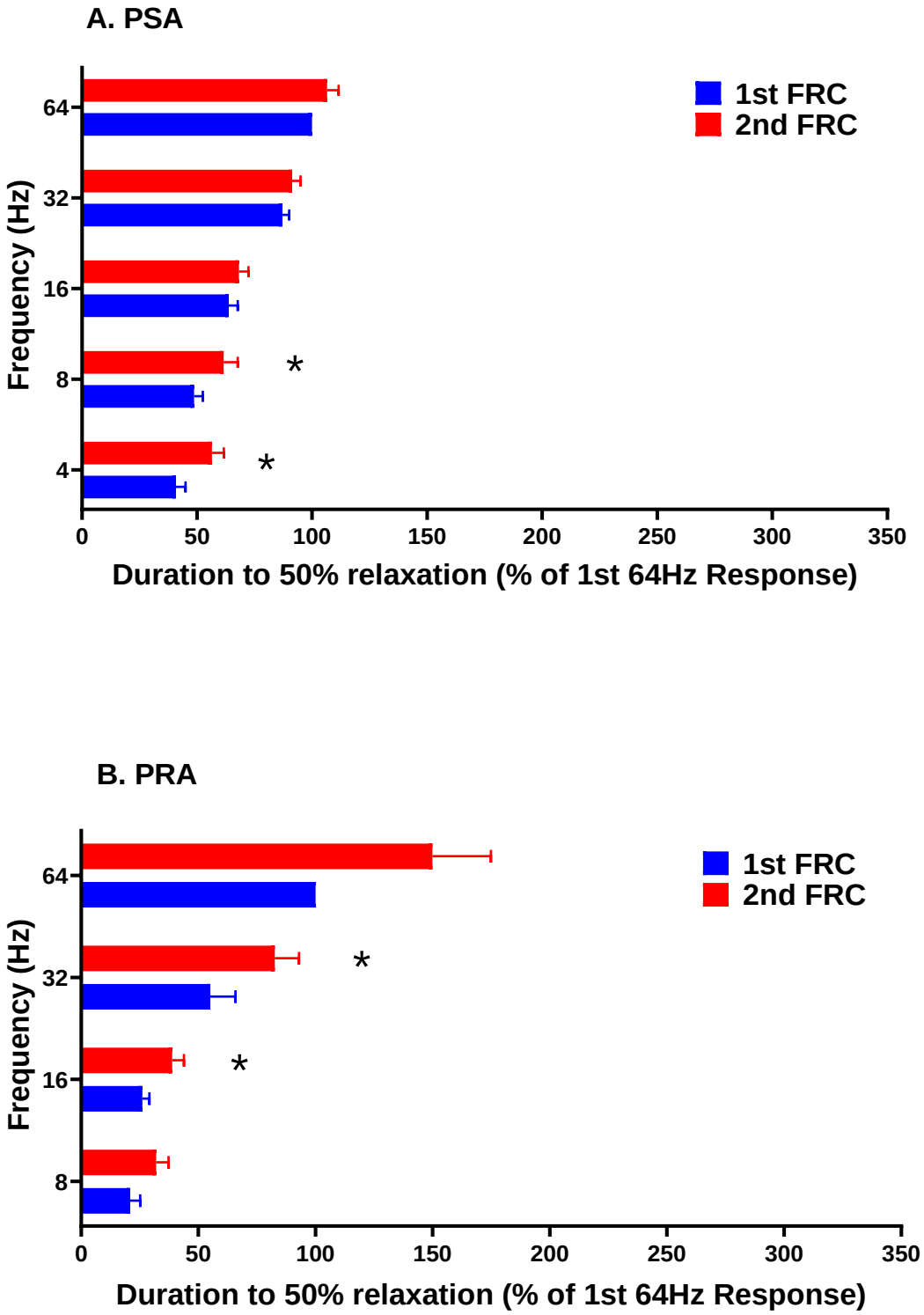


Figure 5.7: Duration to 50% relaxation of the 1st and 2nd electrically stimulated contractions (EFS, 2 to 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in (**A**) porcine splenic arteries (n= 18-21), and (B) porcine renal arteries (n= 5-6). Data are expressed as the percentage of 1st 64 Hz responses and presented as mean ± SEM. *p <0.05 represented significant differences, multiple two tailed t-tests.

5.3.1.1. Effect of NA uptake1 inhibitors on EFS (2 - 64 Hz) in porcine splenic artery:

In the porcine splenic artery, atomoxetine (10 nM) and cocaine (3 µM) produced no significant effect on the magnitude of electrically stimulated contraction at all the frequencies (2 - 64Hz) (Figure 5.8 and 5.9). Also, no significant changes were observed in the time to 50% relaxation of the electrically stimulated contractions at low frequencies (2 Hz, 4 Hz, and 8 Hz). However, atomoxetine significantly increased the time to 50% relaxation at higher frequencies (16 Hz, 32 Hz, and 64 Hz), and cocaine increased it at 16 Hz and 32 Hz (Figure 5.10).

In separate experiments, two concentrations of reboxetine (100 nM and 300 nM), and desipramine (100 nM) (table 5.3 A) were tested against the control in the electrically stimulated porcine splenic artery, where they didn't affect the magnitude of the contractions at all the frequencies (Figure 5.11 and 5.12). On the other hand, the small concentration of reboxetine 100 nM produced a significant increase in the time duration to 50% relaxation in the electrically stimulated porcine splenic artery at frequencies 4 - 64 Hz, while a higher concentration of 300 nM increased the time duration to 50% relaxation at higher frequencies 32 and 64 Hz only. Desipramine did not affect the relaxation time at all the frequencies except 32 Hz, where it significantly increased the time to 50% relaxation, but the change was smaller than that of reboxetine (Figure 5.11, 5.12, and 5.13).

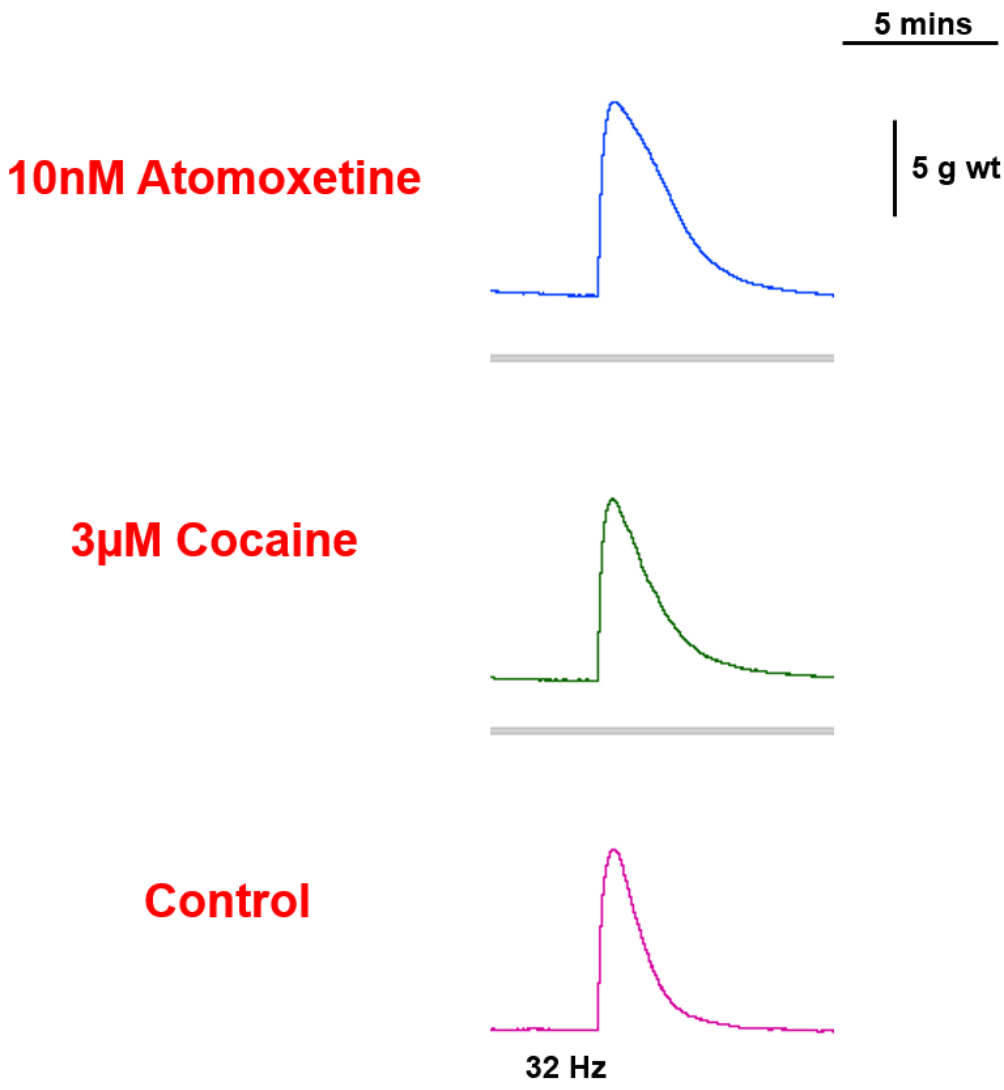


Figure 5.8: Representative traces show the responses of electrically stimulated contractions (EFS 32 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in the absence and presence of 10 nM atomoxetine and 3 µM cocaine in porcine splenic artery.

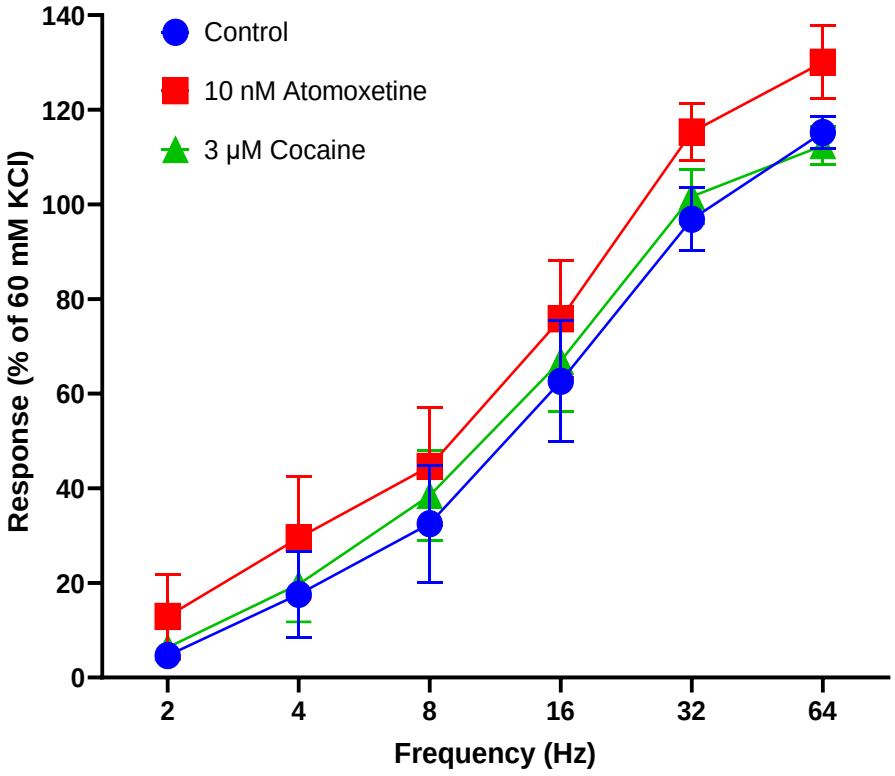


Figure 5.9: The effect of atomoxetine (10 nM) and cocaine (3 µM) on peak responses of the electrically stimulated contractions at frequencies 2 -64 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in porcine splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, control group n=7, atomoxetine group n=9, cocaine n=9.

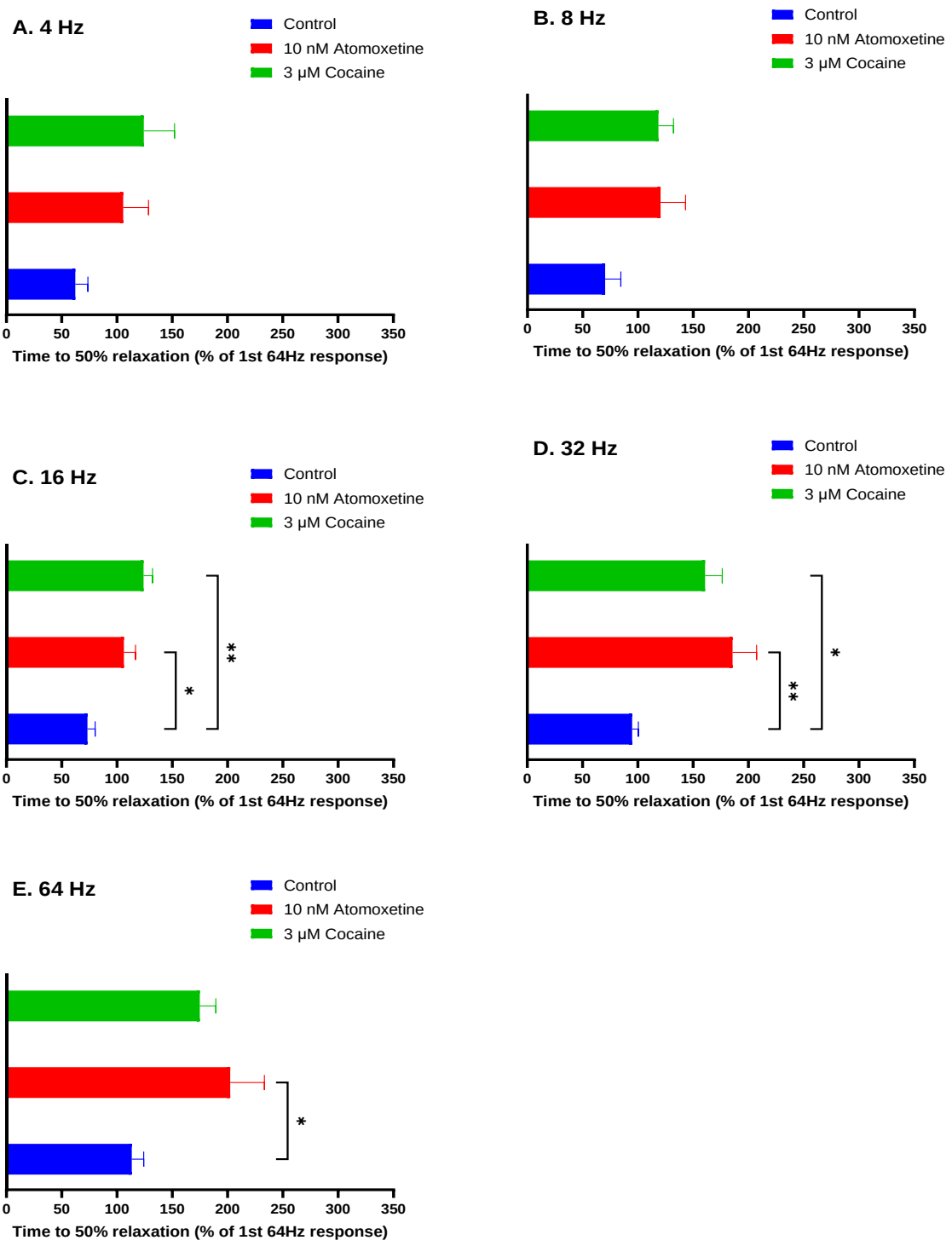


Figure 5.10: The effect of atomoxetine (10 nM) and cocaine (3 µM) on time to 50% relaxation of the electrically stimulated contractions at different frequencies (**A**) 4 Hz, (**B**) 8 Hz, (**C**) 16 Hz, (**D**) 32 Hz, (**E**) 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in porcine splenic arteries. Data are expressed as the percentage of 1st 64 Hz responses and presented as mean ± SEM, n=6-9. *p < 0.05. **p < 0.01 represented significant differences, one-way ANOVA followed by Dunnett's test.

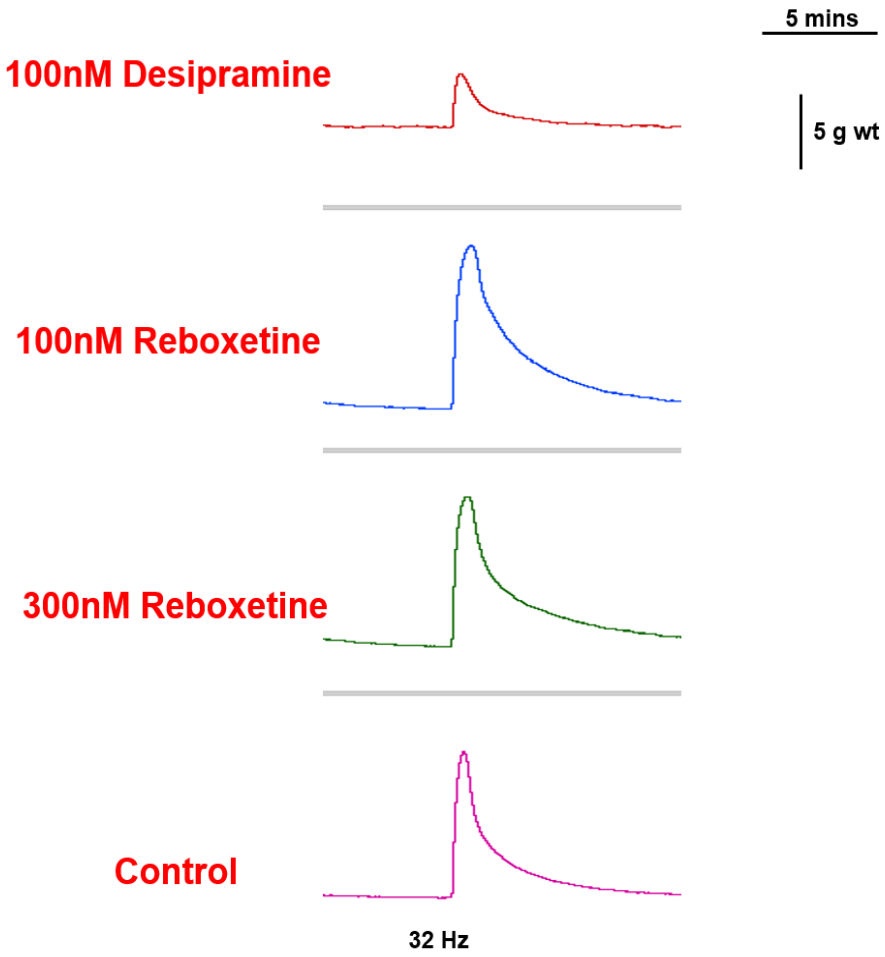


Figure 5.11: Representative traces show the responses of electrically stimulated contractions (EFS 32 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in the absence and presence of 100 nM and 300 nM reboxetine, and 100 nM desipramine in porcine splenic artery.

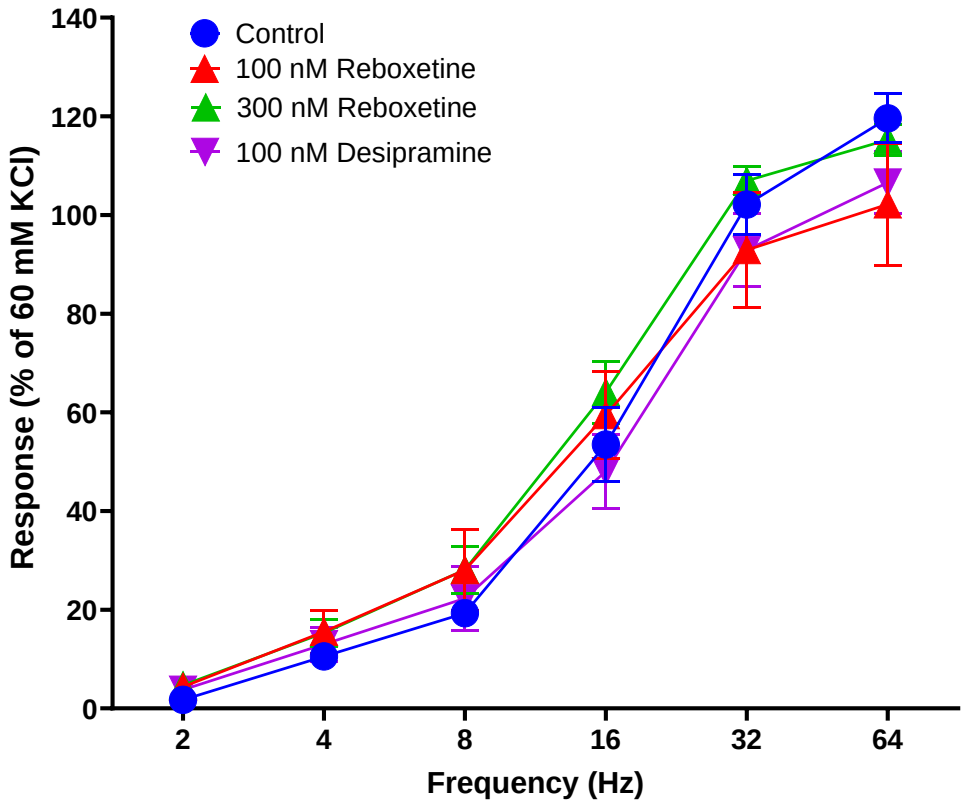


Figure 5.12: The effect of reboxetine (100 nM, 300 nM) and desipramine (100 nM) on peak responses of the electrically stimulated contractions at frequencies 2 - 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in porcine splenic arteries. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM, n=10.

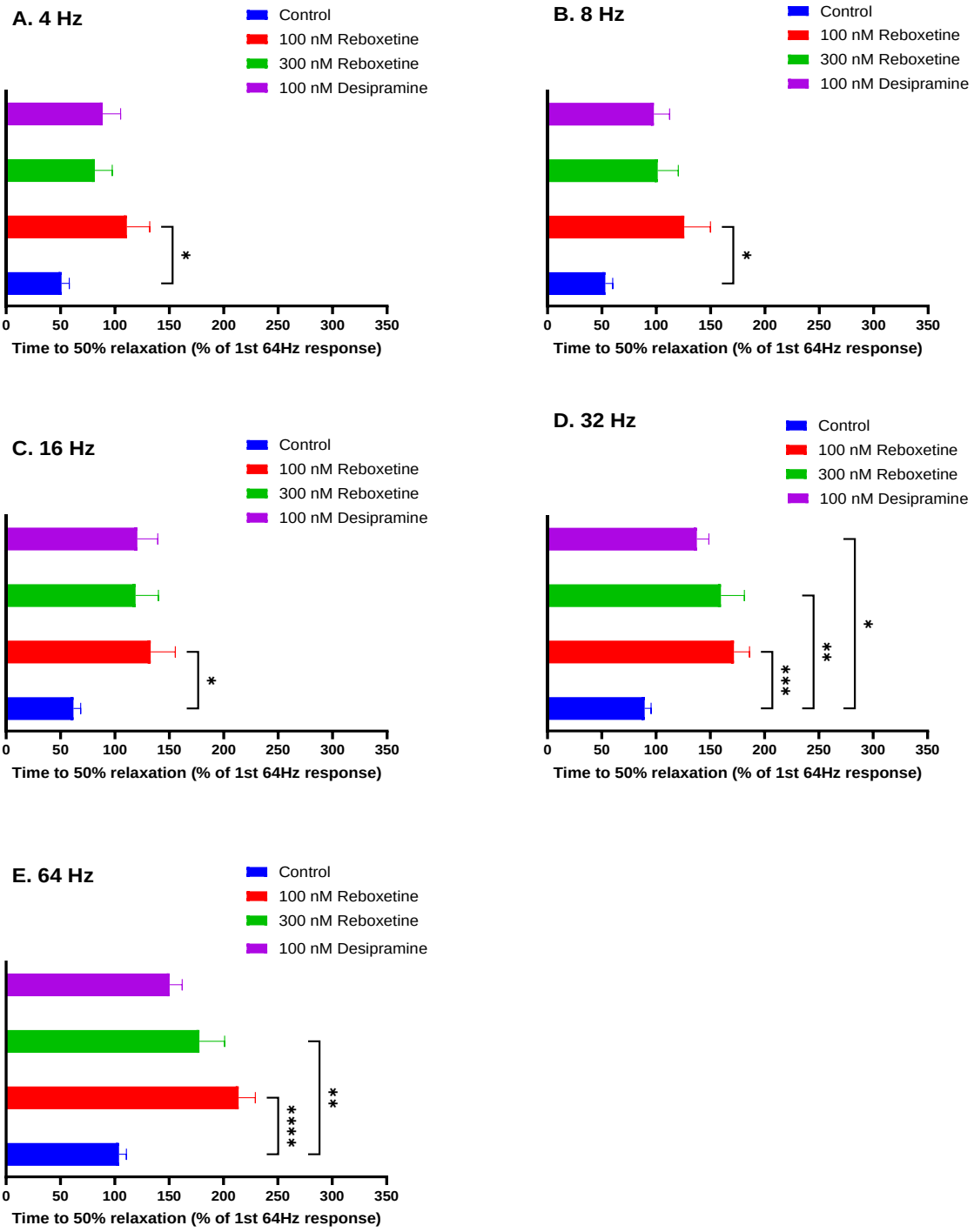


Figure 5.13: The effect of reboxetine (100 nM, 300 nM) and desipramine (100 nM) on time to 50% relaxation of the electrically stimulated contractions at different frequencies (**A**) 4 Hz, (**B**) 8 Hz, (**C**) 16 Hz, (**D**) 32 Hz, (**E**) 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation) in porcine splenic arteries. Data are expressed as the percentage of 1st 64 Hz responses and presented as mean ± SEM, n =7-10. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 represented significant differences, one-way ANOVA followed by Dunnett's test.

5.3.1.2. Effect of atomoxetine and cocaine on EFS (2 - 64 Hz) in porcine renal arteries:

Table 5.5 shows the effect of atomoxetine and cocaine on EFS (8 - 64 Hz, 10 Sec stimulation) in porcine renal arteries. In contrast to their effects in the porcine splenic artery, they both produced significant increases in the magnitude of the contractions in the porcine renal artery. Atomoxetine increased the magnitude at a frequency of 32 Hz (p-value=0.016), and cocaine increased the magnitude at frequencies of 8 Hz (p-value=0.04) and 16 Hz (p-value=0.024). There were no other significant effects on the magnitude of the EFS induced contractions at other frequencies. However, the impacts of atomoxetine and cocaine on the time to 50% relaxation of EFS responses was smaller in the porcine renal artery than that observed in the porcine splenic artery. Contrary to the porcine splenic artery, atomoxetine produced no significant increase in the time to 50% relaxation of EFS responses at 8 - 64 Hz frequencies, while cocaine produced a significant increase at 16 Hz (p-value=0.018) only. However, these results could be due to the small sample size and the mean size of the responses which was much larger than that in porcine splenic artery resulting in large SEM values, particularly at high frequencies.

Table 5.5: The effect of atomoxetine (10 nM) and cocaine (3 µM) on peak responses and time to 50% relaxation of EFS induced contractions (8 - 64 Hz, 0.3 ms, 200 mA, 10 sec stimulation) in the porcine renal artery. Data are presented as mean ± SEM. *p < 0.05 represented significant differences in comparison to control, one-way ANOVA followed by Dunnett's test.

Conditions	Parameters from the 2nd EFS	Frequency of EFS			
		8 Hz	16 Hz	32 Hz	64 Hz
Control n= 5-6	Peak contraction (% to 60 mM KCl)	16.2 ± 3.4	43.7 ± 3.9	111.2 ± 3.4	154.9 ± 7.7
	Time to 50% relaxation (% of 1st 64 Hz EFS)	32.0 ± 5.2	38.8 ± 5.0	82.7 ± 10.3	149.9 ± 24.9
Atomoxetine (10 nM) n= 7	Peak contraction (% to 60 mM KCl)	25.9 ± 3.7	59.3 ± 3.6	133.6* ± 4.9	173.3 ± 14.6
	Time to 50% relaxation (% of 1st 64 Hz EFS)	55.3 ± 10.1	84.0 ± 16.1	177.6 ± 30.4	227.5 ± 38.5
Cocaine (3 µM) n= 7	Peak contraction (% to 60 mM KCl)	31.4* ± 4.6	63.0* ± 5.7	117.6 ± 6.5	140.9 ± 9.8
	Time to 50% relaxation (% to 1st 64 Hz EFS)	136.0* ± 42.9	130.3* ± 28.8	211.4 ± 59.3	240.0 ± 57.2

5.3.2. The responses of EFS using a repeated, single frequency (16 Hz) in isolated porcine blood vessels:

The reproducibility of the responses induced by the repeated EFS at a single frequency of 16 Hz was assessed in the porcine splenic artery to ensure the consistency of the responses produced in these experiments. Figure 5.14 shows representative traces of the repeated EFS (16 Hz, 0.3 ms, 200 mA, 30 sec stimulation time) with 10 minutes intervals between stimulations in porcine splenic and renal arteries without adding drugs. In the porcine splenic artery, the results showed higher peak responses at the beginning of the EFS, which intend to consistently reduce and stabilise after the 4th EFS. The time to 50% relaxation was nearly constant starting at the 3rd EFS response and remained constant over time for up to 2 hours. In the porcine renal artery, the trace recording showed more constant responses from the beginning of stimulation and lasted for at least 2 hours. Table 5.6 has shown that there is a significant reduction (p-value < 0.0001) in the magnitude of the EFS induced contractions in the porcine splenic artery when comparing the 3rd EFS (control) to the 9th and 12th EFS. However, no significant changes were observed in the time to peak and to 50 % relaxation of the EFS induced contractions over the 2 hours of stimulation.

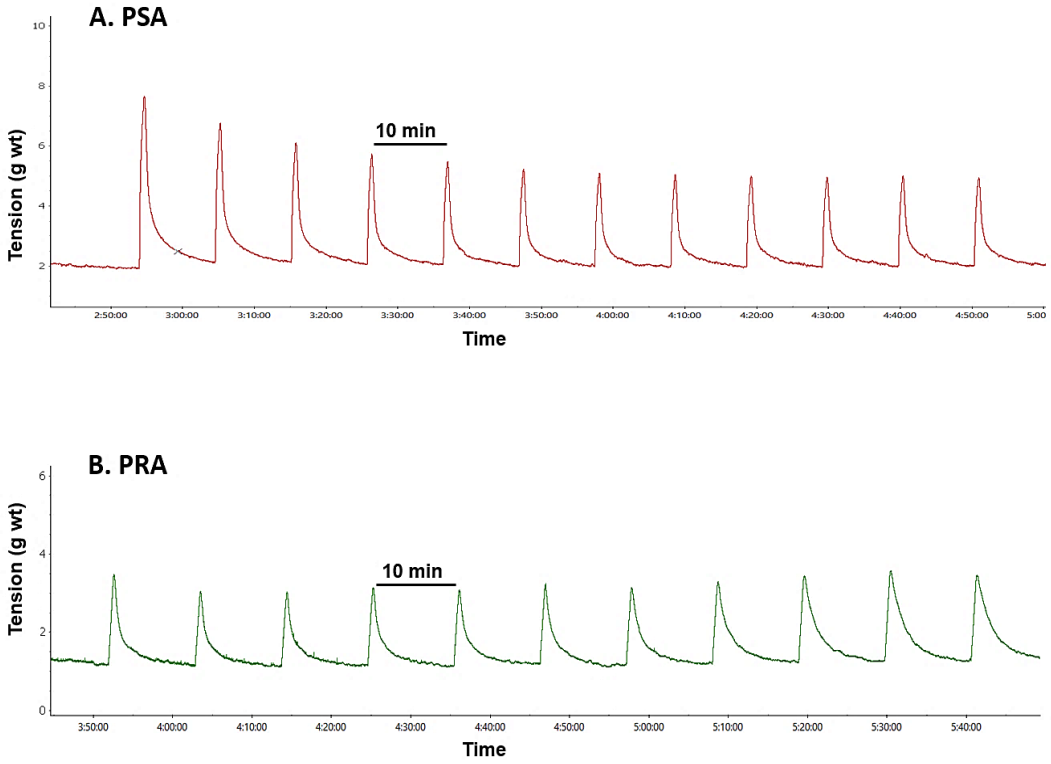


Figure 5.14: Representative traces show the responses of electrically stimulated contractions (EFS, 16 Hz, 0.3 ms, 200 mA, 30 sec stimulation) in (**A**) porcine splenic artery and (**B**) porcine renal artery.

Table 5.6: Reproducibility of the EFS responses showing the peak responses, time to the peak and 50% relaxation of 3rd EFS (control), 9th EFS, and 12th EFS (16 Hz, 30 sec, 0.3 ms, 200 mA) in porcine splenic artery. Data are expressed as the percentage of the 6th EFS response and presented as mean ± SEM, n=16. The ****p-value < 0.0001 was considered statistically significant, one-way ANOVA followed by Dunnett's test.

Parameters (% of the 6th EFS)	3rd EFS	9th EFS	12th EFS
Magnitude	117.3 ± 2.5	91.9 ± 2.6****	86.8 ± 4.7****
Time to peak responses	107.9 ± 3.0	107.6 ± 3.4	109.4 ± 3.3
Time to 50% relaxation	98.7 ± 2.8	102.1 ± 2.9	110.5 ± 3.9

5.3.2.1. Effect of atomoxetine on EFS (16Hz, 30 or 10 sec stimulation) in porcine splenic arteries:

These experiments aimed to compare the effect of different concentrations of atomoxetine on responses induced by repeated EFS at a single frequency (16 Hz) in porcine splenic and renal arteries. Two different stimulation duration of EFS was used 30 and 10 seconds (Table 5.3 C and D). In the porcine splenic artery, atomoxetine (10 nM, and 100 nM) produced no significant increase in the peak responses of EFS (16 Hz, 30 sec stimulation). However, a high concentration of atomoxetine (1 µM) significantly reduced the magnitude of the peak responses after 60 minutes of stimulation as shown (Figure 5.15). Nonetheless, atomoxetine produced a significant increase in the relaxation time of the electrically stimulated responses. Atomoxetine increased time to 50% relaxation after 60 minutes, while the higher concentrations (100 nM and 1 µM) increased the relaxation duration after 30 minutes of exposure to the drug as well as after 60 minutes (Figure 5.16).

In the porcine splenic artery, atomoxetine did not significantly increase the magnitude of EFS (16 Hz) responses even when the stimulation duration was decreased to 10 seconds (Figure 5.17). Again, the most effects were observed on the time duration of the relaxation, where atomoxetine 10 nM and 100 nM increased the time to 50% relaxation after 30 and 60 minutes, and atomoxetine 1 µM after 60 minutes (Figure 5.18). The contractile responses were partially attenuated or completely abolished after adding prazosin (100 nM) to the porcine splenic artery.

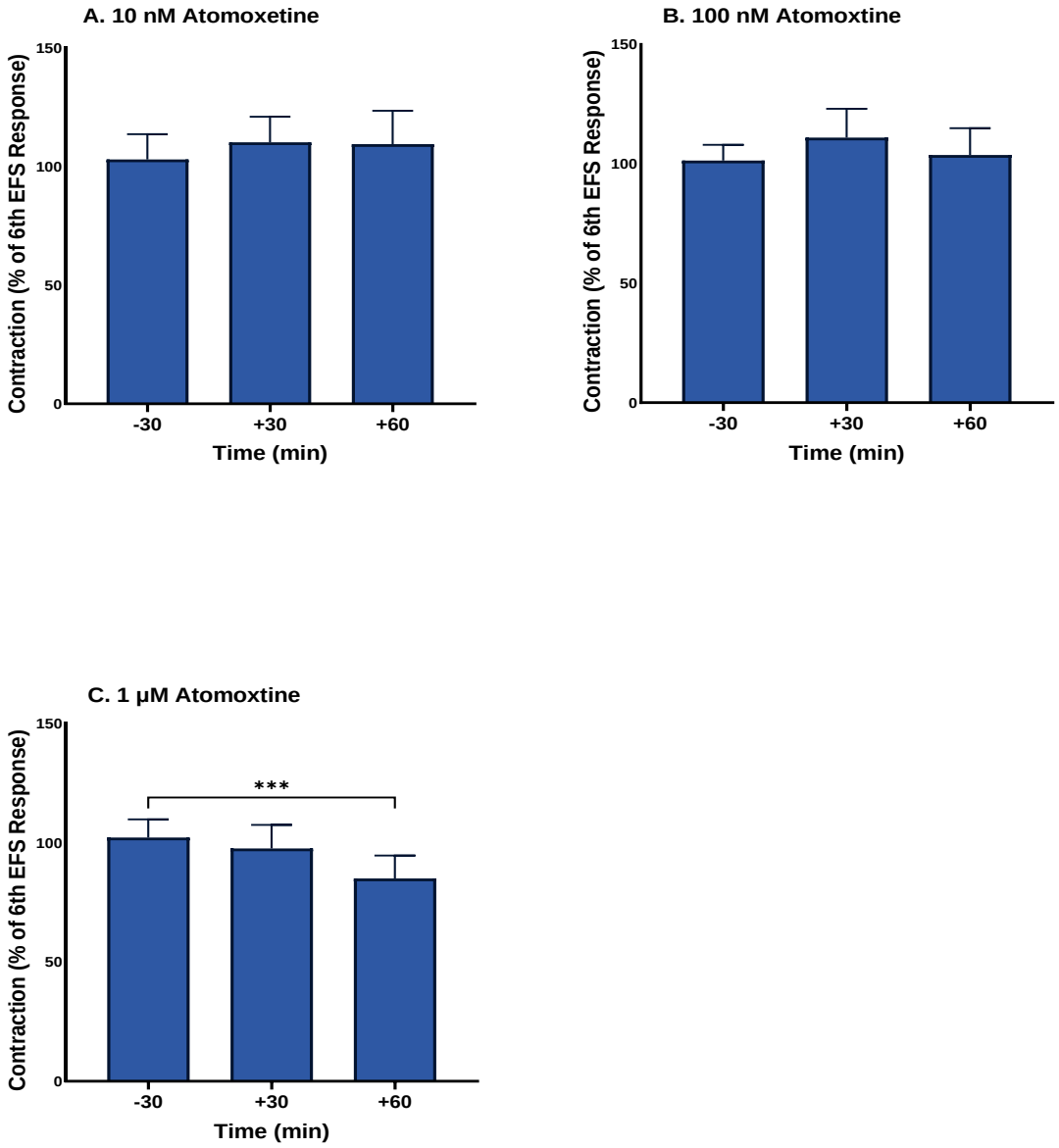


Figure 5.15: The effect of atomoxetine (**A**) 10 nM, (**B**) 100 nM, and (**C**) 1 µM on peak responses of EFS (16Hz, 0.3ms, 200mA, 30sec stimulation duration) in porcine splenic artery. Data are expressed as the percentage of 6th EFS responses and presented as mean ± SEM, n=9. ***p < 0.001 represented significant differences, one-way ANOVA followed by Dunnett's test.

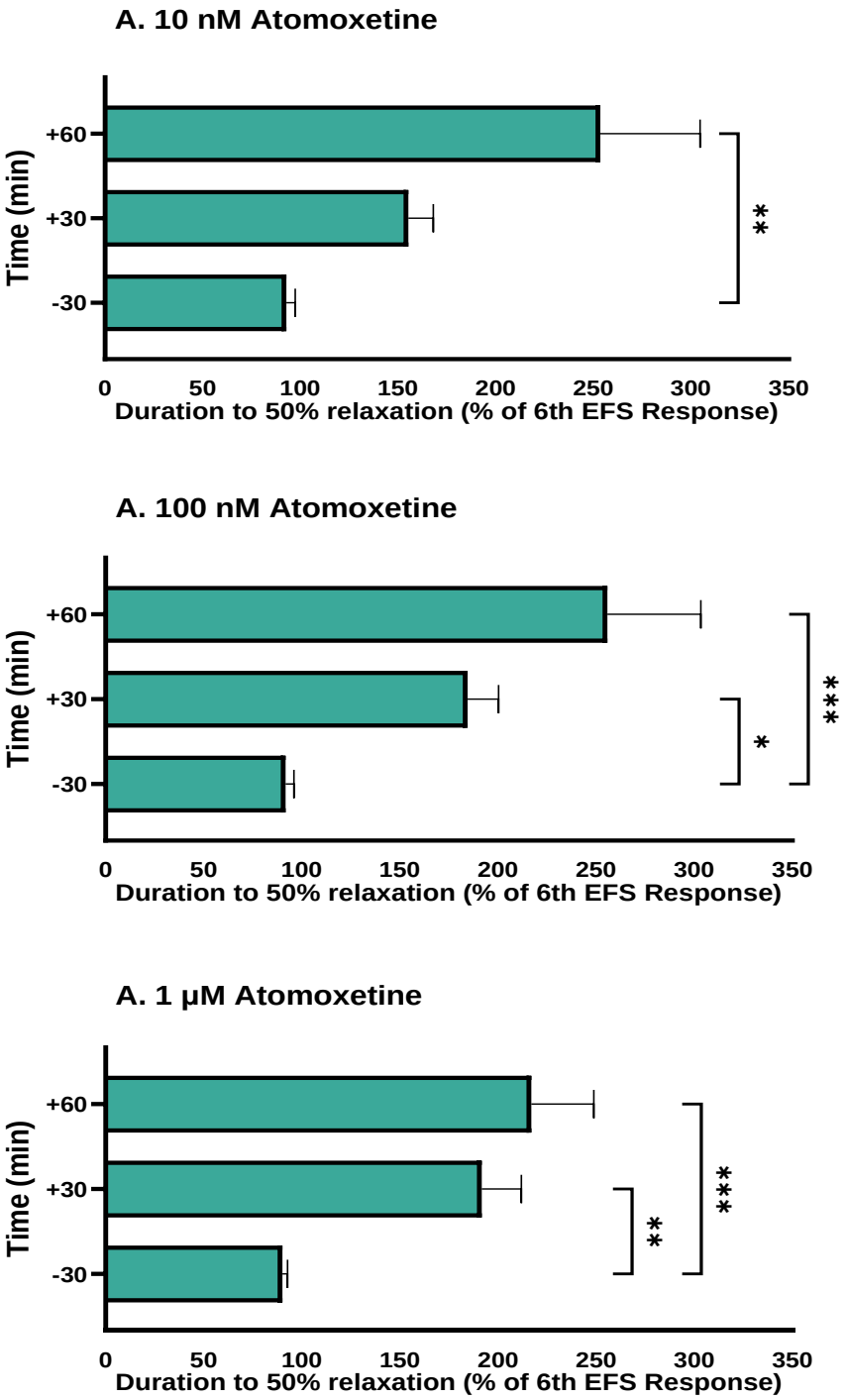


Figure 5.16: The effect of atomoxetine (**A**) 10 nM, (**B**) 100 nM, and (**C**) 1 µM on time to 50% relaxation of EFS (16 Hz, 0.3 ms, 200 mA, 30 sec stimulation duration) in porcine splenic artery. Data are expressed as the percentage of 6th EFS responses and presented as mean ± SEM, n=9. *p < 0.05, **p < 0.01, ***p < 0.001 represented significant differences, one-way ANOVA followed by Dunnett's test.

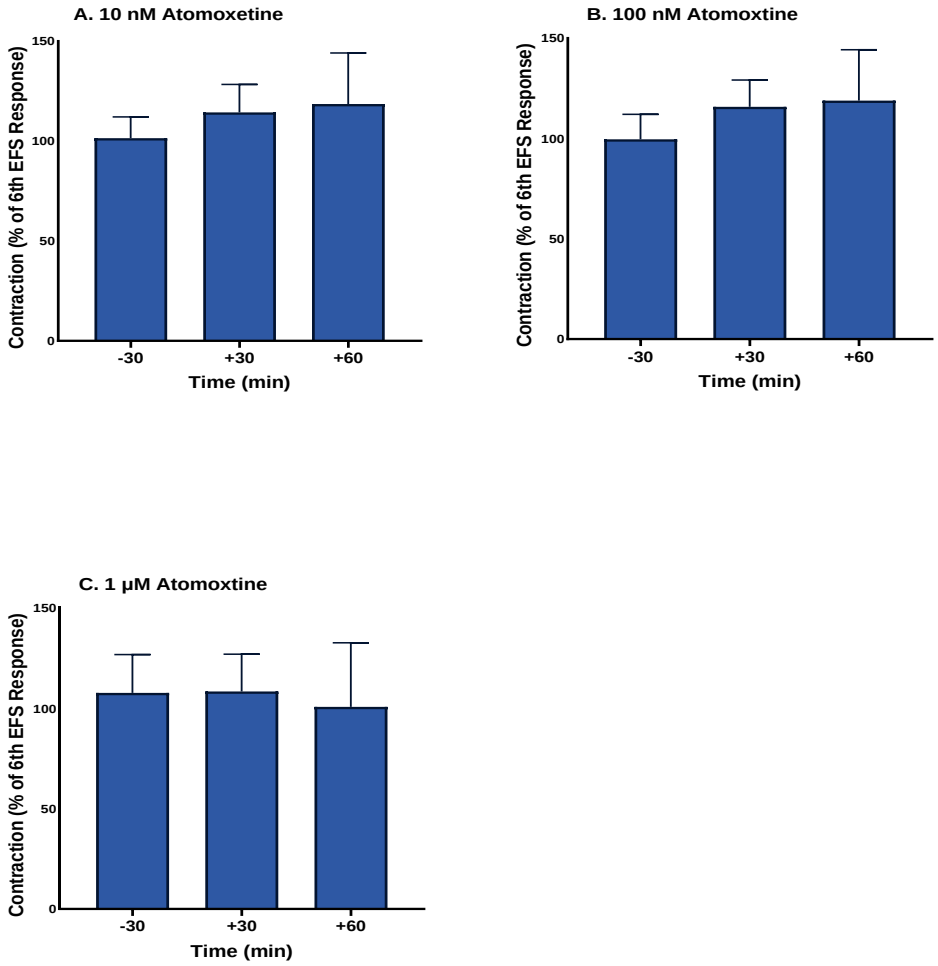


Figure 5.17: The effect of atomoxetine (**A**) 10 nM, (**B**) 100 nM, and (**C**) 1 µM on peak responses of EFS (16 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in porcine splenic artery. Data are expressed as the percentage of 6th EFS responses and presented as mean ± SEM, n=7.

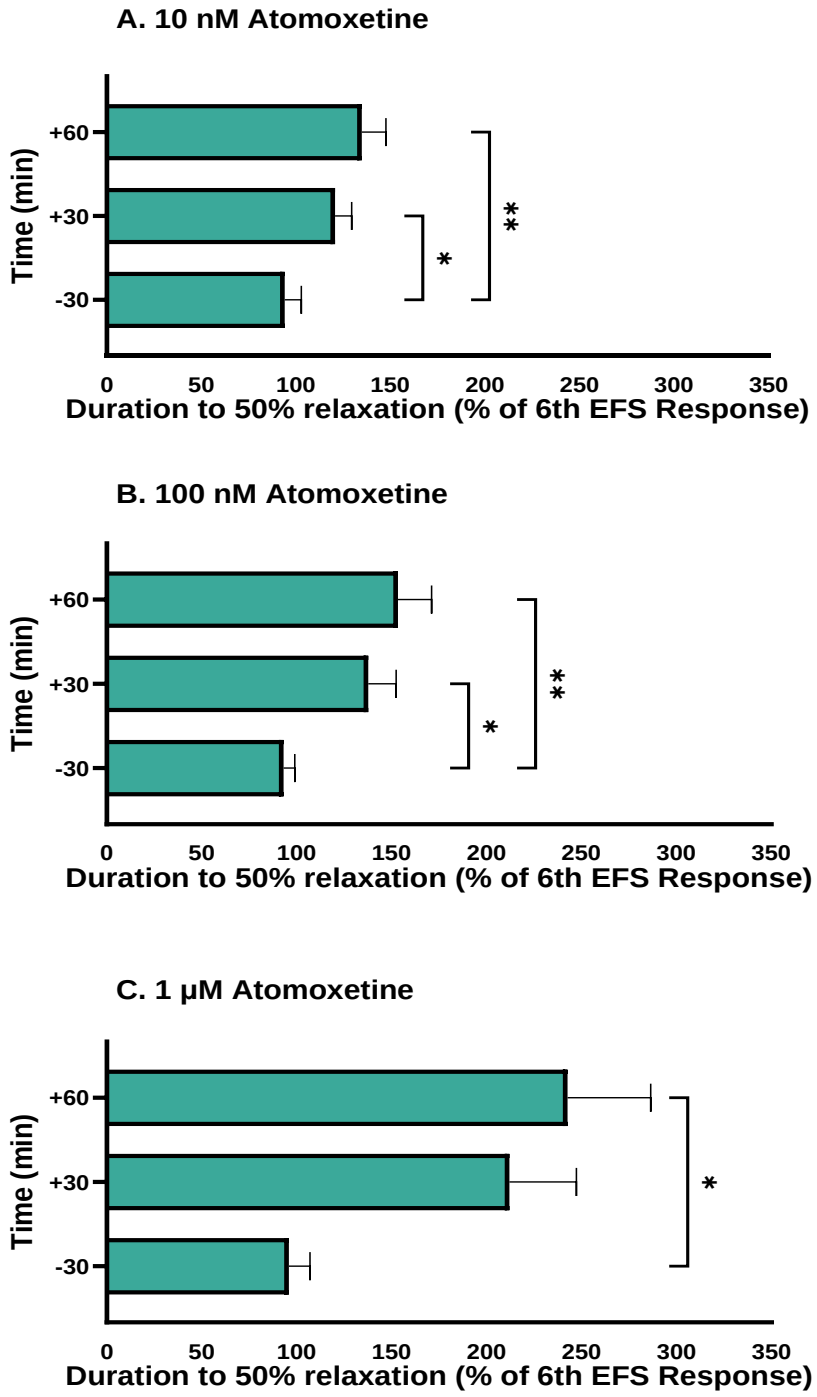


Figure 5.18: The effect of atomoxetine (**A**) 10 nM, (**B**) 100 nM, and (**C**) 1 µM on time to 50% relaxation of EFS (16 Hz, 0.3 ms, 200 mA, 10 sec stimulation duration) in porcine splenic artery. Data are expressed as the percentage of 6th EFS responses and presented as mean ± SEM, n=7. *p < 0.05, **p < 0.01 represented significant differences, one-way ANOVA followed by Dunnett's test.

5.3.2.2. Effect of atomoxetine on EFS (16Hz, 10 sec stimulation) in porcine renal arteries:

In the porcine renal artery, atomoxetine (10 nM, 100 nM and 1 µM) had no significant effect on the magnitude of EFS (16 Hz) responses when stimulated for 30 seconds, but significantly increased the time to 50% relaxation (figures not shown). Of note, there was a notable increase in the baseline in the presence of atomoxetine when compared to the control. The contractile responses were partially attenuated or completely abolished after adding prazosin (100 nM) (Figure 5.19). Atomoxetine affects the magnitude of the electrically stimulated responses when the pulse stimulation period was reduced to 10 seconds (Table 5.3 E). 100 nM Atomoxetine significantly increased the magnitude of EFS (16 Hz) responses stimulated for 10 seconds, while 1 µM atomoxetine significantly reduced it after 60 minutes. Furthermore, all concentrations of atomoxetine increased the time to 50% relaxation of EFS (16 Hz) responses stimulated for 10 seconds (Table 5.7).

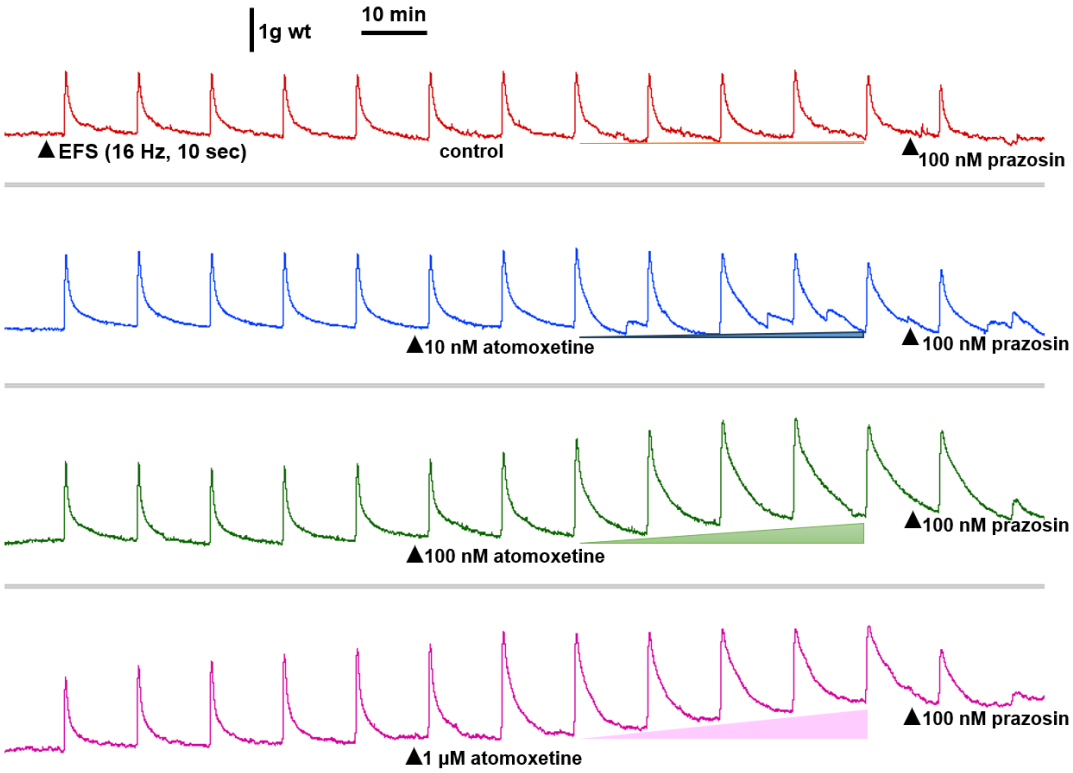


Figure 5.19: Representative traces of four channels showing the effect of atomoxetine (10 nM, 100 nM, and 1 µM) and prazosin (100 nM) on EFS (16 Hz, 10 sec) induced responses in the porcine renal artery.

Table 5.7: The effect of atomoxetine (10 nM, 100 nM, 1 µM) on peak responses and time to 50% relaxation of electrically stimulated contractions (16 Hz, 0.3 ms, 200 mA, 10 sec stimulation) in porcine renal artery. Data are presented as mean ± SEM, n=5. *p < 0.05, **p < 0.01, ***p < 0.001 represented significant differences in comparing to control, one-way ANOVA followed by Dunnett's test.

Conditions	Parameters	% of 6th EFS responses		
		Control	+ 30 min	+ 60 min
Atomoxetine (10 nM) n=5	Peak contraction	98.5 ± 4.0	111.4 ± 4.4	111.6 ± 5.7
	Time to 50% relaxation	85.1 ± 4.6	163.2 ± 20.6	324.4** ± 79.5
Atomoxetine (100 nM) n=5	Peak contraction	97.3 ± 2.1	118.7** ± 4.0	110.5* ± 3.2
	Time to 50% relaxation	85.1 ± 5.4	219.7* ± 10.8	344.8*** ± 59.1
Atomoxetine (1 µM) n=5	Peak contraction	97.8 ± 2.8	97.2 ± 5.6	81.9* ± 4.9
	Time to 50% relaxation	85.2 ± 2.7	225.2* ± 28.1	269.8* ± 44.8

5.3.2.3. Effect of cocaine on EFS (16Hz, 10 sec stimulation) in porcine renal arteries:

In the porcine renal artery, cocaine (0.3 µM) potentiated the electrical stimulated peak response starting at 30 minutes with no significant change in the duration to 50% relaxation of the responses. However, the higher concentration of cocaine (3 µM) significantly potentiated the peak responses at 60 minutes and time to 50% relaxation starting at 30 minutes (Table 5.8).

Table 5.8. Effect of cocaine (0.3 µM and 3 µM) on peak responses and time to 50% relaxation of electrically stimulated contractions (16 Hz, 0.3 ms, 200 mA, 10 sec stimulation) in the porcine renal artery. Data are presented as mean ± SEM, n=6. *p < 0.05, **p < 0.01, ***p < 0.001 represented significant differences in comparing to control, one-way ANOVA followed by Dunnett's test.

Conditions	Parameters (% of the 6th EFS)	Control	+ 30 min	+ 60 min
Cocaine (0.3 µM) n=6	Peak contraction	92.0 ± 3.2	113.2*** ± 1.7	114.9*** ± 1.7
	Time to 50% relaxation	80.4 ± 7.2	124.1 ± 4.1	195.7 ± 20.3
Cocaine (3 µM) n=6	Peak contraction	92.5 ± 3.4	123.6 ± 8.5	140.6** ± 18.1
	Time to 50% relaxation	80.1 ± 7.9	261.4* ± 31.9	377.8*** ± 73.5

5.3.3. NA sparing effect of NA uptake1 inhibitors in porcine splenic artery:

These experiments aimed to examine the additive effects of neuronal reuptake inhibitors on the contraction responses induced by small concentrations of NA (1 nM - 200 nM). The findings showed that NA mediated contractions were enhanced in the presence of 100 nM atomoxetine (Figure 5.20). The maximum contraction of NA was not affected when the porcine splenic artery was precontracted with 1 µM cocaine and 10 nM atomoxetine. The contractions were enhanced in the presence of higher concentrations of cocaine (3 µM) and atomoxetine (100 nM) (Figure 5.21). The addition of 10 nM dexmedetomidine to 10 nM atomoxetine and 10 nM dexmedetomidine to 100 nM atomoxetine produced tones of about 6.9% and 5.8% to 60 mM KCl, respectively. The combination of 10 nM dexmedetomidine with 10 nM atomoxetine did not affect NA mediated contractions, but the contractions were enhanced by combining 10 nM dexmedetomidine with 100 nM atomoxetine (Figure

5.22 and 5.23). Figure 5.24 shows that concentration response curve of NA was enhanced in the presence of 10 nM dexmedetomidine plus 100 nM atomoxetine. Table 5.9 shows significant changes in the potency (-Log $EC_{30\ KCl}$) values of NA in pre-contracted porcine splenic arteries with cocaine (10 µM), atomoxetine (100 nM), and dexmedetomidine (10 nM) combined with atomoxetine (100 nM).

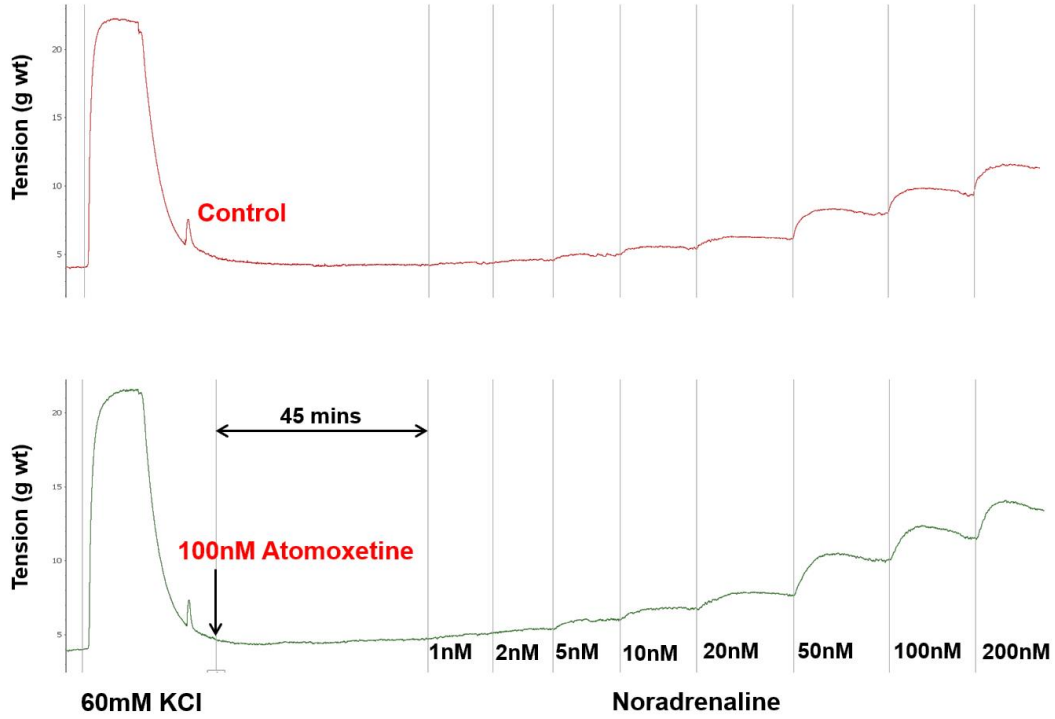


Figure 5.20: Representative traces show the cumulative responses of NA in the presence of atomoxetine (100 nM) in the porcine splenic artery.

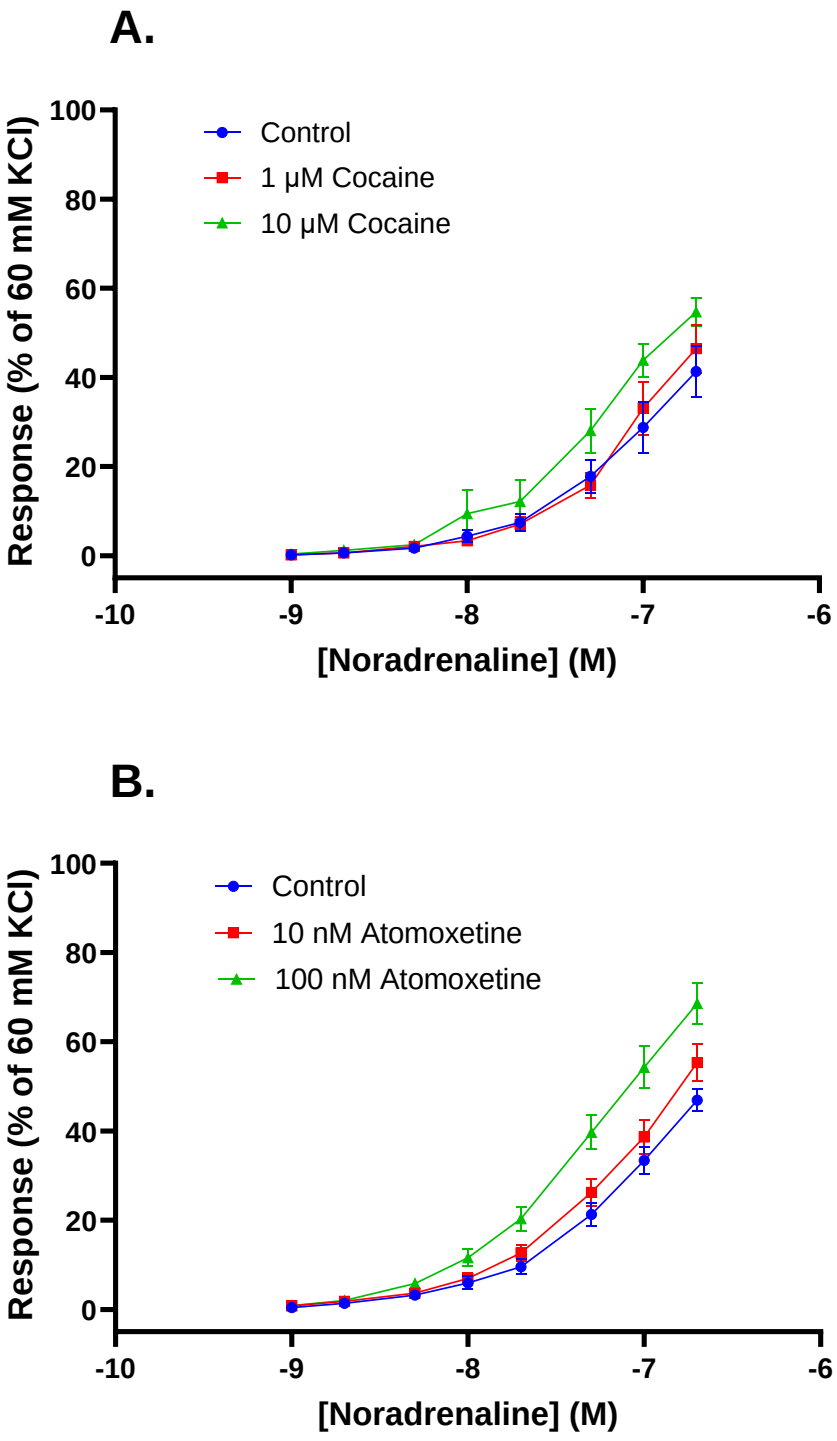


Figure 5.21: Cumulative concentration-response curves of noradrenaline (NA) in the presence of (**A**) 1 μM and 10 μM cocaine (**B**) 10 nM and 100 nM atomoxetine in porcine isolated splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM.

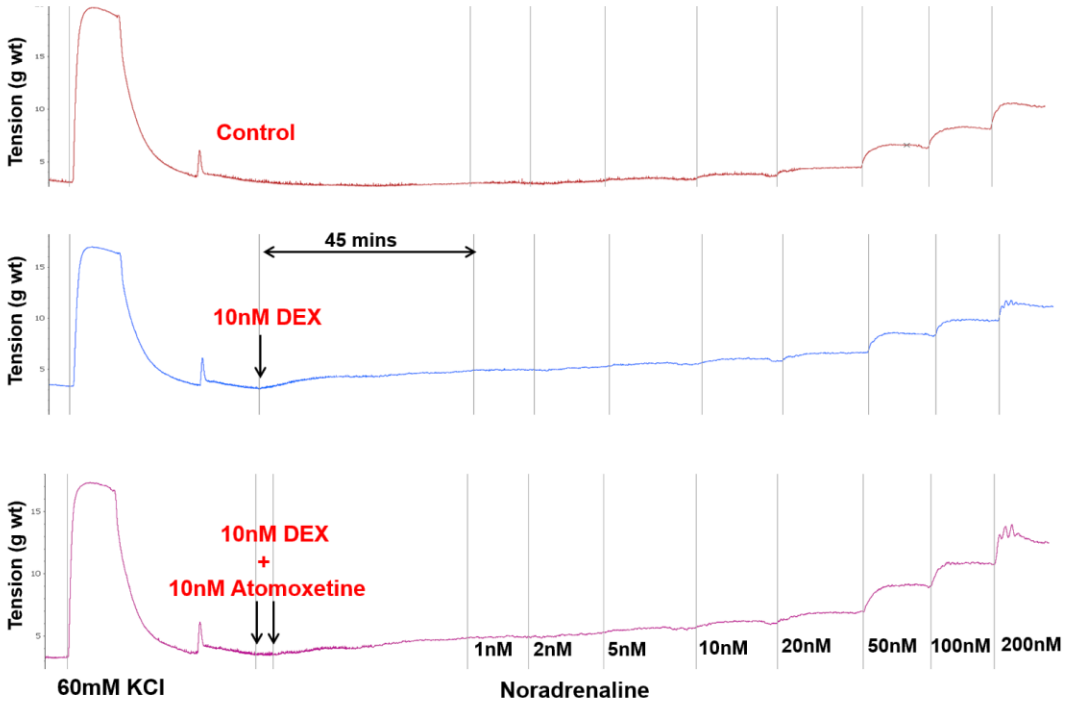


Figure 5.22: Representative traces show the cumulative responses of NA in the presence of 10 nM dexmedetomidine alone or combined with 10 nM atomoxetine in the porcine splenic artery.

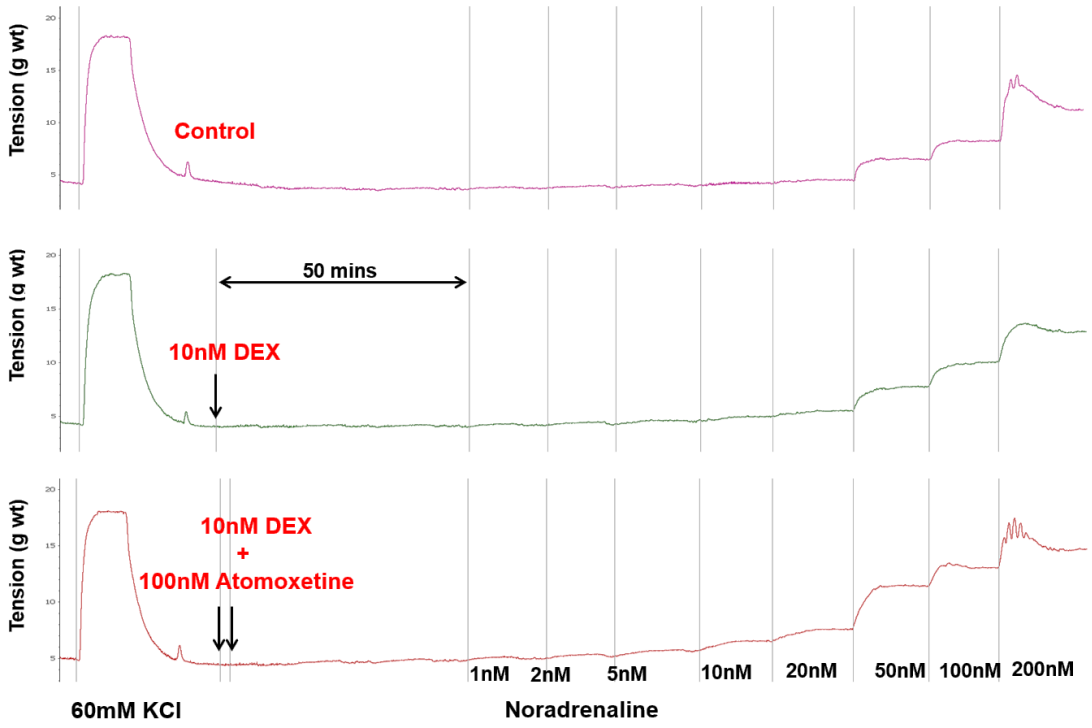


Figure 5.23: Representative traces show the cumulative responses of NA in the presence of 10 nM dexmedetomidine alone or combined with 100 nM atomoxetine in the porcine splenic artery.

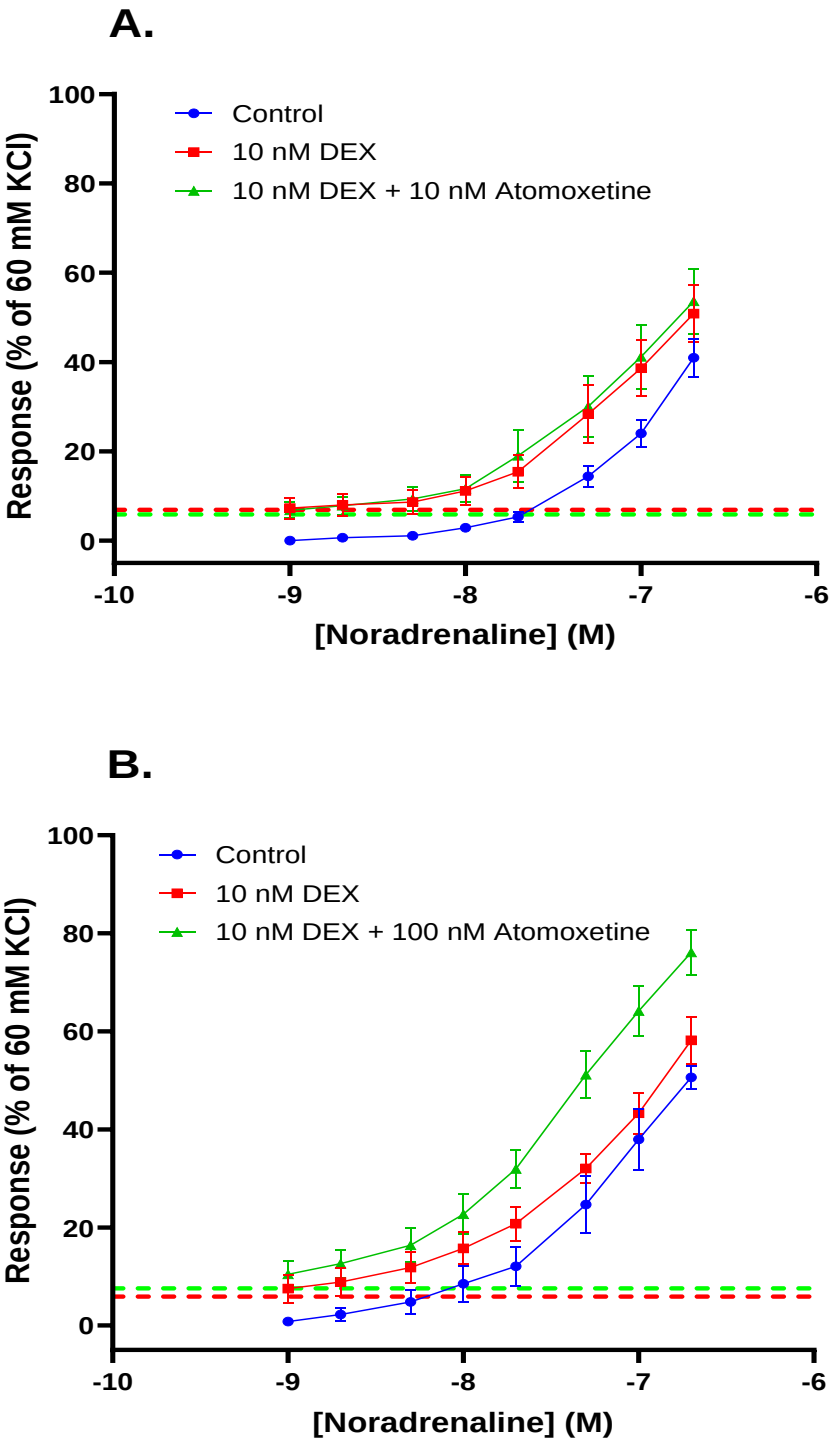


Figure 5.24: Cumulative concentration-response curves of noradrenaline (NA) in the presence of 10 nM dexmedetomidine or combined with (**A**) 10 nM atomoxetine or (**B**) 100 nM atomoxetine in porcine isolated splenic artery. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM. Grid lines (---) indicate the tones of drugs and are expressed as % of 60% KCl.

Table 5.9: Comparison of the potency (-Log$EC_{30\ KCl}$) of noradrenaline (NA) in the absence and presence of cocaine, atomoxetine alone or combined with dexmedetomidine. Data are expressed as the percentage of 60 mM KCl responses and presented as mean ± SEM. *p-value < 0.05, and **p-value < 0.01 were considered statistically significant, one-way ANOVA followed by Dunnett's test. N: number of animals.

Protocols	N	-Log $EC_{30\ KCl}$
NA (control)	12	6.9 ± 0.10
NA + 1 µM Cocaine	11	6.9 ± 0.08
NA + 10 µM Cocaine	10	7.3 ± 0.10*
NA (control)	22	7.1 ± 0.06
NA + 10 nM Atomoxetine	14	7.2 ± 0.07
NA + 100 nM Atomoxetine	16	7.4 ± 0.07**
NA (control)	6	6.9 ± 0.07
NA + 10 nM Dexmedetomidine		7.2 ± 0.16
NA + 10 nM Dexmedetomidine + 10 nM Atomoxetine		7.2 ± 0.18
NA (control)	8	7.2 ± 0.13
NA + 10 nM Dexmedetomidine		7.4 ± 0.11
NA + 10 nM Dexmedetomidine + 100 nM Atomoxetine		7.8 ± 0.11**

5.4. Discussion

The purpose of using the electrical field stimulation in this study was to simultaneously stimulate the endogenous release of NA at the sympathetic nerves of the porcine isolated blood vessels. The findings of the present study showed that neither of the examined neuronal uptake1 inhibitors significantly affected the magnitude of electrically stimulated contractions (2 - 64 Hz) in porcine splenic arteries, but they increased the time to 50% relaxation, particularly at higher frequencies. In the porcine renal artery, both atomoxetine and cocaine enhanced the EFS mediated contractions by increasing the magnitude and time to 50% relaxation. However, the electrically stimulated porcine renal artery at a single frequency of 16 Hz showed that atomoxetine did not significantly increase the magnitude of EFS responses while cocaine (3 µM) significantly potentiated the peak responses and time to 50% relaxation

5.4.1. Electrical field stimulation (EFS):

In the present study, the EFS induced contractions immediately developed after the start of stimulation followed by recovery to the baseline. It has been found that with stimulation of the dog mesentery artery at 1Hz for 50 seconds, purinergic and adrenergic mediated contractions were similar in peak magnitude, but the adrenergic contraction developed slowly to become more pronounced at high frequency (10 Hz, 5 seconds). This suggested that purinergic receptors are involved in the early phase of the total response and the adrenergic components contribute to the latter phase (Muramatsu *et al.,* 1989). The animal study on rat animal tails has shown that the release of NA induced by the nerve stimulation diffuses across the nerve junction and binds to α_1-adrenoceptors on vascular smooth muscle cells producing contraction. The elevation in measured NA overflow was faster than the onset of the arterial

contraction, and this could be due to the time required for NA to reach the necessary concentration and to diffuse through the smooth muscle layers (Msghina *et al.,* 1999). The diameter of the rat artery was reduced by nearly 28% after 20 Hz electrical stimulation, in addition to an increased concentration of NA at the surface of the arteries. The oxidation current value decreased slowly to the pre-stimulation level after reaching the maximum due to a reduction in the flux of NA to the electrode. The vascular relaxation is believed to result from the degradation process of the neurotransmitters at the junctional synaptic cleft. It is known that the neuroeffector junctions at sympathetic nerves are influenced by diffusion, neuronal uptake, enzymatic degradation, and receptor desensitisation. This would clear the NA leading to the relaxation of the vascular smooth muscles (Park *et al.,* 2006).

In this study, two protocols of electrical field stimulation in two different isolated blood vessels were conducted to study the additive effects of NA uptake1 inhibitors on endogenous NA induced contractions. The experiments of EFS at increasing the frequencies (2 - 64 Hz) for 10 seconds stimulation time porcine splenic and renal arteries showed that the peak responses increased in magnitude proportionally with the frequency. It is reported that there is a wide range of sympathetically innervated tissues, and the electrical stimulations could activate the post-junctional adrenoceptors by eliciting the release of two sympathetic neurotransmitters NA and adenosine 5-triphosphate (ATP). The present study showed some differences in EFS mediated contractions between porcine splenic and renal arteries. Both the neuronal uptake and autofeedback processes play important roles in determining the relative contributions of α_1 and α_2-adrenoceptors in the response of blood vessels to neurotransmission. The α_2-adrenoceptor mediated autofeedback was demonstrated

in both the purinergic and noradrenergic components of the nerve-induced responses in the isolated rabbit artery preparations (MacDonald *et al.,* 1992). In the present study, the electrical stimulation didn't result in the depression of contraction magnitude by increasing the frequency from 2 Hz up to 64 Hz when stimulated for 10 second pulses.

It has been found that the excitation and depression of ATP and NA are similar in the response magnitude and time course in rat tail artery. The amplitude of the NA oxidation current grew almost linearly with train pulses from 4 to16, indicating the constant release of NA, but there was train length dependant depression in the growth of the amplitude of the NA oxidation current when the pulses exceeded 20, and this continued up to 100 pulses. This depression can be due to clearance or decrease in the output of NA (Msghina *et al.,* 1999). Our findings agreed with previous studies that suggested the differences in the pattern and distribution of the sympathetic nerves and NAT proteins between different tissues. For instance, there is a higher sympathetic innervation and NAT uptake system in the cardiac muscle than renal muscle resulting in more efficient neuronal uptake of NA in the cardiac cells (Kopin *et al.,* 1998). The current results have shown that the magnitude of the peak responses and time to 50% relaxation were bigger in splenic than renal arteries proposing a greater sympathetic innervation in splenic arteries.

Also, the responses in veins could vary from that in arteries. A study on rat mesentery showed that there are quantitative differences in the kinetics of NA release and clearance, and constriction and decay times between veins and arteries. It was reported that veins are more sensitive to NA than arteries due to the differential

distribution and arrangement of the perivascular sympathetic nerves. The NA oxidation current and the vascular constriction of the electrically stimulated mesenteric vein were detected at a lower frequency than in the mesenteric artery. The NA oxidation currents in the veins were bigger than that of arteries and the frequency response curve of constriction in the veins was left-shifted in comparison to that in the arteries. The vascular contraction in the veins was blocked by yohimbine but unchanged in the arteries suggesting that α_2-adrenoceptors contributed to the NA-induced constriction in veins but not in arteries (Park *et al.,* 2007). For future work, porcine splenic veins could be used to test the effect of atomoxetine and reboxetine on NA mediated contractions.

The second experimental protocol was conducted by using a single frequency of 16Hz for either 10 seconds or 30 seconds of stimulation to exclude the possibility that the distance between the nerve terminal and tissue smooth muscle might affect the EFS evoked responses. To identify the consistency of the electrical-evoked stimulations in the absence of the drugs, the isolated splenic arteries were stimulated 12 times with a time interval of 10 minutes between stimulations. The magnitude of the responses was gradually depressed over time, where the reduction was statistically significant after 90 and 120 minutes. This depression could be due to the saturation of the post-junctional α_1-adrenoceptors or α_2-adrenoceptors mediated inhibition. No obvious changes were observed in the time to peak response and recovery time. In comparing the representative traces of the porcine splenic artery to the porcine renal artery (Figure 5.14), the responses were more consistent in the porcine renal artery, where no reduction was observed in the magnitude of the responses.

It is known that electrical stimulation evoked contraction in the dog mesentery artery involved postjunctional p2-purinoceptors and α_1-adrenoceptors, which were blocked by 100 nM prazosin (Muramatsu *et al.,* 1989). In the present study, the blockage of the responses evoked by electrical stimulation at a frequency of 16 Hz was observed shortly after adding prazosin, a selective α_1-adrenoceptor antagonist. This finding ensures that the EFS evoked contraction is mediated through NA acting on α_1-adrenoceptors.

It is important to know the concentration of NA evoked by EFS and compare it to the physiologically relevant plasma concentration in humans. In an animal study on a rat tail artery, the comparison of the oxidation current resulting from the electrical stimulation 5 Hz for 100 seconds pulse with the oxidation current from the exogenous NA showed that the increase in oxidation current during the electrical stimulation was proportional to the NA concentration of 10 - 300 nM (Mermet *et al.,* 1990). Furthermore, the maximum contraction evoked by 5 Hz stimulation corresponded to NA concentration of 48 ± 19 nM (Gonon *et al.,* 1993). These results could help to estimate that the concentration of NA released during the EFS is within the range of the plasma concentration of NA observed in healthy volunteers (Ensinger *et al.,* 1992), and septic patients (Abboud *et al.,* 2009).

5.4.2. The effect of NA uptake1 inhibitors on NA mediated contractions:

The concentrations of NA uptake1 inhibitors used in this study were chosen based on their plasma concentrations as reported in humans (mentioned in Table 6.1). 3 µM Cocaine did not potentiate the magnitude of responses in porcine splenic arteries at all the frequencies, while 0.3 and 3 µM cocaine potentiated the peak magnitude in

porcine renal arteries at stimulation of 16 Hz. The time required for 50% relaxation was prolonged in both tissues in the presence of cocaine. In prior research involving dog mesenteric artery, cocaine potentiated the magnitude and duration of adrenergic responses but attenuated the purinergic responses evoked by the electrical stimulation (Muramatsu *et al.,* 1989). Moreover, inhibition of NA uptake by cocaine 10 µM enhanced the magnitude and duration of the response evoked at 20 Hz for 16 pulses (Gonon *et al.,* 1993) and this agreed with current results in the porcine renal artery. In rat mesenteric artery, cocaine resulted in an increased oxidation current at both a low frequency of 3 Hz and a high frequency of 10 Hz, while the response was increased at low frequency and unaffected at high frequency (Park *et al.,* 2006).

Also, the application of various concentrations of cocaine may produce different effects on NA induced responses. In this study, the application of a low concentration of cocaine 0.3 µM potentiated the peak magnitude in only 30 minutes which was faster than that of 3 µM cocaine in the porcine renal artery, while the duration of relaxation was significantly reduced in the presence of both concentrations. It has been reported that the membrane potential and membrane resistance of the guinea pig mesenteric artery showed that the application of cocaine at the small concentration (100 nM) did not significantly depolarise the membrane or increase the membrane resistance. However, the higher concentration of cocaine (10 µM) depolarised the membrane. Also, cocaine produced dose-dependent reduction in the magnitude of responses in the presence of phentolamine suggesting that its effects could not be due to interaction with pre-junctional α_2-adrenoceptor but mainly on the post-junctional receptors (Kuriyama and Suyama, 1983).

Cocaine and all other NA uptake1 inhibitors tested did not significantly impact the contraction magnitude evoked by (2 - 64 Hz) EFS in the porcine splenic artery. However, atomoxetine like cocaine potentiated the EFS responses in the porcine renal artery. The duration of 50% relaxation was affected in the presence of NA uptake1 inhibitors. The changes were not significant at low frequencies but increased in line with increasing the frequencies. The elevation of duration in the presence of a smaller concentration of atomoxetine was greater than that of cocaine at higher frequencies. These findings suggested that atomoxetine is likely more potent and effective in blocking the presynaptic NAT proteins than cocaine. Furthermore, the current results supported the previous studies stating that atomoxetine increases orthostatic hypotension due to its neurogenic vasoconstriction actions (Shibao *et al.,* 2007).

Reboxetine showed dose-dependent vasoconstrictive action where the small concentration increased the time to 50% relaxation and this change was bigger than that of higher concentration. Desipramine showed some reduction but not significant in the magnitude and increase in the time duration of relaxation. These findings agreed with the previous results on the isolated human cerebral arteries, where cocaine produced small contractions, followed by significant relaxation of the human arteries at higher concentrations. It is believed that cocaine interferes with the role of Ca^{++} in the maintenance of arterial tone resulting in the blockage of Ca^{++} entry through the membrane (Salom *et al.,* 1996). Similarly, animal studies on rabbit carotid arteries have shown that the small concentrations of reboxetine and cocaine but not desipramine enhanced the contractions evoked by EFS. However, they inhibited the neurogenic contractions at higher concentrations. The mechanism behind this could be the activation of inhibitory α_2-adrenoceptors by released NA. They found that

reboxetine and desipramine at a concentration of 10 nM -1 µM reduced the NA uptake, and that reboxetine 100 nM blocked the contraction induced by the exogenous phenylephrine and NA, which was antagonised by higher concentrations of reboxetine (Rasmussen and Nedergaard, 2003).

After the first findings testing the effect of 10 nM atomoxetine on (2 - 64 Hz) EFS, various concentrations were chosen to be further investigated using EFS at a single frequency (16 Hz) in porcine splenic and renal arteries. Atomoxetine has similar activity to cocaine, where an increase in the magnitude was observed with 100 nM atomoxetine, and with both concentrations of cocaine in the porcine renal artery. Although atomoxetine demonstrated some inhibitory activity at higher concentrations, the time required to achieve 50% relaxation was notably prolonged. This suggests that atomoxetine has additive vasoconstrictor effects on the endogenous NA.

In this study, the NA sparing actions of atomoxetine in the porcine splenic artery were also examined. The potency was measured using the EC_{30} relative to the maximum KCl contraction ($EC_{30\ KCl}$) because a small range of NA concentrations was used to match plasma levels of atomoxetine, and hence the maximum effect of NA was not reached to calculate EC50. The $EC_{30\ KCl}$ of NA significantly decreased when combined with atomoxetine (100 nM), indicating their sparing activity for NA. Previously, the clinical study showed that combining vasopressors such as L-NAME with NA in patients with severe sepsis increased MAP, vascular resistance, and pulmonary arterial pressure while reducing catecholamine dosage, particularly at early stages of infusion (Avontuur *et al.,* 1998). Additionally, the coadministration of methylene blue an inhibitor of NOS, within the first 24 hours as adjuvant therapy for septic patients

could reduce the time to vasopressor discontinuation and increase vasopressor-free days, and reduce the length of stay in ICU (Ibarra-Estrada *et al.,* 2023), and this could potentially due to the reduction of lethal risk associated with high dose of NA. Our findings support the hypothesis that atomoxetine alone or combined with dexmedetomidine could enhance the vasoconstriction action of NA. These findings agreed with the previous clinical study on healthy volunteers, where atomoxetine enhanced the pressor activity of NA infusion (Zerbe *et al*., 1985).

In conclusion, therapeutically relevant concentrations of neuronal uptake1 inhibitors might enhance the endogenous and exogenous NA mediated vasoconstriction. Same as cocaine, other neuronal uptake1 inhibitors such as atomoxetine could have neurogenic vasoconstriction actions by blocking the NA uptake1. In the present study, the enhancement of EFS contractions was remarkably noted by increasing the time duration of tissue recovery rather than the magnitude of the responses. Atomoxetine also produced some NA sparing activity. These results will support the potential hypothesis that these clinically approved drugs might improve the hypotension associated with septic patients in the ICU. Further investigations are required to determine the effect of atomoxetine and reboxetine in animal models of sepsis and on the haemodynamic parameters such as blood pressure and heart rate of the healthy volunteers.

6. Chapter Six: Identifying potential changes in blood cell counts in ICU patients treated with clonidine infusion.

6.1. Introduction

6.1.1. The role of α_2-adrenoceptor agonists (dexmedetomidine and clonidine) in ICU:

The α_2-adrenoceptor agonists have been used for decades to treat different medical conditions such as hypertension, ADHD, and symptoms of opioid, benzodiazepine, and alcohol withdrawal (Lowenthal *et al.,* 1988, Giovannitti *et al.,* 2015). Table 2.1 in Chapter 2 lists the dosages and main indications of α_2-adrenoceptor agonists. In recent years, some α_2-adrenoceptor agonists have increasingly been used as adjuncts to maintain sedation and to manage delirium in critically ill patients with or without sepsis. A selection of α_2-adrenoceptor agonists has been trialled in ICU settings but only two, dexmedetomidine and clonidine, are regularly used (Lankadeva *et al.,* 2021).

Clonidine was synthesised as the first α_2-adrenoceptor agonist in the 1960s to be used as a nasal decongestant. However, it exhibited unexpected side effects, including sedation and severe cardiovascular depression. These findings led to further studies and the introduction of clonidine as an antihypertensive drug in 1966. Over the years, clonidine became widely accepted not only for treating high blood pressure but also for managing alcohol and drug withdrawal, serving as an adjunctive medication in myocardial ischaemia, and providing pain relief in intrathecal anaesthesia (Tamsen and Gordh, 1984).

The subsequent studies showed that complete anaesthesia is possible by application of a new, more potent α_2-adrenoceptor agonist such as dexmedetomidine, which was approved by the Food and Drug Administration at the end of 1999 for use in humans

as a short-term medication for analgesia and sedation in ICU. The findings showed that dexmedetomidine is a suitable sedative and analgesic agent during the perioperative and postoperative periods with the same actions as benzodiazepines (Gertler *et al.,* 2001) and the administration of dexmedetomidine to ICU patients resulted in the same quality of sedation as propofol with no respiratory depression (Venn and Grounds, 2001). Dexmedetomidine provided a safe and effective sedation with reduced usage of opioids, other sedative agents and NA (Gertler *et al.,* 2001, Herr *et al.,* 2003).

Those highly selective α_2-adrenoceptor agonists act centrally in the locus coeruleus and inhibit the release of NA. They are currently used as anaesthetic adjuvant and sedative agents for mechanically ventilated patients in the ICU (Pichot *et al.,* 2012, Fraser *et al.,* 2013, Giovannitti *et al.,* 2015, Fernandes *et al.,* 2018). A recent study showed that the administration of dexmedetomidine to ICU sepsis patients without shock resulted in less hypotension compared to propofol in a retrospective cohort study (Benken *et al.,* 2020). Critically ill patients requiring ICU treatment are a heterogeneous population with a wide variety of clinical conditions (Cuadrado *et al.,* 2021). However, some conditions have a high prevalence among critically ill patients including septic shock (Shankar-Hari *et al.,* 2017). Those patients are at high risk of developing delirium, especially when supported with mechanical ventilation. dexmedetomidine is licensed for the continuous sedation of ICU patients and has been intensively investigated to reduce the incidence of delirium (Zhang *et al.,* 2022, Lankadeva *et al.,* 2021). In a clinical study comparing dexmedetomidine to midazolam as sedative agents in ICU, the findings showed that dexmedetomidine treated patients

spent less time on the ventilator, experienced less delirium, and developed less tachycardia and hypertension (Riker *et al*., 2009).

The Society of Critical Care Medicine (SCCM) suggests the use of short-acting agents such as propofol and dexmedetomidine over benzodiazepines. However, the ideal sedative agent is patient specific, which depends on the pharmacokinetic and pharmacodynamic properties of the drug and patient. Dexmedetomidine has been shown to reduce post-operative delirium, as well as help in agitated delirium in non-intubated patients (Pandharipande *et al.,* 2017). However, a recent clinical study reported that the outcomes of critically ill patients who received dexmedetomidine were not different from those who received propofol (Hughes *et al.,* 2021).

Clonidine is also regularly used in ICUs throughout the UK for the management of agitation, although this is an unlicensed indication (Tran *et al.,* 2018). It has been found that α_2-adrenoceptor agonists could stabilise the haemodynamic activities in critically ill patients. Administration of clonidine attenuated the stress-induced sympathoadrenal responses, improved the intraoperative hemodynamic stability, and decreased anaesthetic requirements during the surgery (Matot *et al.,* 2000b). The preoperative use of dexmedetomidine is associated with reduced cardiac surgical complications, along with the reduction in arterial BP and HR in postsurgical patients (Bhana *et al.,* 2000).

Dexmedetomidine also reduced the length of ICU stays, adverse effects, and the score of the sequential organ failure assessment (SOFA) (Huang *et al.,* 2021, Yuan *et al.,* 2022). It reduced the duration of the mechanical ventilation without affecting the

mortality and other clinical outcomes (Wang *et al.,* 2021a). However, a clinical trial of SPICE III stated that early administration of dexmedetomidine was associated with more adverse effects and there was no significant effect on the 90-day mortality rate of critically ill patients in ICU (Shehabi *et al.,* 2019).

The α_2-adrenoceptors mediate a variety of physiological functions in the central nervous system and peripheral tissues. The activation of presynaptic α_2-adrenoceptors induces sympatholytic effects by inhibition of endogenous NA, resulting in hypotension. However, the activation of vascular postsynaptic α_2-adrenoceptors distributed in the vascular smooth muscle of veins and arteries can also cause vasoconstriction and an increase in blood pressure (Starke *et al.,* 1975, Talke *et al.,* 2003). Moreover, α_2-adrenoceptors are associated with other tissues, including macrophages, platelets, kidney, pancreas, and brain, causing a variety of different effects in addition to those associated with blood pressure. The receptor ligand binding studies have shown that neutrophils and platelets contain α_2-adrenoceptors which were measured by using yohimbine as radioligand (Motulsky *et al.,* 1980, Panosian and Marinetti, 1983). In addition, the NA mediated stimulation of these receptors promotes renal inflammation and interstitial fibrosis in the progression of the chronic kidney diseases (Jang *et al.,* 2019). The α_{2A}-adrenoceptors on the surface of macrophages contribute to the production of proinflammatory mediators and increase the release of TNF-α (Miksa *et al.,* 2009).

Recently, several clinical studies have suggested that dexmedetomidine may have the role of anti-inflammatory and immune regulation during excessive cytokine release in sepsis and infection in critically ill patients. It reduced the TNF-α, IL-1β and IL-6 (Memis

et al., 2007), HMGB1 (Chang *et al.,* 2013), and improved the CRP, procalcitonin and albumin levels in ventilated septic patients (Ohta *et al.,* 2020). It has been found that IV dexmedetomidine decreased TNFα and IL-6, improved survival, and decreased lung neutrophil infiltration over 8 hours in septic rats. Also, dexmedetomidine regulates the inflammatory actions in ventilator induced lung injury dogs, where there was a dose-dependent reduction in lung TNFα, iNOS, NFκB, and Myeloperoxidase and Polymorphonuclear neutrophils (Flanders *et al.,* 2019). In contrast, an in vitro study on human neutrophils has shown that clinical plasma concentrations of α_2-adrenoceptor agonists such as clonidine and dexmedetomidine did not significantly affect the neutrophil functions, suggesting the safety of co-administration of these agonists with anaesthetics in patients with infection such as septic patients (Nishina *et al.,* 1999). Moreover, clonidine did not change the neutrophil activation of healthy patients with an ischemic forearm during reperfusion but increased the platelet activation in their systemic circulation (Gourdin *et al.,* 2012).

In critically ill patients with septic shock, the overproduction of inflammatory cytokines such as TNF-α and interleukins could result in microvascular thrombosis due to activation of coagulation cascade in the vascular endothelial walls. This ultimately causes organ damage and multiple organ failure (Mallat *et al.,* 2019). The meta-analysis of 19 randomised controlled trials, reported that the use of dexmedetomidine in sepsis was associated with a significant decrease in the level of TNF-α and IL-6 compared to other sedatives (Zhang *et al.,* 2022). The anti-inflammatory effects of dexmedetomidine in several clinical trials on patients who underwent surgical operations are listed previously in Table 2.4. dexmedetomidine has a possible protective effect on various systems including renal, pulmonary, and liver functions,

particularly in postoperative patients. It showed a protective action against acute renal failure in patients who underwent cardiac bypass surgery. The dose dependant protection of dexmedetomidine was detected in the early postoperative period and was determined by measurements of neutrophil gelatinase-associated lipocalin levels, which were significantly lower in high dose dexmedetomidine group compared to placebo and low dose dexmedetomidine group (Balkanay *et al.,* 2015, Cui *et al.,* 2020). Moreover, dexmedetomidine improved the liver functions of patients with liver cancer undergoing hepatectomy. It suppressed the production of reactive oxygen species and the consequential hepatocyte apoptosis, resulting in lower expression of peripheral immune cells causing liver protection (Li *et al.,* 2023).

There is variation in platelet responses to catecholamines across different mammalian species and this could be due to the relative expression and sensitivity of α- and β-adrenoceptors on their platelets. The animal models demonstrated that the excitatory response of rat platelets to adrenaline is mediated by α_2-adrenoceptors which enhanced the activation and aggregation of platelets in the presence of other aggregatory agents. While the inhibitory response of rabbit platelets to adrenaline is mediated by β_2-adrenoceptors. In human platelets, adrenaline can prompt an aggregatory response in the absence of other excitatory agents suggesting that there are differences in the stimulus coupling mechanism of aggregation between species (Kerry *et al.,* 1984). Adrenaline inhibited the adenylate cyclase and hence the platelets aggregation in human platelets via α_2-adrenoceptors, however, clonidine acted as a partial agonist on these receptors and reversed the inhibition action of adrenaline (Steer and Atlas, 1982). NA induces platelet aggregation through stimulation of α_{2A}-adrenoreceptors coupled with the Gi family on the surface of the

platelets (Yang *et al.,* 2002). A clinical study on healthy volunteers reported that the therapeutic concentrations of dexmedetomidine did not affect platelet functions (Kose *et al.,* 2013), and clonidine also did not significantly affect platelet aggregation in patients receiving anti-platelet drugs (Adam, 2014). Moreover, the increased platelet reactivity during and after the surgery was not affected by the administration of clonidine, suggesting that the use of clonidine does not increase the risk of platelet-induced perioperative arterial thrombosis (Rosenfeld *et al.,* 1993). In contrast, an in vitro study on human platelet functions indicated that dexmedetomidine has bidirectional effects; enhancement and suppressive actions on platelet functions (Kawamoto *et al.,* 2015). Furthermore, brimonidine, a selective α_2-adrenoceptor agonist restored the platelet aggregation through inhibition of cAMP (Porta Bonete *et al.,* 2020). A pilot study of 322 patients by William Baker, Martin Beed and Vince Wilson, looking at changes in blood counts and serum biochemical markers during ICU stay, identified a possible association between clonidine use and a rising platelet count" (Unpublished data, 2018).

6.1.2. The usage of clonidine in Nottingham University Hospitals- NHS:

Nottingham University Hospitals NHS Trust is a UK tertiary-referral teaching hospital with a catchment of between 2 and 4 million people (depending upon the referring condition). It has 1700 beds, including three general ICUs, which collectively care for approximately 2800 patients annually, covering most medical, surgical and trauma conditions, except for transplant surgery (other than renal), cardiac surgery, and extracorporeal membrane oxygenation (ECMO). A significant proportion of these patients fall under the Level 3 category, which means they have chronic impairment

of one or multiple organ systems, experience delirium and agitation, and require advanced ventilatory or multiple-organ support (Society, 2021). In common with other UK ICUs, the primary causes for ICU admission are sepsis, trauma, and post-operative elective or emergency surgeries (Harrison *et al.,* 2004).

In the acute phase of treatment, many ICU patients at NUH require cardiovascular support in the form of inotropes and vasopressors. Clonidine and dexmedetomidine are regularly used for sedation and the management of agitation within NUH ICUs. Because dexmedetomidine is new and more expensive, it has been used less frequently than clonidine. A pilot study on 20 patients has shown that transitioning from dexmedetomidine to clonidine is potentially efficacious, safe, and less costly in the management of sedation ICU. The potential cost savings from drug acquisition ranged from $819 to $2338 per patient over 3 months (Gagnon *et al.,* 2015). As mentioned previously, clonidine and dexmedetomidine can induce hypotension and bradycardia, therefore the NUH use them with caution in critically ill patients, typically only started when patients are in their recovery phase. They are commonly used to facilitate weaning from sedation and ventilation in patients who become agitated and delirious when they are recovering from an acute infection (Malbrain *et al.,* 2022).

This study aimed to demonstrate the association of clonidine infusion with the levels of white blood cells, neutrophils and platelets to demonstrate its potential anti-inflammation and anti-coagulation actions in ICU. The study focused on the possibility of collecting the data from well-matched (paired) patients, meanwhile, most previous studies relied on the data collected from patients of different ages and conditions.

6.1.3. Aims of the study

Primary outcomes:

1. To compare changes in neutrophils, white blood cells and platelet counts before and after clonidine infusion in ICU patients.

2. To compare the changes in the clonidine group with the well-matched control (non-clonidine) group of ICU patients.

6.2. Methods

6.2.1. Data Collection:

The study is a retrospective observational cohort study on patients (over 18 years old) in Intensive Care Units (ICUs) of Nottingham University Hospital (NUHs). The approval of the submitted proposal of the current clinical study is in Appendix 4. NUH is a UK NHS tertiary referral teaching hospital providing a range of emergency, specialist, and planned services to more than 2.5 million residents of Nottinghamshire and its surrounding communities. The critical care facilities cover all major specialist medical, surgical and trauma disciplines, except for cardiac surgery. There are 66 mixed level 2 and level 3 critical care beds.

Patients who had received clonidine infusions lasting greater than twenty-four hours within an ICU between August 2020 and November 2023 were identified using records kept by the Omnicell® automated drug cabinet system of three ICU areas in NUH: City Hospital, Critical Care Department, Queens Medical Centre, Adult Intensive Care Unit, and Ward E12. A matched set of patients was also identified who had not received clonidine infusions during ICU admissions within the same date range (this will be referred to as the matched control group). Pseudonymised data were collected by the Trust Data Warehouse Team in line with hospital Information Governance (IG) guidelines and National General Data Protection Regulation (GDPR) 2018 and the Data Protection Act 2018. The ethical approval was provided by the NHS Research Ethics Committee (REC) (IRAS project ID: 328769, NUH R&I number 23AN001), and the confirmation of capacity and capability was provided by NUH Research and Innovation before data collection.

6.2.2. Protocol:

The data collected included the basic demographic profiles using on-site databases of the Critical Care Minimum Dataset (CCMDS) and the Intensive Care National Audit & Research Centre Case Mix Programme (ICNARC CMP). The demography of the patients includes age, sex, date of hospital admission and discharge, date of critical care admission and discharge, critical care and hospital survival, dates of any transfer between NUH Critical Care wards, the Acute Physiology and Chronic Health Evaluation II (APACHE II) scores on admission to Critical Care, primary and secondary diagnoses on admission to critical care (planned or emergency admission).

The patients were excluded if they were never admitted to adult intensive care, or if complete data was unavailable (for example, some patients were initially admitted as unknown patients, making it impossible to link admission data to later medical records. Only the first episode of clonidine infusion was included for those patients treated with multiple episodes of clonidine infusions during a single ICU admission. Also, only the first admission was included for patients treated with multiple episodes of clonidine infusion during multiple ICU admissions.

The records of blood tests including neutrophils, white blood cells and platelet counts of both the clonidine and control (non-clonidine) were performed for comparison. Blood results were analysed from the day before clonidine was administered (T1), until the day after the clonidine infusion had been discontinued (T2). The blood results analysed from the control group were those taken from identical time periods during their ICU stay to the time during which clonidine was administered in the clonidine group. For example, if a patient had received clonidine between days 2 and 6 for their

ICU admission then blood results from around that time were analysed, and their matched (non-clonidine) control would also have blood results analysed from around day 2-6 of their admission. The infusion rate of the clonidine used throughout this study was 0-2 µg/kg/hour.

The control group patients were matched on a one-to-one basis with patients who had received clonidine. Control group patients were not necessarily admitted to an ICU area at the same time as their counterparts, although in some cases they were but were cared for in the same study period 2020-2023. The control group was matched using the following criteria: sex, length of ICU stays, age at the time of admission (+/- 5 years), primary admitting diagnoses for ICU admission including overdose (OD), pneumonia (including COVID), trauma, cardiac arrest, haemorrhage (non-trauma), intracranial pathology, cancer, autoimmune disease, other vascular diseases. Whenever primary admitting diagnoses could not be matched, secondary admitting diagnoses were used. Figure 6.1 is the flow chart for identifying the number of patients excluded and included in the study, and the subsequent steps in the identification and analysis of matched data.

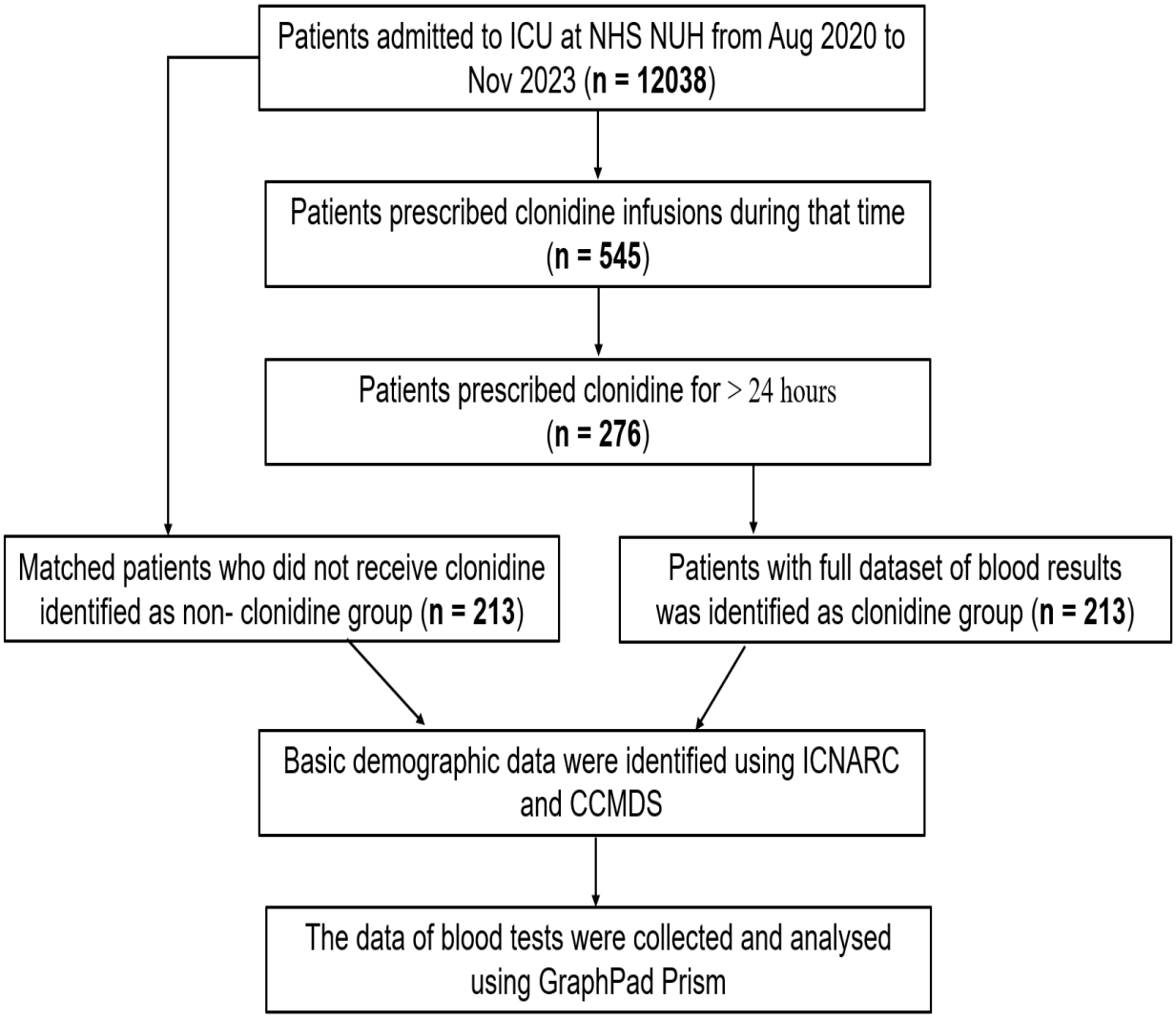


Figure 6.1: Flow chart illustrates the number of patients included in the study and the protocol for identification and analysis of matched data.

6.2.3. Data Analysis and Statistics:

The matched data were identified and entered in an Excel sheet plotted as figures and analysed using Prism version 10.0.3 (GraphPad, USA). The final matched data were tested for normality distribution by D'Agostino & Pearson test and subsequently analysed by Student's paired t-test (Wilcoxon matched pairs) to compare the data of the clonidine group before and after clonidine infusion or unpaired t-test (Mann-Witney Test) to compare the data between the control group and clonidine group. The Chi-square test was used to compare the survival rates between the control group and the clonidine group.

6.3. Results

6.3.1. Demographic analysis and survival rate of the participants:

From August 2020 to November 2023, 12038 ICU-admitted patients were initially identified, where a total of 426 matched patients with the full dataset of the blood results were included. The participants were divided into two groups: 213 patients in the control (non-clonidine) group and 213 patients in the clonidine group who received clonidine for more than 24 hours (Figure 6.3). There were no significant differences in age, sex, APACHE scores, ICU length of stay (LOS), and hospital length of stay (LOS) between the groups (Table 6.1). The survival rate was assessed using a chi-square test, which confirmed that there is no significant difference between ICU deaths and survivors. However, a significant difference in survival rates was observed between deaths and survivors in the hospital (p-value = 0.0038) (Table 6.2).

Table 6.1: Demographic data of the participants in control and clonidine groups. LOS: length of stay, ICU: intensive care unit, APACHE: acute physiology and chronic health evaluation. Values are expressed as numbers or median (interquartile range IQR).

Parameters		Control (n= 213)	Clonidine (n= 213)	P-value
Sex	**Male**	162	162	
	Female	51	51	
Age		49 (35.24 - 57.90)	46.28 (34.38 - 56.15)	0.406
Patients with a primary diagnosis		177	190	
Patients with a secondary diagnosis		153	134	
APACHE		14 (10 - 18)	13 (9 -17)	0.088
LOS in ICU (Days)		16 (9 - 25)	16 (9 -25)	1.0
LOS in Hospital (Days)		33.71 (21.24 - 56.7)	30.18 (18.23 - 49.00)	0.098

Table 6.2: The survival rate of ICU and hospital patients. Values are expressed as the total number of patients. *p < 0.05 represented significant differences using the chi-squared test.

	Control	Clonidine	p-value
ICU Deaths	20	13	0.210
ICU Survivors	201	208	
Hospital Deaths	39	23	0.028*
Hospital Survivors	182	198	

6.3.2. Clonidine infusion in ICU:

This study focused on patients who received a clonidine infusion in the ICU for more than 24 hours. The median day of starting the clonidine infusion was 5 (IQR 3-9), and the median duration of clonidine administration was 3 days (IQR 2-5). After discontinuation of clonidine, the median length of stay in the ICU was 5 days (IQR 1.5-12) (Figure 6.2 A, B, and C).

6.3.3. Comparison of blood results at baseline (T1) with the last measurement (T2) of ICU patients:

In preliminary analysis, the blood results of neutrophils, WBCs, and platelets were identified for all the patients in both groups (n=426) to demonstrate the difference between the first measurement (T1) and the last measurement (T2) of blood results. The results showed that there is no significant difference comparing the blood results of neutrophils, and WBCs of ICU patients at T1 and T2. However, a significant increase was observed in the level of platelets at T2 when compared to T1 (p-value < 0.0001) (Figure 6.3).

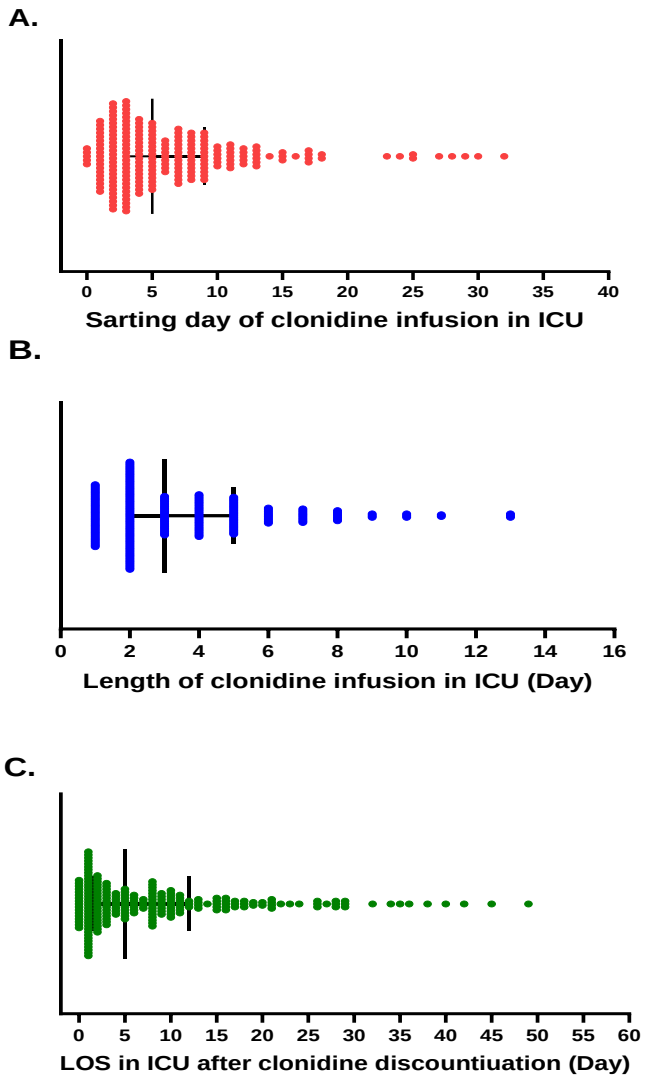


Figure 6.2: Clonidine infusion to ICU patients: (**A**) starting day of clonidine infusion, (**B**) length of clonidine infusion, and (**C**) length of stay in ICU after clonidine discontinuation. Values are presented as median (IQR).

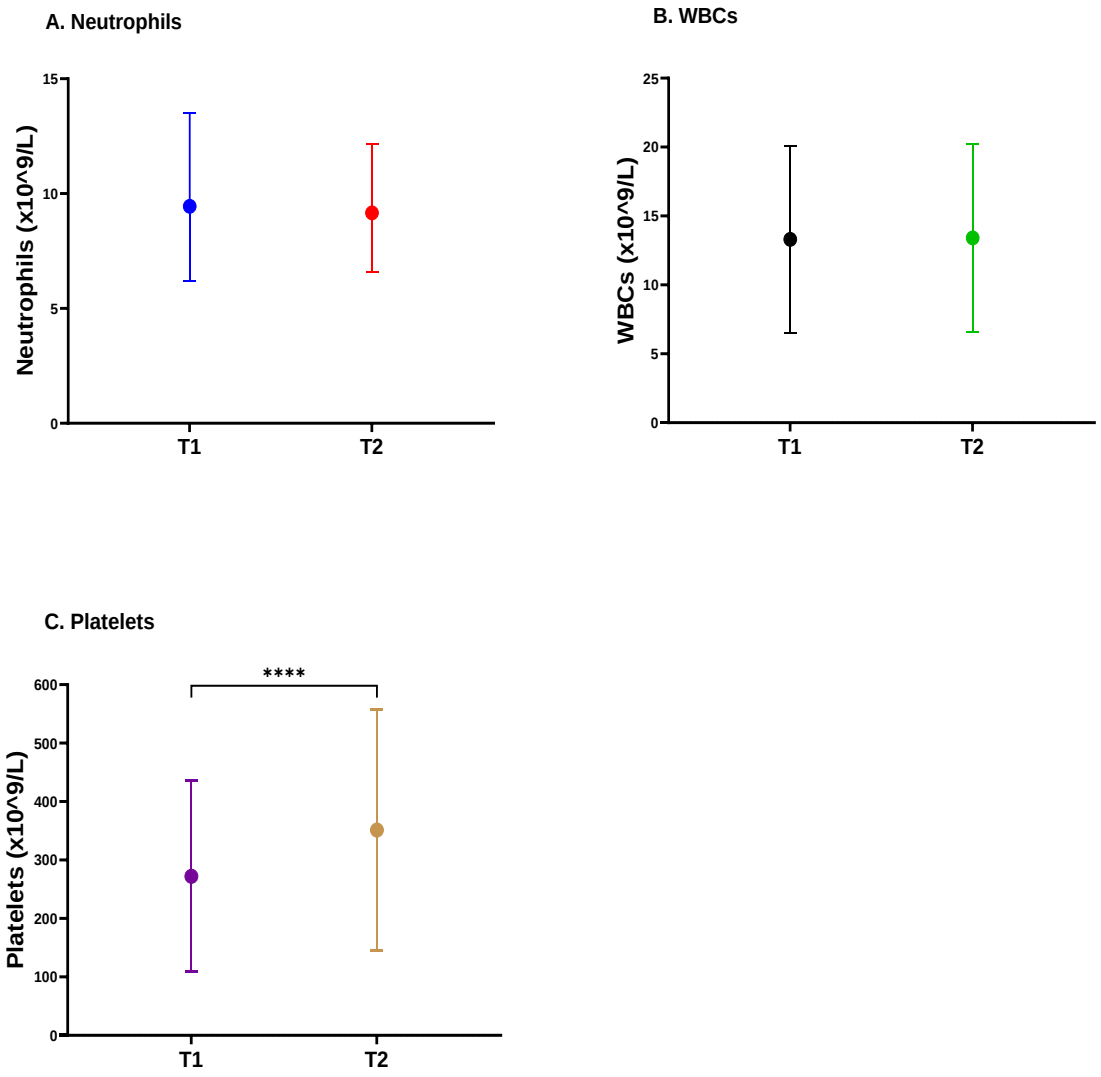


Figure 6.3: Comparison between the first measurement (T1) and last measurement (T2) of blood levels of (**A**) neutrophils, (**B**) WBCs, and (**C**) platelets of all the participants (n=426). Values are expressed as median (IQR). T1: first measurement, T2: last measurement. The ****p-value < 0.0001 was considered statistically significant, paired t-test (Wilcoxon matched pairs test).

6.3.4. Association of clonidine infusion with neutrophil, WBCs and platelet counts:

In the clonidine group (213), there was no significant difference in the level of neutrophils and WBCs at T1 (before clonidine) compared to T2 (after clonidine). However, a significant difference was observed in the platelet count (p-value < 0.0001) (Figure 6.4). Similarly in the control group, no significant change was observed in the levels of neutrophils and WBC, meanwhile, the platelet count was increased by comparing the first measurements (T1) to the last measurements (T2) (Figure 6.5). The percentage of change in the blood results was calculated by subtracting the values at T1 from those at T2. This percentage change was then analysed to compare the rate of change between the control group and the clonidine group. The results showed that there was no significant difference in the percentage of change in the level of neutrophils, WBCs and platelets between the control group and the clonidine group (Figure 6.6).

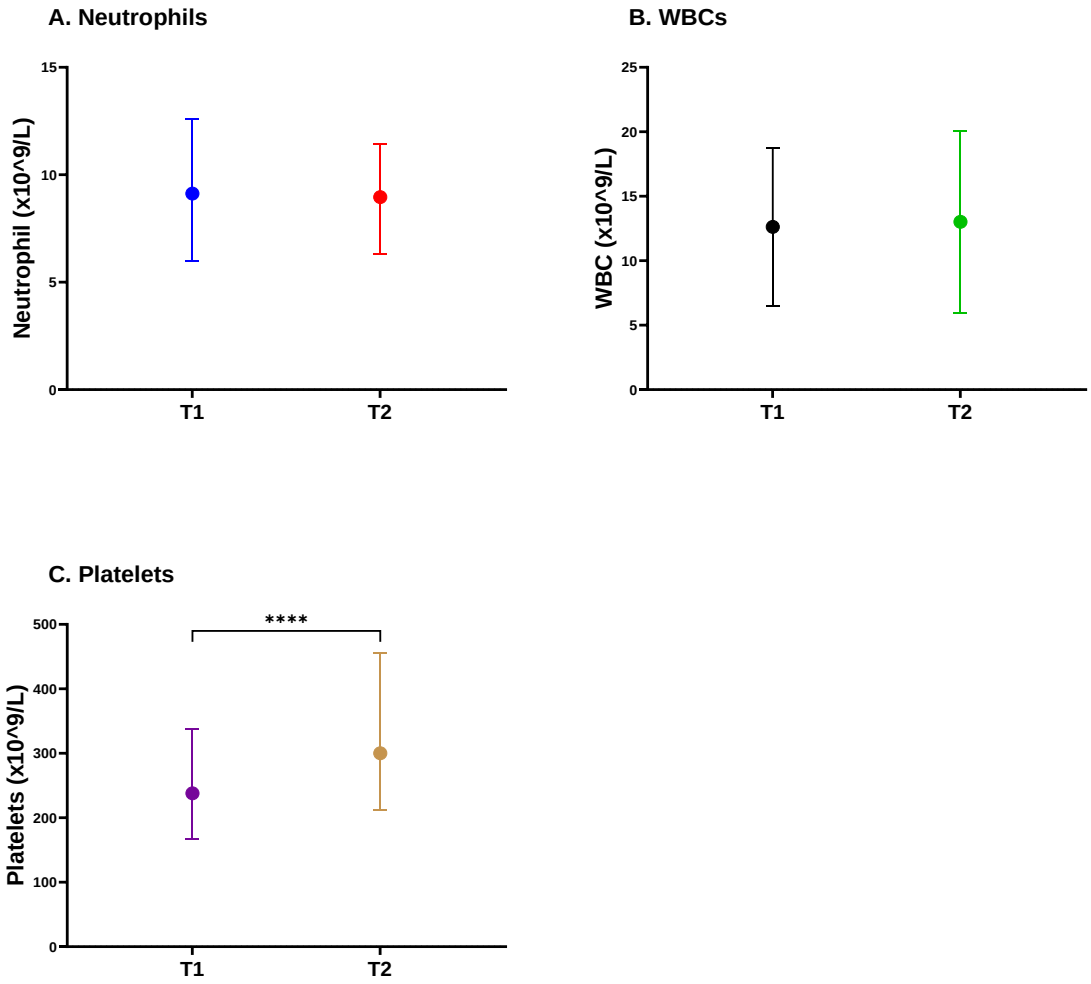


Figure 6.4: Comparison of the first measurement before clonidine (T1) and last measurement after clonidine (T2) of (**A**) Platelets, (**B**) WBCs, and (**C**) platelets in the clonidine group (n=213). Values are expressed as median (IQR). T1: baseline before clonidine infusion, T2: last measurement after discontinuation of clonidine infusion. ****p-value < 0.0001 was considered statistically significant, paired t-test (Wilcoxon matched pairs test).

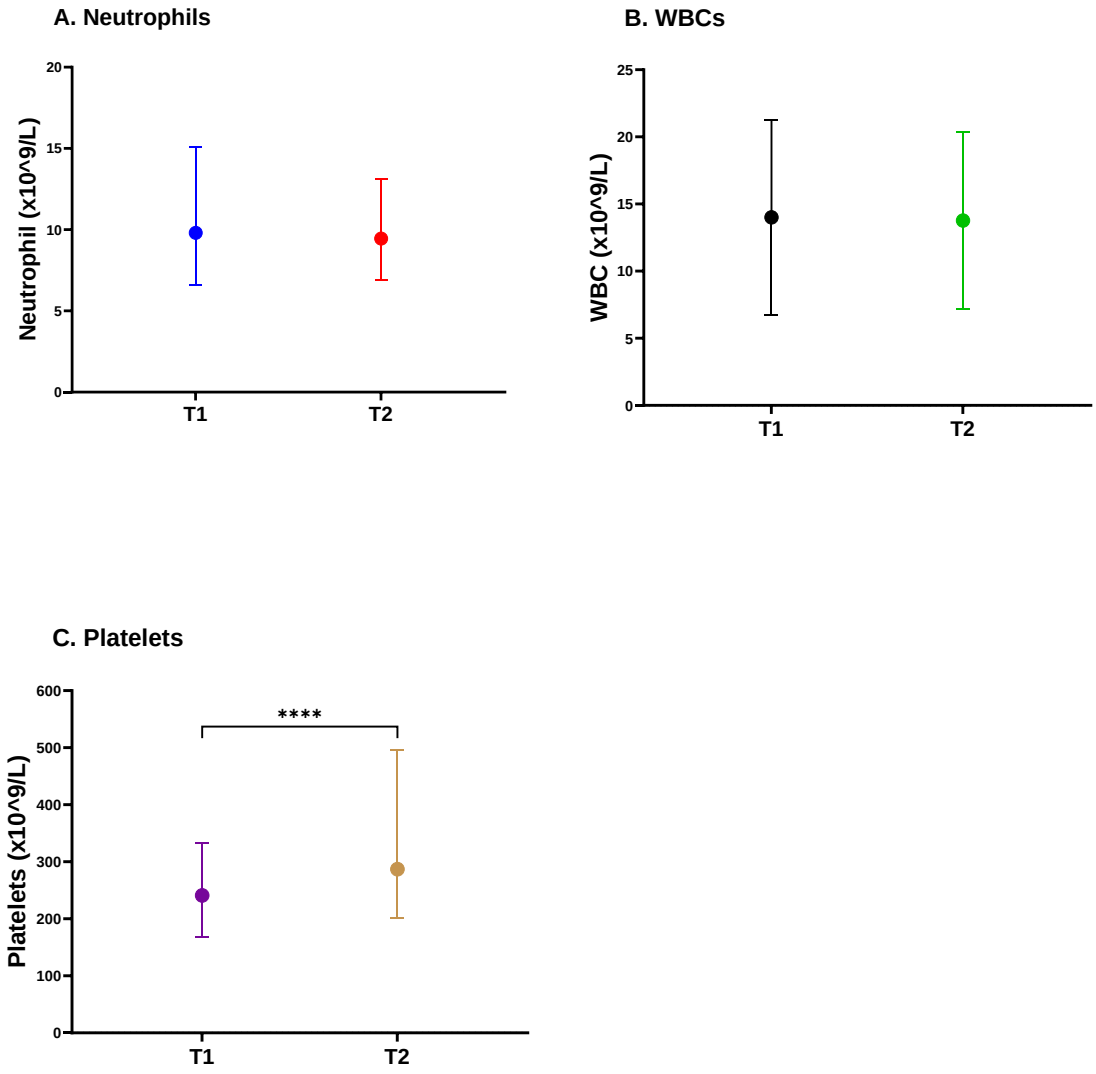


Figure 6.5: Comparison of the first measurement (T1) and last measurement (T2) of (A) Platelets, (B) WBCs, and (C) platelets in the control group (n=213). Values are expressed as median (IQR). T1: baseline before clonidine infusion, T2: last measurement after discontinuation of clonidine infusion. ****p-value < 0.0001 was considered statistically significant, paired t-test (Wilcoxon matched pairs test).

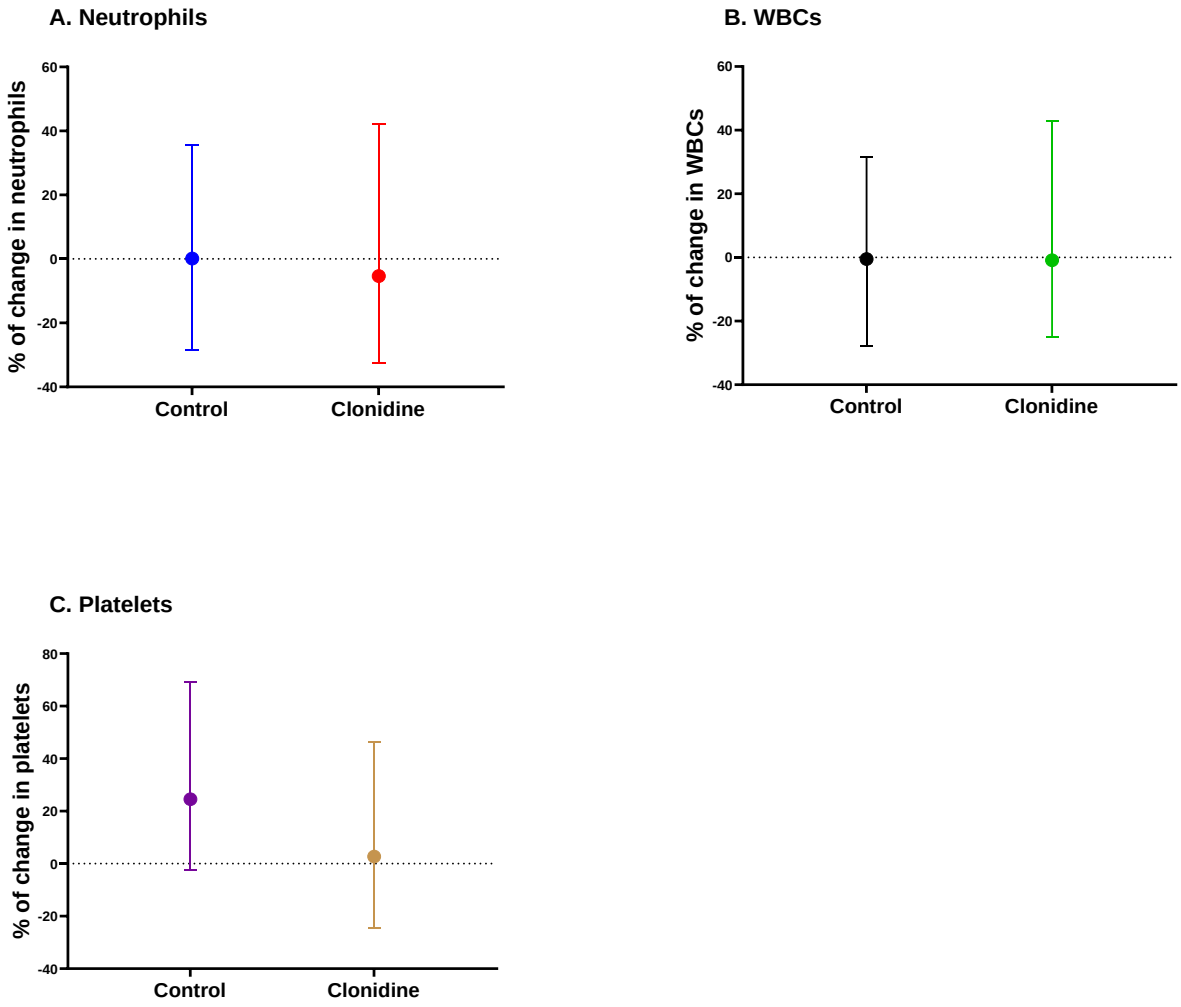


Figure 6.6: Comparison of the percentage of change in the level (A) Neutrophils, (B) WBCs, and (C) platelets between the control group (n=213) and clonidine group (n=213). Values are expressed as median (IQR).

6.4. Discussion

This study demonstrated the association of clonidine infusion with ICU patients by analysing the changes in specific blood parameters. The results showed that the participants in both groups were similar in terms of age, sex, APACHE scores, ICU LOS, and hospital LOS, indicating that the participants were perfectly matched. Therefore, the outcomes of this study could be more confidential due to the intervention of clonidine rather than demographic variation. The survival rate between hospital deaths and survivors was significantly higher in the clonidine group, but there was no association between clonidine use and ICU survival, thus clonidine may not impact immediate ICU mortality rate, but it could influence long-term hospital outcomes. There are conflicting results regarding the influence of α_2-adrenoceptor agonists on the mortality rate of critically ill patients. Dexmedetomidine improved the survival rate in critically ill patients with sepsis (Kawazoe *et al.,* 2017, Zhang *et al.,* 2022, Zhao *et al.,* 2024). However, dexmedetomidine reduced the duration of mechanical ventilation and inflammatory response without affecting the mortality rate (Wang *et al.,* 2021a). Also, the SPICE III clinical trial found that early administration of dexmedetomidine was associated with more adverse effects with no significant impact on the 90-day mortality rate of critically ill patients in the ICU (Shehabi *et al.,* 2019).

The outcomes of this study showed that the blood levels of neutrophils, WBCs, and platelets were unaffected by clonidine infusion. As mentioned before, both dexmedetomidine and clonidine have anti-inflammatory effects, which may improve clinical outcomes in critical conditions like sepsis (Flanders *et al.,* 2019). Dexmedetomidine reduced concentrations of various cytokines and inflammatory markers such as IL-18 and IL-2Rα in healthy volunteers (Kallioinen *et al.,* 2019, Bosch

et al., 2020b). Furthermore, the administration of dexmedetomidine during cardiac surgery could significantly alleviate the surgery-induced increase in cytokines such as IL-1, IL-6, TNF-α, and INF-gamma, and reduce postoperative complications (Bulow *et al.,* 2016, Wu *et al.,* 2004, Wu *et al.,* 2018, Xu *et al.,* 2021). Therefore, we hypothesised that clonidine could exert some anti-inflammatory function in critically ill patients by determining the effect of clonidine on the level of neutrophils and WBCs. Our findings suggested that clonidine might exert some anti-inflammatory effects by reducing the cytokines as reported by other studies without changing the counts of neutrophils and WBCs. It is known that neutrophils are the first responders to be involved in the host inflammation system. An in vitro study on human neutrophils found that the clinically relevant concentrations of clonidine and dexmedetomidine failed to modulate the neutrophils' function, where they did not affect the chemotaxis, phagocytosis, or production of superoxide by human neutrophils (Nishina *et al.,* 1999). Their results agreed with our findings, where no change was observed in the neutrophil counts. However, an in vitro study has shown that dexmedetomidine significantly suppressed the phagocytic activity of human neutrophils after Escherichia coli stimulation (Chen *et al.,* 2016). In a recent prospective randomised clinical study, the continuous perioperative dexmedetomidine infusion significantly decreased the production of neutrophils and tumour metastasis biomarkers in postoperative lung cancer patients. Additionally, dexmedetomidine reduced inflammation, preserved cellular immune function, and enhanced the quality of postoperative recovery (Ren *et al.,* 2023).

In the present study, the significant increase was only observed by comparing the platelet count between the first and last measurements of blood results in all ICU patients. However, this change was not observed by comparing the percentage of

change at two-time points of the clonidine group with that of the control group, indicating that an increase in the platelets existed in all ICU patients regardless of clonidine infusion which could be due to other ICU factors. A retrospective cohort study showed that during the first seven days following ICU admission, platelet counts decreased initially but later increased above the admission value in survivors, whereas platelets decreased without upturn in non-survivors (Li *et al.,* 2022). Previously, different studies showed the opposite effect of catecholamines on platelet activity. It was reported that adrenaline induced platelet aggregation in human platelets via α_2-adrenoceptors, while clonidine reversed the aggregation action of adrenaline (Steer and Atlas, 1982). Also, NA enhanced the platelet aggregation through α_{2A}-adrenoreceptors coupled with the Gi family on platelets (Yang *et al.,* 2002). However, an increase in the platelet reactivity induced within 48 hours of surgery was not affected by clonidine administration (Rosenfeld *et al.,* 1993). This observation agreed with the present study, suggesting the safety of clonidine infusion in ICU, and a decrease in the risk of arterial thrombosis.

To our knowledge, this is the first study that focused on collecting the data from the carefully paired patients which eliminates any differences related to the ages or other ICU circumstances. In conclusion, this study provides an important insight into the effects of clonidine infusion on ICU patients, where clonidine did not significantly alter the neutrophil, WBC and platelet counts. Further clinical studies with a larger number of patients are required. Also, an improvement in the hospital survival rates in the clonidine group can be a potential area for further clinical research.

7. Chapter Seven: General discussion and conclusion

7.1. General Discussion

The pathophysiological features of sepsis-induced organ dysfunction include excessive production of inflammatory mediators, overactivation of the sympathetic system, vascular dysfunction leading to vasoplegia and persistent hypotension (Levy *et al.,* 2010). Despite decades of extensive research, there is no specific therapy for sepsis yet. Early diagnosis and treatment with appropriate antibiotics, fluids, and vasopressors are crucial for minimising the organ damage and reducing the mortality rate of sepsis and septic shock (Font *et al.,* 2020). The growing interest in α_2-adrenoceptor agonists for the management of ICU critical conditions, particularly septic shock, is due to their multiple pharmacodynamic actions. Recently, α_2-adrenoceptor agonists such as dexmedetomidine and clonidine have increasingly been used to maintain sedation and manage delirium in critically ill patients with or without sepsis (Lankadeva *et al.,* 2021). Meanwhile, these agonists can also influence the peripheral systems, potentially stabilising the haemodynamic responses (Matot *et al.,* 2000b), attenuate the inflammation (Bulow *et al.,* 2016, Wu *et al.,* 2004, Wu *et al.,* 2018, Xu *et al.,* 2021), reduce the NA requirements (Geloen *et al.,* 2013, Lankadeva *et al.,* 2015, Lankadeva *et al.,* 2019) and improve the ICU outcomes (Kawazoe *et al.,* 2017, Zhang *et al.,* 2022, Zhao *et al.,* 2024).

Additionally, the other important factor that prioritises dexmedetomidine over other sedatives is the ICU related costs. The sedation with dexmedetomidine in ICU patients who need long term sedation (more than 24 hours) was associated with ICU cost savings of $9679 compared with midazolam due to less ICU stay (Dasta *et al.,* 2010). Moreover, several clinical trials have compared the costs of dexmedetomidine with other sedatives such as midazolam and propofol used for short-term sedation in ICU.

Dexmedetomidine is associated with shorter ICU stays and decreased mechanical ventilation times, resulting in more than 20% cost reduction (Aggarwal *et al.,* 2020). However, dexmedetomidine is still considered an expensive sedative agent, and therefore clonidine is mostly used in UK ICUs (Gagnon *et al.,* 2015).

By comparing the pharmacodynamic actions and lipophilic properties of different α_2-adrenoceptor agonists, the agonists with lower lipophilicity are considered less permeable to CNS and better redistribute the blood in the peripheral cardiovascular system. We found that there are other α_2-adrenoceptor agonists such as brimonidine and mivazerol with less lipophilicity than dexmedetomidine and could potentially have peripheral vasoconstriction actions with less sedation actions. However, brimonidine has local clinical uses only, and mivazerol has not been clinically approved yet. The other α_2-adrenoceptor agonist that drew our interest is guanfacine, which could be used as an alternative sedative agent to dexmedetomidine or clonidine in the ICU based on its central and peripheral vasoconstriction action. The peripheral vasoconstriction actions of α_2-adrenoceptor agonists can potentially maintain blood pressure, improve endothelial permeability and reduce the NA requirement in ICU.

The vasoconstriction action and NA sparing activity of α_2-adrenoceptors:

The current study showed that α_2-adrenoceptor agonists can mediate vasoconstriction with varying degrees of potencies in different blood vessels. The potency of α_2-adrenoceptor agonists was higher in the splenic vein and artery compared to the tail artery. In comparison to NA, the maximum contraction induced by α_2-adrenoceptor agonists was smaller than that of NA in the porcine splenic artery. Previous studies showed that dexmedetomidine induced vasoconstriction in multiple species, including

dog and porcine coronary arteries, as well as human gastroepiploic and brachial arteries (Seyrek *et al.,* 2011). Experimental results on porcine splenic arteries revealed that α_2-adrenoceptors mediate vasoconstriction, but less prominently than α_1-adrenoceptors. Contractions induced by phenylephrine were about 60% greater than that of clonidine in the porcine splenic artery (Barbieri *et al.,* 1998). Additionally, this study demonstrated that the vasoconstriction of dexmedetomidine and mivazerol was significantly reduced in the presence of SNP in the porcine splenic artery, indicating that NO mediated vasorelaxation could affect the contractile responses of α_2-adrenoceptor agonists in sepsis. SNP induced vasodilatation by increasing cyclic GMP through NO release, causing vascular smooth muscle relaxation in both peripheral arteries and veins (Ranadive *et al.,* 2017).

This study aimed to investigate the vasoconstrictive actions of α_2-adrenoceptor agonists in LPS mediated septic model. However, this was not possible due to the failure to develop a suitable septic model in porcine splenic arteries despite of various justifications in the protocols. The previous studies from this lab used porcine isolated coronary arteries to design a sepsis model, where the overnight exposure to 1 µg/ml and 100 µg/ml LPS lowered KCl and U46619 vascular contractions and impaired the endothelium-mediated relaxation induced by substance P (Wei *et al.,* 2008, Al-Shalmani *et al.,* 2011). This study was the first to use porcine splenic arteries to create a sepsis model. However, the main issue encountered was infection, which occurred when the tissue segments were incubated overnight at 37 ºC. Several justifications were followed to solve the problem of infection such as using sterilised dissection tools, incubation in antibiotics solution plus antiproliferative agents, washing the spleens with the 0.1% chlorus solution before dissection. However, this was

insufficient to control the infection in isolated porcine splenic arteries. The spleens, often contaminated with animal faeces due to their abdominal location, were difficult to separate without contamination. This explains the reason why splenic arteries are more susceptible to infection than coronary arteries. Our results indicated that bacterial infection during the overnight incubation of the porcine splenic artery can impair the contraction of vascular smooth muscles and the integrity of the vascular endothelium. Therefore, the usage of encapsulated organs such as kidneys and lungs will likely be more suitable to build a sepsis model.

This study used a unique approach to demonstrate the additive vasoconstriction action of α_2-adrenoceptor agonists when combined with NA. It is known that prolonged exposure to high doses of catecholamines resulted in irreversible cardiac injuries and lethal complications (Stolk *et al.,* 2020, Bode *et al.,* 2024). Several studies approved that coadministration of catecholamines with other vasoactive agents such as L-NAME, vasopressin, angiotensin II, and methylene blue could improve blood pressure and reduce the required dosage of catecholamines (Avontuur *et al.,* 1998, Annane *et al.,* 2018, Patel *et al.,* 2002). In the present study, the preliminary experiments tested the NA sparing activity of BAYK8664, an L-type voltage-gated calcium channels agonist. The results showed that the presence of BAYK8664 significantly enhanced the potency of NA, where the concentration-response curve shifted to the left and the pD2 of NA increased from 5.8 ± 0.1 to 6.3 ± 0.1 (Appendix 5). Consequently, this approach was taken to investigate the NA sparing activities of other vasoactive agents such as U46619, a potent and selective agonist of thromboxane A2 receptors, and α_2-adrenoceptor agonists such as dexmedetomidine and guanfacine. The novelty of the present study is that we focused on small concentrations of NA (1 nM - 200 nM)

to perform the cumulative concentration response curves measuring $EC_{30\ KCl}$ rather than EC_{50}. This range of NA concentrations fall within its clinically relevant plasma concentrations (Beloeil *et al.,* 2005).

This study also demonstrated that the vasoconstriction actions of α_2-adrenoceptor agonists initiate at low range of concentrations, typically within 1-100 nM. Therefore, these concentrations of α_2-adrenoceptor agonists such as dexmedetomidine and guanfacine were chosen to study their NA sparing activity. dexmedetomidine which is commonly used as a sedative agent in ICU, was reported that its plasma concentration ranged from 1 to 70 nM (Dyck *et al.,* 1993, Ebert *et al.,* 2000). Moreover, the plasma concentration of guanfacine exhibit sedation was nearly 34 nM (Dollery and Davies, 1980). The present findings showed that dexmedetomidine and guanfacine have NA sparing actions by reducing the NA dosage by 50%. This reduction in the required doses of NA could improve the outcomes of ICU patients (Patel *et al*., 2002, Ibarra-Estrada *et al*., 2023). Hence, this supports the hypothesis that dexmedetomidine may enhance the peripheral vasoconstriction and reduced the catecholamines requirements in ICU patients. It has been previously reported in that there is a potential synergistic contraction mediated through vascular α_1- and α_2- adrenoceptors (Daly *et al*., 1988, Sporkova *et al*., 2010). The present findings suggest that there is a potential benefit of co-administering the α_2-adrenoceptor agonists with NA, which could improve the clinical outcomes of septic patients. This study presents new opportunities for more therapeutic approaches of α_2-adrenoceptor agonists in septic shock, which may help minimising the undesired effects of catecholamine treatment.

The effect of dexmedetomidine on LPS-induced endothelial cells hyperpermeability

The endothelial barrier dysfunction is an essential factor in the onset of multiple organ failure in sepsis (Arina and Singer, 2021). As mentioned in Table 4.1, various in vitro studies demonstrated that LPS induces hyperpermeability or dysfunction of the endothelial barrier. Although most studies reported that 1 µg/ml LPS results in endothelial hyperpermeability, in this study, significant FITC-dextran barrier leakage was only observed at the higher concentration (10 µg/ml). These findings support the study by Zheng *et al*. (2018), which found that LPS at high concentrations only (10 and 100 µg/ml) increased the monolayer permeability of the primary HPMVEC barrier function (Zheng *et al.,* 2018). Several in vitro studies reported that dexmedetomidine has a potentially protective action against vascular leakage following sepsis or cellular ischaemia by inhibiting ferroptosis. Our findings showed that dexmedetomidine could potentially have protective action against LPS induced hyperpermeability of HUVECs. These findings agreed with the previous in vivo study, where dexmedetomidine alleviated endothelial barrier dysfunction in septic rats (She *et al.,* 2021). It also alleviated the cytotoxicity and hyperpermeability in HBMVECs (Zhao *et al.,* 2021). However, the limitation of our study was the shortage of inserts used for the permeability experiments on endothelial cells, which prevented us from repeating the experiments more than four or five times. Also, the concentration of dexmedetomidine used in the permeability study was higher than its clinical plasma concentration in humans. Further experiments are needed to investigate the protective action of dexmedetomidine at therapeutic related concentrations.

NA uptake1 inhibitors and their effect on EFS mediated vasoconstriction:

This study also investigated the effects of NA uptake1 inhibitors such as atomoxetine, reboxetine, and desipramine on EFS vasoconstriction in porcine splenic and renal arteries. These drugs are known to enhance the postsynaptic adrenoceptors in CNS and hence used to treat a variety of mental disorders such as depression and ADHD (Zhou, 2004). Previous animal studies reported that cocaine can significantly enhance vasoconstriction and raise blood pressure (Kuriyama and Suyama, 1983, Kuhn *et al.,* 1990, Gonon *et al.,* 1993). Furthermore, a randomized clinical study on patients with impaired central and peripheral autonomic nervous systems showed that atomoxetine significantly increased blood pressure in seated patients with central autonomic impairment (Shibao *et al.,* 2007), and increased SBP and HR in ADHD children and adolescents (Hennissen *et al.,* 2017).

Surprisingly, cocaine and neither of the other NA uptake1 inhibitors used in this study increased the magnitude of EFS responses in the porcine splenic artery, but it enhanced noradrenergic vasoconstriction by increasing the time duration of vasoconstriction in EFS responses. This partly agreed with the previous animal studies, which demonstrated that cocaine potentiated the duration of adrenergic responses in addition to the magnitudes evoked by EFS (Muramatsu *et al.,* 1989, Gonon *et al.,* 1993). Atomoxetine (100 nM) significantly decreased the $EC_{30\,KCl}$ of NA, suggesting that atomoxetine has NA-sparing activity, and could enhance the vasoconstrictive action of NA. Our findings support the hypothesis that co-administration of these clinically approved NA uptake1 inhibitors with NA could enhance the clinical outcomes of ICU patients. However, further investigations are

required to study the NA sparing activity of atomoxetine and reboxetine in the sepsis model. For future work, there is a plan for the clinical study on healthy volunteers to investigate the effects of reboxetine on physiological vital signs such as HR and BP. The present findings also raise the suggestion to investigate the combination of atomoxetine and guanfacine, as they may work better together.

The potential effect of clonidine on the inflammatory-immune system and platelet activity in ICU patients:

The participants in the retrospective cohort study were well-matched in age, sex, APACHE scores, ICU length of stay, and hospital length of stay, enhancing the reliability of the results. Both clonidine and dexmedetomidine have known anti-inflammatory effects, which may improve outcomes in critical conditions like sepsis (Flanders *et al*., 2019). The current findings suggest that the anti-inflammatory effect of clonidine is possibly due to decreasing cytokine levels as reported by previous studies without affecting neutrophil and WBC counts. Our results agreed with an in vitro study which found that clinically relevant concentrations of clonidine and dexmedetomidine did not modulate the functions of human neutrophils (Nishina *et al*., 1999). Previous studies have shown varying effects of catecholamines on platelet activity (Steer and Atlas, 1982, Yang *et al*., 2002). The present results showed that the change in the platelet activity of ICU patients is not related to the clonidine infusion, indicating the safety of using clonidine infusion for critically ill patients with no or minimum adverse effects including post-surgery haemorrhage or risk of arterial thrombosis. The hospital survival rate was significantly higher in the clonidine group, suggesting that clonidine may influence long-term hospital outcomes.

7.2. Conclusion

In conclusion, despite conflicting clinical outcomes, many studies support the beneficial role of α_2-adrenoceptor agonists in sepsis management. Dexmedetomidine showed a potential role in reducing vasopressor requirements, mortality rates, mechanical ventilation, and ICU stay duration. Our research suggested the role of α_2-adrenoceptor agonists in raising MAP through peripheral vasoconstriction and that other α_2-adrenoceptor agonists with low lipophilicity such as brimonidine or guanfacine could potentially be better than dexmedetomidine in the management of sepsis related hypotension. Different α_2-adrenoceptor agonists produced contractions with various Emax and pD_2 in central and peripheral porcine vasculature. Moreover, the vasoconstriction action of dexmedetomidine and mivazerol was reduced in the presence of SNP but not completely abolished. Therefore, further studies are needed to confirm the peripheral vasocontraction of α_2-adrenoceptor agonists in LPS treated blood vessels. Moreover, the combination of dexmedetomidine and guanfacine with NA enhanced NA-mediated contraction in the porcine splenic artery resulting in the left shift of the concentration response curve of NA. This supports the potentiality that administration of dexmedetomidine could enhance sepsis outcomes in the ICU by lowering 50% of the NA dosage. Dexmedetomidine had protective effects on LPS-induced hyperpermeability in HUVECs. Investigations on therapeutically relevant doses of α_2-adrenoceptor agonists on endothelial hyperpermeability in various cell lines are required. Neuronal uptake1 inhibitors enhanced the duration of noradrenergic vasoconstriction by increasing the time to 50% relaxation, and atomoxetine produced NA sparing activity in the porcine splenic artery. Clonidine infusion in carefully matched ICU patients did not significantly alter neutrophil, WBC, and platelet counts,

but improved the hospital survival, suggesting a potential area for further clinical research.

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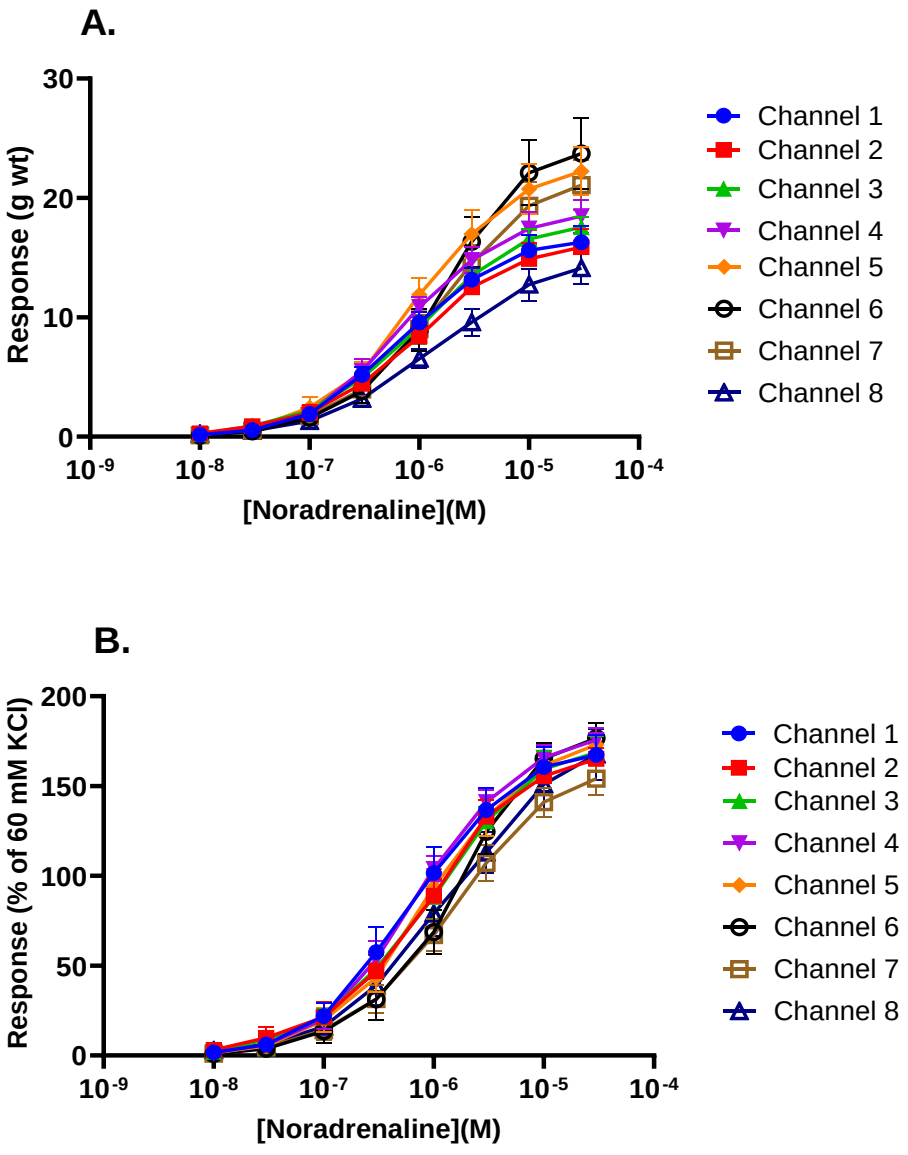
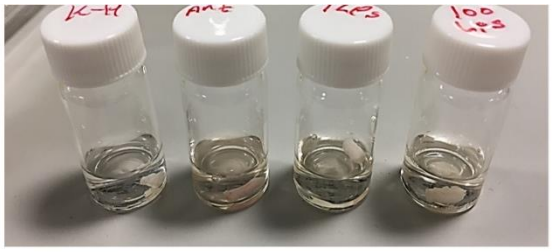
Appendix

Appendix 1: The cumulative concentration response curves of NA in eight segments of the porcine splenic artery. The responses are expressed as (A) Weight in grams and (B) % of 60 mM KCl. The data are presented as mean ± SEM, n=6.



Appendix 2: Comparison of the cloudiness of incubation media (Krebs-Henseleit with antibiotics solution) of porcine splenic arteries throughout the incubation period at 37ºC.



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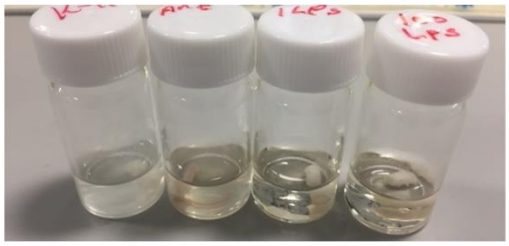
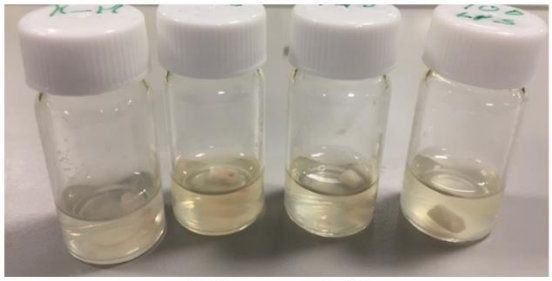
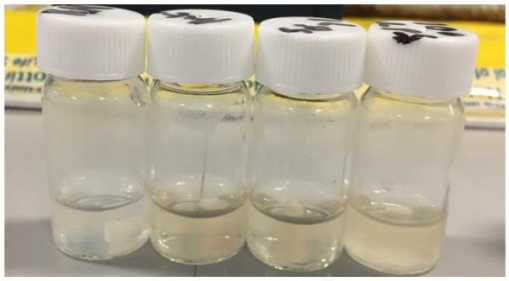


24 hours at 37˚C

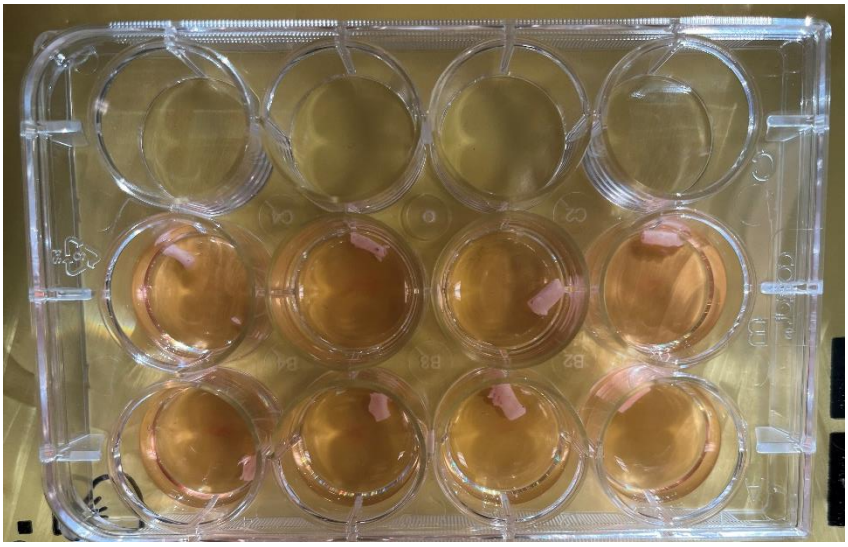


48 hours at 37˚C



168 hours at 37˚C

Appendix 3: Segments of porcine splenic arteries incubated in culture media (ECM) at 37 ºC after 24 hours.



Appendix 4: A copy of the signed approval pages for the submitted proposal of the current clinical study.

Nottingham University Hospitals NHS
NHS Trust

FULL/LONG TITLE OF THE STUDY

Identifying potential changes in blood cell count and liver function in patients treated with clonidine infusions during treatment on the intensive care unit.

SHORT STUDY TITLE / ACRONYM

Clonidine infusion and ICU blood counts RESEARCH REFERENCE NUMBERS

IRAS Number:	328769
SPONSORS Number:	23AN001
FUNDERS Number:	N/A

OTHER RESEARCH REFERENCE NUMBERS:

SPONSOR: Nottingham University Hospitals NHS Trust

PROTOCOL VERSION NUMBER AND DATE: **Version 1.0 - 21/9/2023**

SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the study in compliance with the approved protocol and will adhere to the principles outlined in the Declaration of Helsinki, the Sponsor's SOPs, and other regulatory requirement.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the investigation without the prior written consent of the Sponsor

I also confirm that I will make the findings of the study publicly available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

For and on behalf of the Study Sponsor:

Signature: Date: ...../...../.....

.....

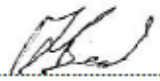
Name (please print):

.....

Position:

.....

Chief Investigator:

Signature:  Date: 21/09/2023

.....

Name: (please print):

.....Dr Martin Beed.....

Appendix 5: The cumulative response curves of NA in the presence of 1 µM BayK8664. The contractions are expressed as % of 60 mM KCl and presented as the mean ± SEM, n= 6.