Title Page

Full Title: The differential *in vitro* effects of clinically used vasoactive drugs on small human pulmonary vessels.

Total number of volumes: One.

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Qualification for which the thesis is submitted: Doctor of Medicine

Institution: University of Hull and the University of York

Department: Hull York Medical School

Month and year of submission: July 2017

Word Count: 35918

Abstract Count: 350

Abstract

Introduction:

Pulmonary hypertension (PH) is an important prognostic factor in cardiac surgery and is associated with increased morbidity and mortality. The mechanism of the underlying pathophysiology is complex and PH may exist prior to surgery or might develop during or after surgery. Different models are used to explore the underlying cellular and molecular mechanism of lung disease especially pulmonary vascular disease. Isolation of human pulmonary artery and measurement of pulmonary vascular tension are vital to understand the pathophysiology of human pulmonary vessels. The aim of this study was to characterize the effects of clinically used pharmacological agents on the human pulmonary vasculature.

Methods:

Patients undergoing lung resection were consulted and consented for their resected lung tissue to be included in the study. Pulmonary arteries (PA) dissected from disease free areas of lung resection and PA rings of internal diameter 2-4 mm and 2 mm long were prepared. Integrity of the endothelium was confirmed with 1µM acetylcholine (ACh) and 37.6 µM (EC_{80}) potassium chloride (KCl) was used to check the contractility of PA rings. Multiwire myograph system was used to mount the PA rings under physiological conditions in modified Krebs solution. Concentration response curves were constructed to pharmacological agents by cumulative addition to the myograph chambers.

Results:

Both the multi-wire myograph and organ bath system have demonstrated identical conclusions and confirmed that the most efficient resting tension for human PA rings of internal diameter 2-4 mm is 1.61 gf Vasopressin had no vasoconstrictive effect on the small human pulmonary arteries and hence may be safe to use for systemic vasoconstriction in patients with pulmonary hypertension. The prostacyclin analogues were the most potent and efficacious vasodilators in the isolated human pulmonary arteries.

Conclusions:

The current study is the only in vitro study that demonstrated the efficacy and potency of clinically used vasopressors, prostacyclin analogues, sodium nitroprusside and phosphodiesterase inhibitors on human pulmonary vascular reactivity. These effects may inform the use of these drugs in the clinical setting as prostacyclin analogues provide better results. Fully blinded randomized control trials are needed to show the potential bench to bed effect of this study.

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Abbreviations

AA: Arachidonic acid

Ach: Acetylcholine chloride

AD: Adrenaline

ALOX5: Arachidonate 5-lipoxygenase

AMPK: Amp activated protein kinase

AVP: Arginine Vasopressin

cAMP: Cyclic adenosine monophosphate

CASMCs: Coronary artery smooth muscle cells

cGMP: Cyclic guanosine monophosphate

CO2: Carbon Dioxide

COPD: Chronic Obstructive Pulmonary Disorder

CPB: Cardiopulmonary bypass

DAG: 1, 2-diacyl-glycerol

(DMSO): Dimethyl sulfoxide

EC: Endothelial cell

EC50: Concentration of agonist required to elicit 50% of maximum response

EDRF: Endothelium derived relaxing factor

EETs: Epoxyeicosatrienoic acids

Emax: Maximum effect of a drug

eNOS: Endothelial nitric oxide synthase

Ep: Epoprostenol

ET-1: Endothelin-1

FDA: Food and Drug Administration

H_2O_2: Hydrogen peroxide

HPAEC: Human pulmonary artery endothelial cells

HPASMc: Human pulmonary artery smooth muscle cells

HPAR: Human pulmonary artery rings

HPAS: Human pulmonary artery strips

HPV: Hypoxic pulmonary vasoconstriction

Ip: Iloprost

IP3: Inositol-1, 4, 5-trisphosphate

KCl: Potassium chloride

KPa: Kilo pascal

Kv: Voltage – dependent K^+ channels

LKB1: Liver kinase B1

L-NAME: N-Nitro-L-arginine methyl ester hydrochloride

Mil: Milrinone

NO: Nitric Oxide

NA: Noradrenaline

NADPH: Nicotinamide adenine dinucleotide phosphate

PA: Pulmonary artery

PDE-3: phosphodiesterase enzyme type 3

PDE-5: phosphodiesterase enzyme type 5

pEC_{50}: Negative logarithm to base 10 of the molar EC_{50} concentration

PGF2α: Prostaglandin F2α

PAH: Pulmonary artery hypertension

PAP: Pulmonary artery pressure

PAEC: Pulmonary artery endothelial cell

PASMC: Pulmonary artery smooth muscle cell

PAWP: Pulmonary artery wedge pressure

ROCK: Rho-associated protein kinase

ROS: Reactive oxygen species

Sd: Sildenafil

SD: Standard deviation

sGC: Soluble guanylate cyclase

SNP: Sodium Nitro-Prusside

Tp: Treprostenil

TPG: Trans-pulmonary gradient

TRP: Transient receptor potential

TRPC6: Channel 6 of canonical subfamily of TRP

TRPV: Vanilloid subtype of transient receptor potential channels

Acquire knowledge and impart it to the people

Holy Prophet Mohammed (Sallallaho Alaihi Wa'Sallam)

Dedication

I dedicate this to my parents, Tahir Hussain and Shamim Tahir, my wife, Alina Azar and to my lovely daughters, Duaa and Izna.

Acknowledgements

I express my warm thanks to my supervisors Professor Alyn Morice and Professor Mahmoud Loubani for their support and guidance and for giving me the opportunity to carry out this research at Castle Hill Hospital. I am using this opportunity to express my gratitude to everyone who supported me throughout the course of this research project. I am thankful for their aspiring guidance, invaluably constructive criticism and friendly advice during the project work. I am sincerely grateful to them for sharing their truthful and illuminating views on a number of issues related to the project.

I would like to thank my Thesis Advisory Panel: Dr Isabel Pires and Mr Mubarak Chaudhry for their invaluable advice and encouragement. I would also like to thank Dr Rob Bennett for making my life easy in laboratory, Mr Michael Cowen, Mr Suhail Qadri for the provision of specimens and the whole cardiothoracic team and all the people who provided me with the facilities and conditions required for my MD project.

Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the Internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymized.

Ethical Approval:

These experiments conformed to the Helsinki Declaration of the World Medical Association and ethical approval from the Local Ethics Committee (Ref no: 15/NW/0808 – 29/09/15) and Local Research and Development Department (Ref no: R1884 – 09/11/15) approval was obtained.

Funding

The funding for this project came from charitable funds donated to Professor Mahmoud Loubani, at the Department of Cardiothoracic Surgery at Castle Hill Hospital.

Publications

Published articles arising from this thesis:

1. **A. Hussain**, R. Bennett, Y. Haqzad, S. Qadri, M. Chaudhry, M. Cowen, M. Loubani, A. Morice. The differential effects of systemic vasoconstrictors on human pulmonary artery tension. ***European Journal of Cardio-Thoracic Surgery***, Eur J Cardiothorac Surg. 2017 May 1; 51(5): 880-886.

2. **A. Hussain**, M.S. Suleiman, S.J.George, M. Loubani, A. Morice. Hypoxic Pulmonary Vasoconstriction in Humans: Tale or Myth. ***The Open Cardiovascular Medicine Journal***, 2017 Jan 24; 11:1-13.

3. **A. Hussain**, R Bennett, M Chaudhry, S Qadri, M Cowen, A Morice, M Loubani. Characterization of optimal resting tension in human pulmonary arteries. ***World Journal of Cardiology***. 2016 Sep; 8(9): 547-552.

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Published abstracts arising from thesis:

1. **A. Hussain**, R. Bennett, Z. Tahir, M. Chaudhry, A. Morice M. Loubani The in-vitro effect of prostacyclin analogues on small human pulmonary artery: comparison with phosphodiesterase inhibitors. ***Journal of the American College of Cardiology*** 69(11):1906, March 2017.

2. **A. Hussain**, R Bennett, K Kotidis, M Chaudhry, S Qadri, M Cowen, A Morice, M Loubani. The in vitro effect of commonly used vasodilators on human pulmonary artery. ***Thorax*** 2016;71:A221-A222.

3. **A. Hussain**. The in-vitro effect of commonly used pharmacological agents on small human pulmonary arteries. ***J Pulm Respir Med*** 2016, 6:6(Suppl) http://dx.doi.org/10.4172/2161-105X.C1.019.

4**. A. Hussain**, R Bennett, M Chaudhry, A Morice, M Loubani. The impact of vasoconstrictors on human pulmonary artery**.** ***Journal of Clinical & Experimental Cardiology*** . 2016, 7:7 (Suppl).

5. **A. Hussain**, R Bennett, M Chaudhry, A Morice, M Loubani. Characterization of optimal resting tension in human pulmonary artery**.** ***Journal of Clinical & Experimental Cardiology*** 2016, 7:7.

Presentations

Invited speaker:

1. **A. Hussain**. Should Vasopressin be used as a first line systemic vasoconstrictor in pulmonary hypertension patients? ***10th Biennial International Conference of Pakistan Society of Cardiovascular & Thoracic Surgeons,*** **Venue Pearl Continental Hotel Lahore, Pakistan,** 12th – 14th October 2017.

2. **A. Hussain.** Characterization of pharmacological effect of clinically used phosphodiesterase inhibitors in comparison to prostacyclin analogues on small human pulmonary arteries. ***International Congress and Expo on Cardiology, Crown Plaza Miami International Airport, Miami, USA,*** September 11-13, 2017.

3. **A. Hussain**. The in vitro effect of commonly used pharmacological agents on small human pulmonary arteries**. *International Conference on Chest, JW Marriott Hotel Dubai, UAE***, 17th -18th November 2016.

International meetings:

1. **A. Hussain**, R. Bennett, Z. Tahir, M. Chaudhry, A. Morice M. Loubani . The in-vitro effect of prostacyclin analogues on small human pulmonary artery: comparison with phosphodiesterase inhibitors. ***American College of Cardiology's 66th Annual Scientific Session***, Friday, March 17 - Sunday, March 19, 2017, Washington, DC, USA.

2. **A. Hussain**, R Bennett, Y.Haqzad, SSA.Qadri, M Chaudhry, M.Cowen, A Morice, M Loubani. Characterization of Agonist Induced Vasoconstriction in Human Pulmonary Artery. ***30th Annual Meeting of the European Association for Cardio-Thoracic Surgery***, CCIB - Centre de Convencions Internacional de Barcelona, Spain, 1st -5th October 2016.

3. **A. Hussain**, R Bennett, Y.Haqzad, A.Habib, M Chaudhry, M.Cowen, A Morice, M Loubani. Comparison of effectiveness of agents commonly used for treatment of pulmonary artery hypertension. ***30th Annual Meeting of the European Association for Cardio-Thoracic Surgery***, CCIB - Centre de Convencions Internacional de Barcelona, Spain, 1st -5th October 2016.

4. **A. Hussain**, R Bennett, M Chaudhry, A Morice, M Loubani. The impact of vasoconstrictors on human pulmonary artery. ***International Conference on Cardiovascular Medicine***, Manchester Airport Marriott Hotel, Manchester UK, 1st – 2nd August 2016.

5**. A. Hussain**, R Bennett, M Chaudhry, A Morice, M Loubani. Characterization of optimal resting tension in human pulmonary artery. ***International Conference on Cardiovascular Medicine***, Marriott Hotel, Manchester UK, 1st – 2nd August 2016.

National meetings:

1. **A. Hussain**, Effects of endogenous versus exogenous vasodilators on isolated human pulmonary arteries: An In-Vitro comparison. ***National Cardiothoracic Research meeting,*** Clinical Education Centre, Glenfield Hospital, Leicester, 28th October 2017.

2. **A. Hussain**, R Bennett, Z Tahir, A Habib, S Qadri, M A Chaudhry, M Loubani, A Morice. Should Vasopressin be used as a first line systemic vasoconstrictor in patients with pulmonary hypertension – an in-vitro study. ***81th Annual Conference of the Scociety for Cardiothoracic Surgery in Great Britain and Ireland***, Belfast Waterfront, Belfast UK, *12th – 14th March 2017.*

3. **A. Hussain**, R Bennett, Y Haqzad, P Ariyaratnam, M Chaudhry, M CowenM Loubani, A Morice. Characterisation of pharmacological effect of clinically used phosphodiesterase inhibitors in comparison to prostacyclin analogues – an in-vitro study. ***81th Annual Conference of the Scociety for Cardiothoracic Surgery in Great Britain and Ireland***, Belfast Waterfront, Belfast UK, *12th – 14th March 2017.*

4. **A. Hussain**, R Bennett, K Kotidis, M Chaudhry, S Qadri, M Cowen, A Morice, M Loubani. The in vitro effect of commonly used vasodilators on human pulmonary artery. ***BTS – British Thoracic Society Meeting***, QEII Centre Broad Sanctuary, Westminster, London, 7th – 9th December 2016.

5. **A. Hussain**, R Bennett, M Chaudhry, A Morice, M Loubani. BNP ameliorates the vasodilatory effect of ANP in human pulmonary artery. ***Belfast, Leicester and Hull Scientific Research Collaborative meeting***, Belmont, Hotel, Leicester, 13th – 14th October 2016.

Local meetings:

1. **A. Hussain**, R Bennett, M Chaudhry, M Cowen, M Loubani, A Morice. The in-vitro effect of commonly used pharmacological agents on small human pulmonary arteries. ***Allam lecture, Hull York Medical School, University of Hull*** 28th April 2017.

2. **A. Hussain**, R Bennett, M Chaudhry, M Cowen, M Loubani, A Morice. The in vitro effect of commonly used vasoconstrictors on human pulmonary artery. ***Allam lecture, Hull York Medical School, University of Hull*** 15 April 2016.

Prizes

(1) **Best oral presentation award** for presenting a study entitled "The in-vitro effect of commonly used pharmacological agents on small human pulmonary arteries" at the Allam Lecture 2017, Hull York Medical School, University of Hull .

(2) **Best poster award** for presenting the poster entitled "The impact of vasoconstrictors on human pulmonary artery" at the International Conference on Cardiovascular medicine, August 01-02, 2016 Manchester.

(3) **Best Young Researcher award** for oral presentation entitled "Characterization of optimal resting tension in human pulmonary artery" at the International Conference on Cardiovascular medicine, August 01- 02, 2016 Manchester.

Chapter 1

Introduction

1.1 Overview Of Pulmonary Circulation:

Pulmonary circulation is the segment of cardiovascular system, which carries the blood to and from the lungs. Its primary function is to oxygenate the deoxygenated blood that has returned to the right side of the heart. The oxygenated blood is then delivered to the left side of heart and thus the systemic circulation (1, 2) (Fig.1).

1.2 Anatomy Of Pulmonary Circulation:

The vascular wall of the pulmonary artery is made up of three layers; tunica intima (internal layer), tunica media (middle layer) and tunica externa (outer layer) (3, 4). Endothelial cells are located in the intima and play an important role in regulating vascular function by responding to neurotransmitters, hormones and vasoactive factors (5). The endothelium and smooth muscle are the vital components for maintenance of arterial tone and regulation of blood pressure. The main purpose of the arteries is to deliver the blood to the organs with high pulse pressure. Arteries can broadly be divided into conducting arteries, conduit arteries (macro-vasculature) and resistance arteries (microvasculature) based on their size, anatomical position and functionality. Conducting arteries are the largest in size and rich in elastic tissues which support the vessels to expand and recoil to accommodate high changes in blood pressure and volume.

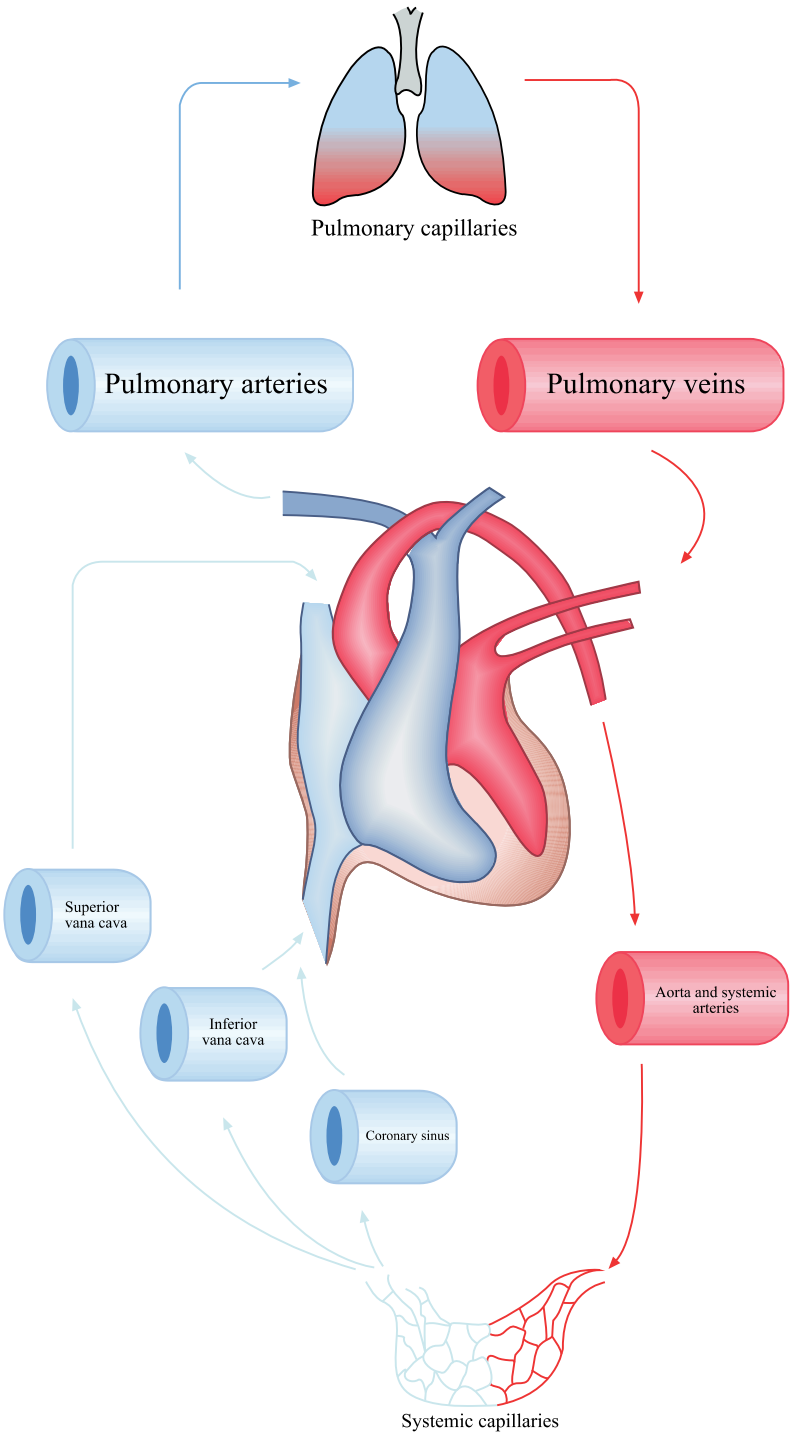

Figure 1: Schematic representation of pulmonary circulation. Red shows oxygen-rich CO2 -poor blood. Blue shows oxygen-poor CO2 -rich blood.

The aorta, pulmonary artery and carotid arteries are the main examples of conducting arteries (6). Pulmonary arteries in particular are less muscular, more distensible and compressible compared to their systemic counterpart and resistance pulmonary arterioles contain the most smooth muscle in the pulmonary vasculature (7, 8). Bronchial circulation is a part of the systemic circulation that provides nourishment to the lungs (9). Pulmonary arteries unlike systemic arteries are always hypoxic as they conduct relatively deoxygenated blood and the gas exchange occurs at capillary level where lungs excrete carbon dioxide (10, 11).

1.3 Regulation Of Pulmonary Circulation:

The right and left ventricles are connected in series as is the case for the pulmonary and systemic circulation (12). The pulmonary circulatory flow is dependent on systemic blood coming to it through right heart as well as the afterload that is determined by the aortic pressure and systemic vascular resistance (13, 14). In addition to this the pulmonary circulation is also influenced by alveolar compression, gravity, body position and lung volume (15).

The regional ventilation and distribution of blood flow in lung varies from the apex to the base in the upright position [16]. At the apex of the lung the alveolar pressure is higher than the pulmonary artery pressure that results in wasted ventilation. On contrary at the base, the pulmonary pressure is higher than alveolar pressure that causes wasted perfusion (16, 17). This difference in matching of blood flow to ventilation determines the regional blood flow and disrupts the gas exchange (18, 19).

At tissue level the release or inhibition of certain factors control regional pulmonary blood flow (20). Agonists of smooth muscle contraction such as endothelin increase the pulmonary artery tone (21, 22). Nitric oxide and acetylcholine are smooth muscle relaxants that are known factors to decrease the pulmonary artery tone (23-25). The pulmonary circulation is a low pressure, low resistance and high flow circuit and depending on age the average resistance is between 1 – 2.5 mmHg.min.L−1 (26). Unlike systemic arterioles that dilate in response to hypoxia, the pulmonary arterioles and venules constrict when exposed to hypoxia (27). This phenomenon is known as hypoxic pulmonary vasoconstriction (HPV) that causes diversion of pulmonary blood from poorly ventilated to well-oxygenated areas of lungs to preserve systemic oxygenation (28). This vasoconstrictive response of human pulmonary artery smooth muscle cells (HPASMc) to hypoxia is further augmented when pulmonary vasculature exposed to hypercapnia and the combined hypoxic and hypercapnic effects are additive not synergistic (29). However if this short term beneficial role of HPV to facilitate perfusion and ventilation persist or alveolar hypoxia become more widespread or if the hypoxic stimulus is not removed as in intrinsic lung disease, this results in increase in pulmonary resistance and subsequent pulmonary hypertension (30, 31).

1.4 Pulmonary Hypertension

1.4.1 Definition:

Pulmonary hypertension is defined as mean pulmonary artery pressure (mPAP) >25 mmHg measured by right heart catheterization (32).

1.4.2 Classification:

The most up to date classification of PH was established during the WHO pulmonary hypertension conference in Nice, France, in 2013 and PH has been classified into the following five subgroups on the basis of similar aetiology or characteristics (33); Group 1: pulmonary artery hypertension, Group 2: pulmonary hypertension due to left heart disease, Group 3: pulmonary hypertension due to chronic lung disease and/or hypoxia, Group 4: chronic thromboembolic pulmonary hypertension and Group 5: pulmonary hypertension due to unclear multifactorial mechanisms.

1.4.3 Pulmonary hypertension and cardiac surgery patients:

PH in cardiac surgery patients is more frequently classified as pre-capillary or post capillary according to the anatomical location of underlying mechanism (34). Post-capillary PH relates to the clinical group 2 (pulmonary hypertension due to left heart diseases) while pre-capillary PH incorporates the clinical groups 1 (pulmonary arterial hypertension), 3 (pulmonary hypertension due to lung diseases and/or hypoxia), 4 (chronic thrombo-embolic pulmonary hypertension) and 5 (pulmonary hypertension with unclear and / or multifactorial mechanisms) (35).

Pre-capillary PH also called as pulmonary artery hypertension (PAH) has been defined as mean pulmonary artery pressure (mPAP) > 25mm Hg, pulmonary artery wedge pressure (PAWP) < 15 mmHg and trans-pulmonary gradient (mPAP-PAWP) >10 mm Hg (36). Post capillary hypertension is also called pulmonary venous hypertension (PVH) has been characterized by mPAP > 25mm Hg, pulmonary artery wedge pressure (PAWP) > 15 mmHg and trans-pulmonary gradient (TPG) <

10 mm Hg (37). PH in cardiac surgery patients is typically group 2 or post-capillary type as it usually associated with left heart disease and the cause is localised after the pulmonary capillary (13).

1.4.4 Factors that can contribute to post cardiac surgery pulmonary hypertension:

Pulmonary hypertension (PH) is an important prognostic factor in cardiac surgery and is associated with increased morbidity and mortality (38). Pulmonary hypertensive patients have more associated comorbidities, which not only increase the EuroSCORE (39) but also result in increase of strokes, bleeding and acute kidney injury (40). PH is an independent risk factor for peri-operative morbidity, which ranges between 14 - 42% and includes arrhythmia, heart and respiratory failure, septicaemia and myocardial infarction (41, 42). Patients are usually diagnosed with PH prior to surgery but cardiac surgery itself can precipitate the condition. The mechanism of the underlying pathophysiology is complex and PH may exist prior to surgery or might develop during or after surgery (35).

Following cardiac surgery, the myocardium of heart especially of left ventricle is at risk either due to ischemia during the operation or due to inadequate myocardial protection (43). The underperforming left ventricle results in back filling of lungs that can leads to pulmonary odema and subsequently pulmonary hypertension (44). During cardiopulmonary bypass (CPB) the heart and lungs are turned off so surgery can be performed on a motionless heart while maintain the circulatory support (45). This period of lung hypoxia causes global pulmonary vasoconstriction and can lead to pulmonary hypertension also called hypoxic pulmonary vasoconstriction (HPV)

(46).

Vasopressor drugs are commonly used perioperatively to treat low systemic arterial blood pressure and maintain organ perfusion while inotropes are used to manage patients with low cardiac output syndrome The choice of vasopressor and inotropes in patients having cardiac surgery should take into account their effects on pulmonary vascular resistance because these drugs could trigger pulmonary vasoconstriction, which, if persistent, could progress to pulmonary hypertension (47).

1.5 Hypoxic Pulmonary Vasoconstriction:

Hypoxic pulmonary vasoconstriction (HPV) describes the physiological adaptive process of lungs to preserves systemic oxygenation. It has clinical implications in the development of pulmonary hypertension which impacts on outcomes of patients undergoing cardiothoracic surgery.

1.5.1 Acute hypoxic pulmonary vasoconstriction:

HPV in humans appears to be have several components (48), the first acute phase occur within 5 minute with a mean time constant of 151-160 +/- 24.8 - 42 seconds followed by a plateau phase of at least 20 minutes (49, 50). A second phase also known as the sustained phase start after a latency period of 30 min and plateau at 2 hours (50) followed by a third chronic phase taking upward of 8 hours (48, 51). During the sustained hypoxic phase an initial temporary vasodilation response is seen followed by secondary vasoconstriction period (52).

The precise underlying mechanism of HPV is still uncertain but cells in the endothelium and/or smooth muscle cells are involved as HPV can be seen in isolated pulmonary artery (53-55). Fishman describe the role of the nervous system and humoral agents as modulatory rather than primary causes of HPV (56). Both adrenergic and cholinergic nerve fibres are found in human lung tissue. α adrenergic receptors are predominant both functionally and numerically in lungs as compared to β adrenergic receptors (57). The adrenergic system only contributes to maintain the initial resting tone needed for HPV while the cholinergic system was found to play no role in the control of pulmonary circulation (58, 59). This concept of a little role of the nervous system modulatory role is further strengthened by the fact that HPV still persists in transplanted and denervated lungs (60).

1.5.1.1 Role of Calcium in hypoxic pulmonary vasoconstriction:

Pulmonary artery smooth muscle cells (PASMCs) are found in large arteries as well as in small arterioles (8) and are believed to cause vasoconstriction in response to hypoxia by increasing the intracellular calcium (Ca^{2+}). Intracellular concentration of Ca^{2+} is mainly regulated by release of sarcoplasmic reticulum stored Ca^{2+}, extracellular Ca^{2+} influx through voltage gated Ca^{2+} channels and receptors or store operated Ca^{2+} channels (61). PASMCs membrane potential is regulated by voltage gated K^{+} channels, which control the cytoplasmic Ca^{2+} concentration. Voltage gated K^{+} channels cause vasoconstriction when exposed to acute hypoxia by increasing cytoplasmic Ca^{2+} concentration and may have a role in some of the components of HPV response (62, 63). Hypoxia depolarizes the membrane by reducing the outward K^{+} current and leads to vasoconstriction by increasing the influx of Ca^{2+} through voltage gated Ca^{2+} channels.

Michelakis et al demonstrated that pulmonary artery smooth muscle cells have the ability to sense oxygen and react to hypoxia without any influence from surrounding parenchyma (64). They also conclude that dissimilarities in systemic and pulmonary circulation response to hypoxia is due to the manifestation of different K^+ channels that trigger hyperpolarization and vasodilation in systemic circulation and depolarization and vasoconstriction in pulmonary circulation.

Tang et al. showed that the increase of calcium in PASMCs after acute hypoxia is due to voltage gated calcium channels to some extent but largely due to transient receptor potential (TRP) channels (65). TRP channels are cations channels that are present in cellular membranes and involved in various cellular activities e.g. pain, touch, temperature and osmolarity (66, 67). Channel 6 of Canonical subfamily of TRP (TRPC6) are extensively expressed in HPASMc and on activation by its mediators such as epoxyeicosatrienoic acids (EETs) induced hypoxic pulmonary vasoconstriction by increasing intracellular Ca^{2+} (65).

Vanilloid subtype of transient receptor potential channels (TRPV) also act as sensory channels for heat, pain touch and osmotic stress (68). TRPV channels are present in endothelium, perivascular nerves and vascular smooth muscle cells and its sub-class TRPV4 is expressed highly in pulmonary endothelial cells and human PASMCs (69). Acute hypoxia increase the EETs levels that activates the TRPV4, as depletion of EETs attenuated the TRPV6 induced HPV (70). Goldenberg et al. demonstrated that acute hypoxia induced TRPV4 to trigger HPV by increasing Ca^{2+} influx and phosphorylation of myosin light chain in human PASMCs (71). EETs activated TRCP6 and TRPV4 work in parallel to each other and form heteromers when exposed to hypoxia, which increase their surface expression and calcium

conductance capacity (72, 73).

Meng et al. demonstrated that arachidonic acid (AA) – a membrane phospholipid, attenuates the hypoxia induced rise in intracellular Ca^{2+} and related vasoconstriction in human PASMCs (74). Inhibition of endogenous AA production by diacyglycerol, fatty acid hydrolysis and phospholipase A augments pulmonary vasoconstriction and increase intracellular Ca^{2+} level through TRP channels, voltage gated Ca^{2+} channel and Na^{+} - Ca^{2+} exchanger.

Beside Ca^{2+} other important mediators such as reactive oxygen species generated during hypoxia. Under normoxic conditions ROS are predominantly produced in mitochondria of pulmonary cells, suggesting that mitochondria might play a role in HPV.

1.5.1.2 Role of mitochondria / reactive oxygen species (ROS) in hypoxic pulmonary vasoconstriction:

Mitochondria are essential organelle, which contains iron-sulphur complexes, and proteins within the inner mitochondrial membrane that generate energy through electron shuttle called electron transport chain (75). During oxygen metabolism stable substances like hydrogen peroxide and unstable toxic substances such as hydroxyl and superoxide radicals are produced. These substances are collectively called reactive oxygen species and are produced from I and III complexes of electron transport chain and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (76). Mitochondrial electron transport chain is believed to act as an oxygen sensor and facilitates hypoxic induced vascular smooth muscle cells contraction by controlling different kinases and ion channels through ROS (77). ROS act as an

intercellular and intracellular secondary messenger system and reacts with protein residue like cysteine to regulate different signalling pathway (78).

Mehta et al. established that hypoxia attenuates the production of ROS in human PASMCs (75). These reduce ROS augments pulmonary vasoconstriction and subsequent pulmonary hypertension through rise in intracellular Ca^{2+}. In addition to PASMCs, coronary artery smooth muscle cells (CASMCs) also show reduction in ROS production during hypoxia. However these cells dilate instead of constricting and the reason for this differential contractile response is not clear. Wang et al. suggested that this differential response to hypoxia in pulmonary and systemic vasculature might be attributed to the way that ROS regulates their ion channels (79).

Waypa et al. also showed that hypoxia reduce the mitochondrial production of ROS that result in hypoxic pulmonary vasoconstriction due to inhibition of voltage gated plasma membrane K^+ (K_V) channels (80). Closure of K_V channels cause membrane depolarization and subsequent vasoconstriction though increases influx of Ca^{2+}.

Freund-Michel et al. described the role of hypoxia induced mitochondrial alterations and mitochondrial dysfunction that shift the oxidative phosphorylation energy production to glycolysis (81). The resultant hyperpolarized mitochondria reduced the production of ROS and the metabolic shift to increased glycolysis increased the concentration of non-oxidized sugars, lipids and amino acids that are pre requisite to smooth muscle cells proliferation. Similarly Evans et al. proved that inhibition of oxidative phosphorylation in mitochondria through LKB1 (liver kinase B1) – AMPK (amp activated protein kinase) signalling pathway triggers hypoxic pulmonary vasoconstriction (82). This demonstration of hypoxia induced

mitochondrial dysfunction in pulmonary artery endothelium, smooth muscle and adventitia of pulmonary hypertension patients suggests that production of targeted mitochondrial therapies will provide effective therapy for this life threatening disease (83, 84).

In contradiction to the above some studies have shown that hypoxia enhanced the production of ROS instead of reducing it. Perez-Vizcaino et al. demonstrated that pulmonary vascular smooth muscle cells augment the production of ROS particularly hydrogen peroxide when exposed to hypoxia (85). Hypoxia induced ROS increase the intracellular Ca^{2+} concentration and PASMCs contraction through modulation of protein kinase C, Rho kinases, ryanodine receptors and voltage gated potassium K^{+} channels. In addition to vascular mitochondria and NADPH oxidases, ROS are also produced from endothelial nitric oxide synthase (eNOS), arachidonic acid metabolism and xanthine oxidase.

This controversy regarding ROS during hypoxia might be related to the duration of hypoxia. ROS production by human PASMCs attenuates initially when exposed to brief period of hypoxia (75) but increases subsequently (86).

Figure 2 provides an overview of mechanisms involved in acute hypoxic pulmonary vasoconstriction.

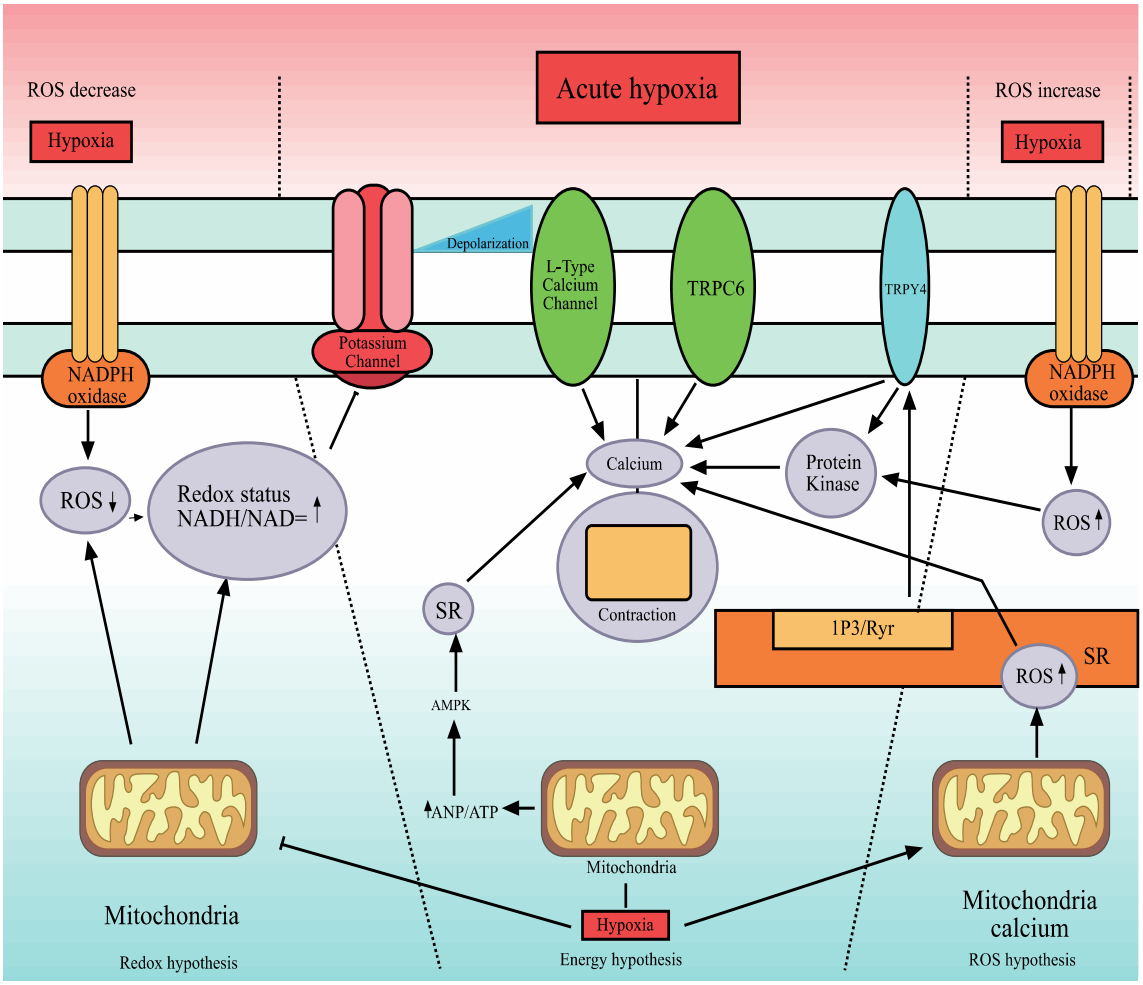

Figure 2: Mechanism of acute hypoxic vasoconstriction in human pulmonary artery. ROS= reactive oxygen species, SR= sarcoplasmic reticulum, TRPC=transient receptor potential channels. Redox hypothesis: hypoxia decreases the ROS level and cause vasoconstriction through inhibition of K+ channels. Energy hypothesis: hypoxia shifts the energy production cycle and produces more AMP that increases the intracellular Ca2+ through SR. ROS hypothesis: hypoxia augments the production of ROS that cause HPV through SR induced release of Ca2+.

1.5.2 Chronic hypoxic pulmonary vasoconstriction:

Chronic hypoxia is coupled with refractory vasoconstriction and attenuated NO mediated vasodilation that expedites human PASMc medial hypertrophy and subsequent pulmonary hypertension (87). Production of reactive oxygen species such as hydrogen peroxide and superoxide and hydroxyl radicals in vascular smooth muscle cells is involved in physiological regulation of vascular tone and vascular remodelling (88).

1.5.2.1 Role of ROS and different ions in chronic hypoxic pulmonary vasoconstriction:

Wu et al. demonstrated that chronic hypoxia (PO_2 = 30 mmHg for 48 h) cause increased ROS level in PASMCs (86). This increased ROS production induce a pathophysiological response that cause pulmonary vascular remodelling and subsequent chronic hypoxia associated pulmonary hypertension (89). Similarly Porter et al. in his experiments showed that hypoxia for more than 72 hours significantly induce the hydrogen peroxide (H_2O_2) production and proliferation of human pulmonary artery endothelial cells (HPAEC) (90). The hypoxic induced generation of H_2O_2 activates the arachidonate 5-lipoxygenase (ALOX5) pathway that induced HPAEC proliferation and vascular remodelling. Pharmacological blockade of ALOX 5 by zileuton or by MK-886 (inhibitors of 5 lipooxygenase activating protein) attenuates hypoxia-induced proliferation of HPAEC.

Platoshyn et al. showed that chronic hypoxia down regulates the mRNA and protein expression of plasma membrane voltage gated potassium (K_V) channels that are responsible for regulation of membrane potential and intracellular Ca^{2+}

concentration. The resultant membrane depolarization from inhibition of K_V channels results in amplified Ca^{2+} influx through voltage gated Ca^{2+} channels in PASMCs and mediates pulmonary vasoconstriction and vascular smooth muscle cells proliferation (91). Zhao et al. demonstrated that the chronic hypoxia leads to the enhanced expression of Zinc transporter ZIP12 in human endothelial and smooth muscle cells (92). This intracellular rise of Zinc plays a role in hypoxia induced pulmonary smooth cells proliferation as inhibition of ZIP12 attenuates HPASMc proliferation and development of pulmonary hypertension. Pharmacological development of ZIP12 inhibitor can be a novel treatment module for pulmonary hypertension management.

1.5.2.2 Role of Rho A and Rho B in chronic hypoxic pulmonary vasoconstriction:

Rho GTPases is a family of signalling G Proteins, and are regulators of cytoskeletal dynamics, cell migration, cell polarity, neuronal development, vesicle transport and cytokinesis (93). Rho-A was the first identified member of Rho GTPases family in 1985 (94).

Rho-A with its downstream factor, Rho Kinase (ROCK) mediated multiple cellular functions such as proliferation, contraction, adhesion, migration and gene expression (95). Rho-A / ROCK signalling pathway is involved in mediating pulmonary artery hypertension, vasoconstriction and vascular remodelling. Inhibition of Rho-A / Rho kinase pathway by Sildenafil (cGMP specific phospho-diesterase inhibitor) proves beneficial in pulmonary hypertension patients due to its vasodilatory effects (96).

Rho-B is a protein homologous to Rho-A and in response to hypoxia induced pulmonary vasoconstriction and vascular remodelling by inducing actin-myosin contractility, enhanced endothelial permeability and promotion of cell growth (97). Rho-B levels found to be significantly increased in human PASMCs when exposed to hypoxia. PDGF / PDGFR signalling pathway is involved in Rho-B mediated PASMCs proliferation as inhibition of PDGF receptor tyrosine kinase by imatinib attenuates the effects of Rho-B. This shows that Rho-B can be a potential therapeutic target to prevent pulmonary hypertension in humans.

Figure 3 summarizes the role of ROS and Rho GTPases family in human chronic hypoxic pulmonary vasoconstriction.

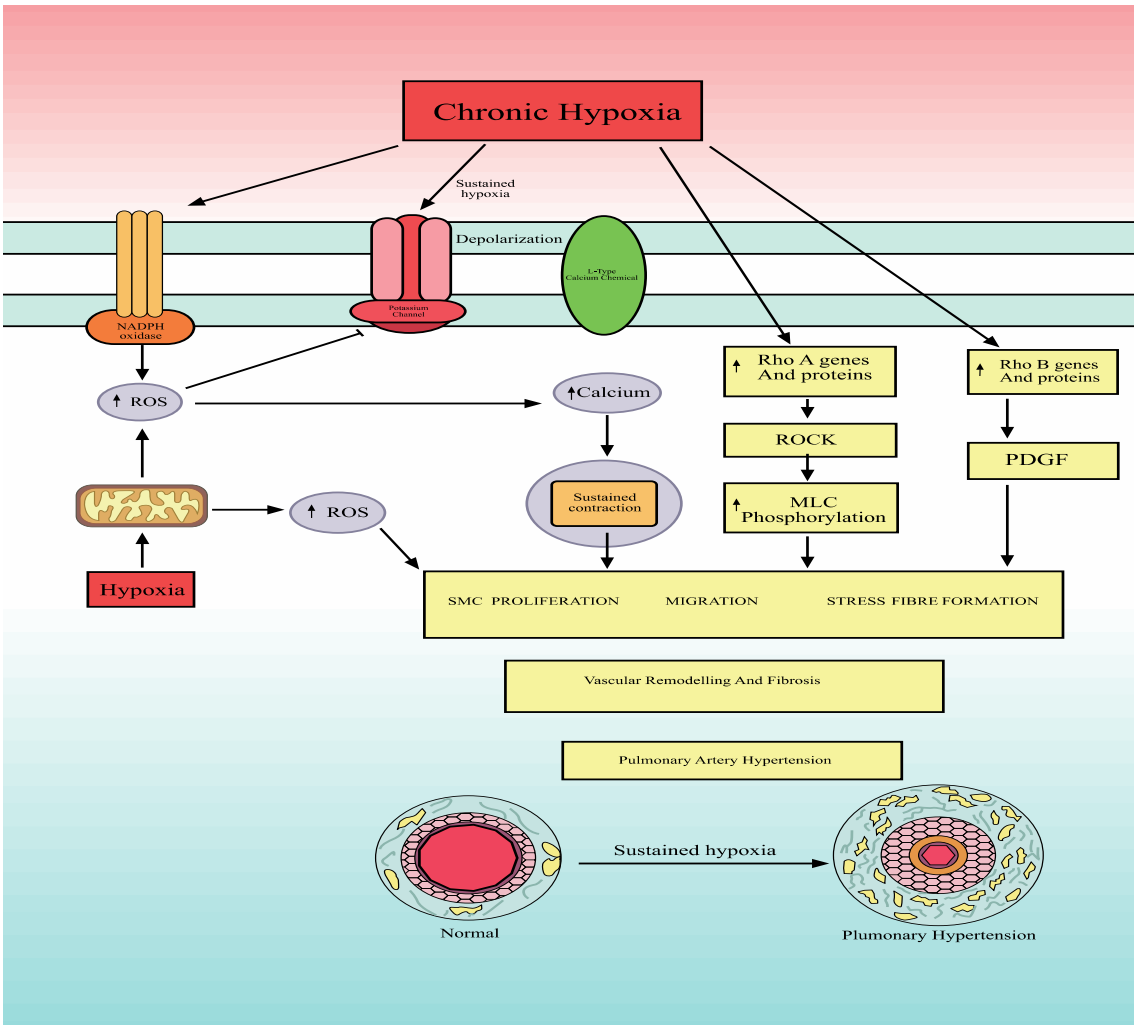

Figure 3: Mechanism of chronic hypoxic vasoconstriction in human pulmonary artery. ROS = reactive oxygen species, Rho A and Rho B are members of Rho GTPases family which is a family of G proteins. Chronic hypoxia induced Rho A and Rho b and ROS production that results in smooth muscle cell proliferation, vascular remodeling and ultimately pulmonary artery hypertension.

1.5.3 Role of pulmonary artery endothelium cells in hypoxic pulmonary vasoconstriction:

Vascular endothelium is a layer of specialized cells between vessel lumen and vascular smooth muscle cells and produces several active compounds including vasodilators and vasoconstrictors (98). Endothelium derived relaxing factor (EDRF-NO), endothelin, lipooxygenase and cyclooxygenase are endothelial vasoactive agents that regulates vascular tone (99).

1.5.3.1 Is Endothelium necessary for HPV?

Hypoxia induced constriction of human pulmonary artery rings dependence on the endothelium was established by Demiryurek et al. as denuding the endothelium markedly abolish the hypoxic vasoconstriction (54). In contrary to this Ohe et al. demonstrated that endothelium is not needed for hypoxia-induced vasoconstriction. They established that human pulmonary artery strips (2mm in diameter) constrict in response to hypoxia and removal of endothelium instead of abolishing actually enhanced the hypoxia-induced vasoconstriction response (55). The reason for this controversial response claimed in these studies is not very clear.

1.5.3.2 Role of endothelial nitric oxide synthase (eNOS) in HPV:

Endothelial nitric oxide synthase (eNOS) is an enzyme that synthesizes nitric oxide (NO) in vascular endothelium (100). Nitric oxide regulates cellular proliferation, vascular tone, platelet aggregation and leukocyte adhesion (101).

Takemoto et al. demonstrated that prolonged hypoxia induced endothelial injury and diminished the production of eNOS via Rho kinase (ROCK) induced cytoskeletal

changes that results in hypoxia induced pulmonary hypertension (102). Hypoxia-induced ROCK expression results in decrease in eNOS mRNA and protein production in pulmonary artery endothelial cells. However Krotova et al. have shown that chronic hypoxia induced NO production in human PAECs and this hypoxia-induced production of NO is further enhanced by inhibition of Arginase II (103). Similarly Beleslin- Cokic et al. showed that hypoxia stimulates NO production, which is independent of hypoxia-induced reduction of eNOS gene expression (104). This increase in NO production in response to hypoxia, appears to be the compensatory mechanism of the body by inducing the production of other nitric oxide synthase (NOS) enzymes e.g. iNOS.

Chovanec also demonstrated that the endothelium augment the production of NO and superoxide when exposed to chronic hypoxia (105). The superoxide with NO facilitates the remodelling of pulmonary vasculature that is characterized by tunica media thickening, augmented muscularity and PASMCs proliferation and migration. Superoxide-NO combination marked the onset of collagen cleavage via peroxynitrite release and the resulted collagen fragments induce pulmonary vessels remodelling.

Ghrelin a peptide hormone produced by ghrelinergic cells in gastrointestinal tract is known to have beneficial effects on human pulmonary artery endothelial cell (HPAECs) function. Yang et al. demonstrated that hypoxia for 24 hours risks the viability of endothelial cells and ghrelin can inhibit hypoxia mediated HPAECs dysfunction by increasing NO production and eNOS phosphorylation (106).

Krotova et al. and Yu et al. demonstrated that human PAECs unlike PASMCs do

not proliferate when exposed to chronic hypoxia (103, 107). This reaction can be due to the fragility of endothelial cells under hypoxic conditions as studies show that hypoxia also increases the permeability of HPAECs (97).

The endothelium seems to play a modularity role in HPV and augments the hypoxic induced HPASMc response by modulating their release of vasoconstrictor and vasodilator factors while PASMc remain the main contractile mechanics as explained by Sylvester (108).

1.5.4 Effect of vessel size on pulmonary vasculature reactivity to hypoxia:

The effect of hypoxia on human pulmonary artery strips (HPASs) prepared from human pulmonary artery of diameter < 5 mm was examined by Hoshino et al. and established that the vessels constrict in response to hypoxia (53). This hypoxia induced contractile response is enhanced when HPASs were pre stimulated with histamine. The response was attenuated by depletion of intracellular and extracellular Ca^{2+} and by HA 1004 – a novel calcium antagonist.

Human pulmonary artery rings - HPARs (2mm in diameter) were demonstrated by Ohe et al. to constrict in response to hypoxia and removal of endothelium enhanced the hypoxia-induced vasoconstriction. Voltage gated Ca^{2+} channels play a role in hypoxic vasoconstriction and pre stimulation is not a prerequisite (55).

Demiryurek et al. in their study on HPARs showed that hypoxia induced vasoconstriction in both smaller (0.38 - 0.68 mm) and larger (2.2 – 4.5 mm) diameter vessels under optimal resting conditions. This hypoxia-induced response is enhanced in both sizes HPARSs when rings were pre constricted and removal of

endothelium diminished the hypoxic vasocontrictive response (54).

In contrary Ariyaratnam et al. demonstrated that larger HPARs (4 mm diameter) dilate in response to hypoxia and constrict when exposed to hyperoxia (95% O2). This hypoxic vasodilation is independent to nitric oxide, because application of N-Nitro-L-arginine methyl ester hydrochloride (L-NAME) had no effect. This study showed hyperoxic vasoconstriction was reliant on voltage gated Ca^{2+} channels as evidenced by inhibition of vasoconstriction when HPARs treated with nifedipine (Ca^{2+} channel blocker) (109).

The reason in variation in response of different size pulmonary vasculature to hypoxia is not clear and whether certain degree of pre stimulation is necessary. More studies need to be performed to confirm whether internal diameter of vessels and pre stimulation alters the reactivity of vessels to hypoxia.

In addition to the hypoxia, the vasopressor agents that used in clinical setting can initiate or exacerbate the pre-existing pulmonary hypertension.

1.6 Vasoconstrictor Responses To Vasopressor Agents In Human Pulmonary Artery:

Vasopressor drugs are commonly used in clinical settings to maintain organ perfusion and to increase the systemic arterial pressure and inotropes to treat low cardiac output (110). During and after weaning from cardiopulmonary bypass (CPB) pharmacological support is often required to optimise the cardiac output (111). Administration of these drugs in pulmonary hypertension patients can further

aggravate their symptoms due to incremental pulmonary vasoconstriction and right ventricular failure (112). Adrenaline, Noradrenaline, Arginine Vasopressin and Potassium Chloride are commonly used to treat the post cardiac surgery patients. Adrenaline and Noradrenaline are sympathomimetic catecholamine and exert their vasopressor effect via activation of vascular smooth muscle cells (113). Post-surgical patients need an ideal vasoconstrictor that only selectively constricts systemic vasculature without affecting the pulmonary vasculature. Little data exist on the direct impact of these drugs on human pulmonary vasculature and this study aimed to fill this gap of knowledge.

1.7 Pharmacologic Management Of Pulmonary Arterial Hypertension:

1.7.1 Introduction:

In the past few decades the treatment of pulmonary hypertension has been dramatically changed which enable clinicians to shift from clinical based treatment approach to evidence based therapy strategy (114). Oral, inhaled, subcutaneous, and intravenous are common routes of administration of PA-specific drugs (115). Patient's symptoms improvement and improve quality of life and survival are the main goals for PH treatment (116). Several other assessment tools are used to measure the treatment response, this includes 6 minutes' walk distance test, cardiopulmonary exercise tolerance test, improving in WHO / NYHA (New York Heart Association) functional classes and improved haemodynamic (117, 118). These measurement tools have significant variability, and still there is no general consensus on which parameter to be used as a primary end point in PH treatment (117, 119, 120).

1.7.2 Current Therapies:

Currently approved therapies for PH include endothelin receptor antagonists (ERAs), phosphodiesterase-5 inhibitors (PDE5i), prostacyclin pathway agents and soluble guanylate cyclase (GC) stimulators. These therapies are approved by FDA (Food and Drug Administration) and are focused at the documented abnormalities within the pulmonary vascular pathways (121). Most of these drugs have been approved only after short trial of 12 – 16 week duration. The mainstay of these trials was improvement in exercise capacity but there was no comparison of different treatment groups (122). In recent years some long term trial were conducted for specific drugs but these studies have some inherent problems (123). There are some trials that explore the efficacy of combination therapy in the treatment of PH, with the potential of paradigm shift in clinical practice (124). To date, Epoprostenol is the only available treatment that demonstrated a 70% mortality risk reduction in PH patients (125). Despite recent advances in PH treatment disease remains deadly that necessitates further research. We need to further strengthen our understanding of right heart failure pathophysiology in PH that has great potential for developing more 'personalized' therapeutic options.

This manuscript will review the current available pharmacological therapies for the management of PH.

1.7.3 Prostacyclin pathway:

Prostacyclin (PGI_2) is a naturally occurring prostaglandin that produced from arachidonic acid by prostacyclin synthase enzyme. It mediates its potent vasodilator, anti-proliferative, anti-thrombotic and antiplatelet effects via intracellular cAMP

(126). Some studies established that lack of prostacyclin contribute to the pathogenesis of PH (127, 128). Epoprostenol, Treprostinil and Iloprost are FDA approved prostacyclin analogues for PH treatment.

Intravenous Epoprostenol (Ep) was the first targeted therapy that has been approved for treatment of PH. Several randomised controlled clinical trials demonstrated that Ep improves exercise capacity, symptoms, haemodynamic and survival in pulmonary artery hypertension patients (129-131).

A large randomised clinical trial (RCT) proved that the continuous subcutaneous administration of Treprostinil significantly improved exercise capacity and haemodynamics (132). An international, multicentre, randomized, double-blind study (FREEDOM-M) proves that the use of oral Treprostinil in isolation for the treatment of pulmonary arterial significantly improved 6-minute walking distance (6MWD) and Borg Dyspnoea Score rating (shortness of breath test) (133). On the other hand oral Treprostinil failed to meet its target (6MWD) when used in combination with PDE-5 inhibitor or ERAs, as evidenced by the two combinations FREEDOM-C and FREEDOM-C2 RCTs () (134, 135).

A multicentre study (AIR) was the first to demonstrate the role of inhaled Iloprost in the treatment of PH patients. Significantly more patients, who received Iloprost versus placebo, achieved the primary end points (after 12 weeks, at least one class improvement in NYHA class, more than 10% improvement in 6MWD and absence of clinical deterioration and death) (136). In another multicentre RCT (STEP trial), addition of inhaled Iloprost to the stable oral monotherapy Bosentan improved 6MWD, WHO functional class, time to clinical worsening, and reduction of pulmonary vascular resistance (137).

Two RCTs were conducted in Europe and US to evaluate the effectiveness of orally active prostacyclin analogue Beraprost in the management of PH patients. Galie et al, randomised 130 patients with PH in a randomised double-blinded study either to the maximal tolerated dose of Beraprost or to placebo for 12 weeks (138). The study demonstrated that the patients treated with Beraprost had improved exercise capacity and symptoms compared with placebo. Similarly to assess the efficacy and tolerability of Beraprost, Barst et al, randomised the patient to receive maximum dose of Beraprost or placebo for 12 months (139). The study shows that the beneficial effects were observed in the early phases of treatment and with time this effect tailed off.

Selexipag is the nonprostanoid diphenylpyrazine derivative, available in oral form acts on prostaglandin I_2 (IP) receptor (140, 141). In comparison to prostacyclin analogues selexipag has higher specificity for IP receptor which resulted in fewer side effects related to activation of other prostanoid receptors that mediate unwanted side effects (142). A placebo controlled RCT demonstrated a significant reduction in pulmonary vascular resistance in patients treated with Selexipag for 17 weeks (143).

1.7.4 Endothelin pathway:

Endothelin -1 (ET-1) is one of the most potent endogenous vasoconstrictor that released from endothelium and exerts its effect via ETA and ETB receptors. ETA receptor is predominantly found on PASMC whereas ETB is found on both endothelium and PASMC and both receptors can mediate smooth muscle constriction and proliferation (144). ETB is also involved in local clearance of ET-1 and release of endothelial NO and PGI2 that can augment the vasodilation (145).

Currently available therapeutic agents exert their beneficial effect through blockage of one or both of Endothelin-1 receptors (146, 147).

Bosentan was the first oral non-selective ET-1 receptor antagonist that was approved for the treatment of PH. It attenuates the development and progression of pulmonary artery hypertension, right ventricular hypertrophy and pulmonary vascular remodelling (148). To confirm the clinical efficacy of Bosentan, a double-blinded RCT (Bosentan Randomized Trial of Endothelin Antagonist Therapy - BREATHE-1) was conducted that enrolled 213 patients (149). The study demonstrated that Bosentan improved the 6MWD, WHO functional class and Borg dyspnoea index. Similar results were found in placebo-controlled trial (BREATHE-5) in patients with congenital heart disease (150-152).

Macitentan is a dual ET-1 receptor antagonist and its clinical efficacy was evaluated with a placebo-controlled RCT - Endothelin Receptor Antagonist in Pulmonary Arterial Hypertension to Improve Clinical Outcome (SERAPHIN) (153). In this study 742 PH patients were randomly assigned to Macitentan at 3 mg once daily dose (250), 10 mg Macitentan once daily dose (242) and to placebo (250) for a period of 100 weeks. Macitentan evidenced significant reduction in both morbidity and mortality but associated with more side effects (nasopharyngitis, anaemia and headache) in comparison to placebo (154, 155).

Ambrisentan is a selective ETA receptor antagonist that was evaluated in the multicentre, placebo-controlled RCTs, Ambrisentan in Pulmonary Arterial Hypertension Efficacy Study 1 and 2 (ARIES-1 and ARIES-2) (156). The study confirmed improvement in NYHA functional class and 6MWD in patients who were

treated with Ambrisentan. The associated risk of liver injury and drug – drug interaction is lower in comparison with Bosentan (157).

1.7.5 Nitric oxide - cGMP pathway:

Nitric Oxide (NO) is biosynthesised from L-arginine, oxygen and NADPH via the enzymatic action of nitric oxide synthase (NOS) (158). Nitric oxide either produced endogenously in pulmonary vascular endothelial cells or exogenously administered, stimulate synthesis of guanosine-3′, 5′-cyclic monophosphate (cGMP) by activating the soluble guanylate cyclase (GC) (159). cGMP activates different protein kinases and calcium gated K^+ channels and relax the vascular smooth muscle cells (160). Several different isoform of phosphodiesterase (PDE) enzymes especially PDE 1, 2, 3, 5, and 9 are present in lungs that can inactivate cGMP (161). Phosphodiesterase inhibitors have been proven to reduce pulmonary vascular resistance and now an approved treatment for pulmonary hypertension (162). Phosphodiesterase-5 (PDE-5) inhibitor (sildenafil), sGC stimulator (Riociguat) and direct administration of NO can manipulate this Nitric Oxide – cGMP pathway.

Sildenafil, the regulator of cGMP degradation and pulmonary vascular smooth muscle tone, was one of the first PDE -5 inhibitor that was approved for the treatment of PAH (163). A placebo controlled RCT (Sildenafil Use in Pulmonary Arterial Hypertension [SUPER-1]) that enrolled 278 was conducted to evaluate the efficacy of Sildenafil in clinical setting (164). The study demonstrated the improvement in functional class, exercise capacity and haemodynamics of PH patients completing one-year treatment with sildenafil as compared to placebo. To evaluate the long term safety and tolerability of sildenafil, patients who completed a

12-week trial of SUPER-1, were entered into open-label extension study (SUPER-2) (165). This study showed that at 3 year of treatment with sildenafil at a dose of 80 mg 3 times a day, majority of patients either improved or maintained their functional status.

Riociguat is a soluble guanylate cyclase (sGC) stimulator, that stabilizes sGC in its active form and also capable of augmenting cGMP production in NO independent fashion (166, 167). sGC is a regulator of NO signalling pathway that produces secondary messenger cGMP, which produces vasodilation and inhibits platelet aggregation and smooth muscle cell proliferation (168, 169). Riociguat clinical efficacy has been studied in a placebo controlled RCT - Pulmonary Arterial Hypertension Soluble Guanylate Cyclase–Stimulator Trial 1 (PATENT-1) (170). The patients who were randomised to treatment group showed improved 6MWD (primary end point), NYHA functional class and pulmonary heamodynamics (secondary end point). These results were also confirmed by another RCT - Chronic Thromboembolic Pulmonary Hypertension Soluble Guanylate Cyclase–Stimulator Trial 1 (CHEST-1) (171). The combined use of Riociguat and Sildenafil provide no clinical benefit but associated with system hypotension as studied in the PATENT-PLUS study (172). Figure 4 summarizes the common vasomotor pathway targeted by approved PH therapeutic agents.

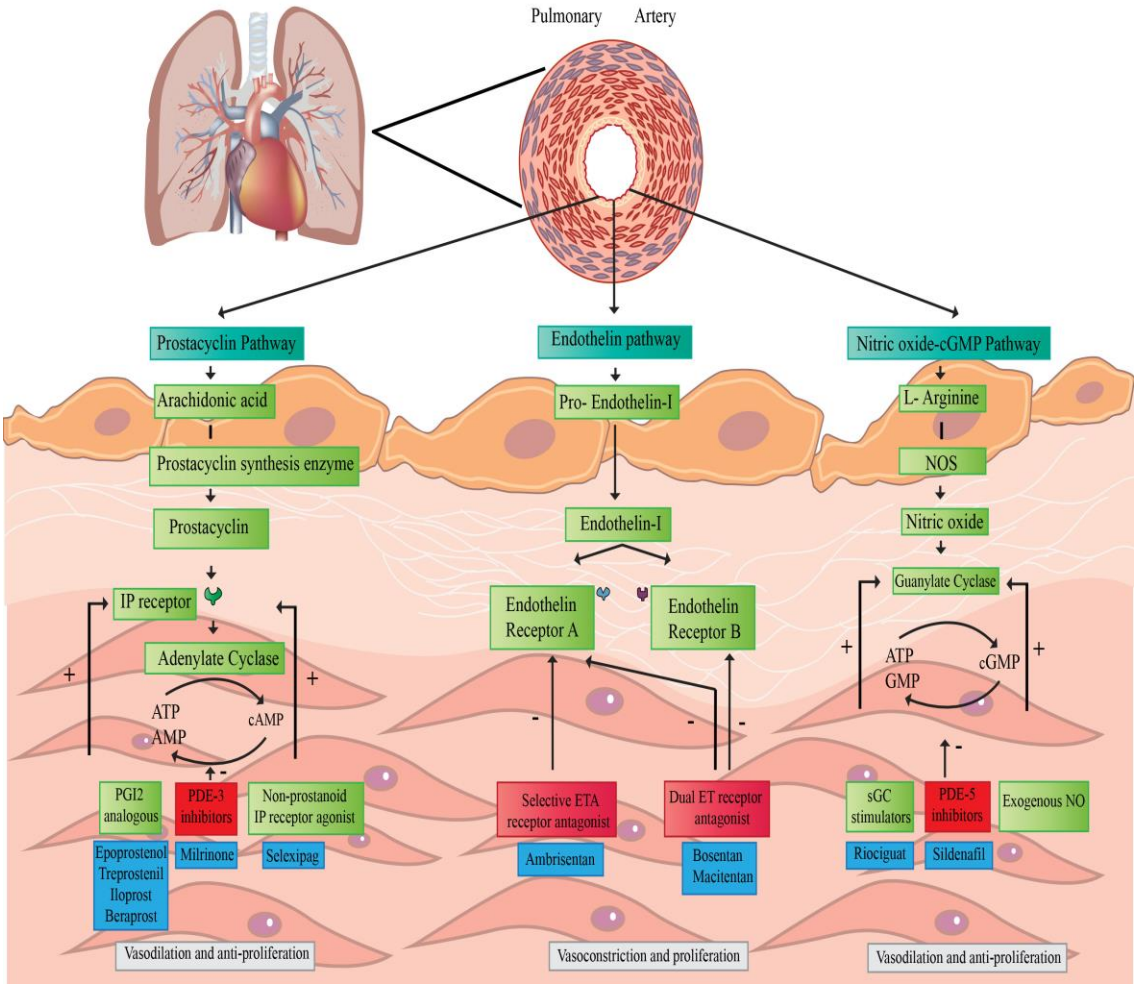

Figure 4: Summary of the common vasomotor pathway targeted by approved PH therapeutic agents. Endothelin-1, nitric oxide, and prostacyclin pathways represent the focuses of currently permitted PAH therapies. PGI2 indicates prostacyclin; PDE, phosphodiesterase enzyme; ET, Endothelin; sGC, soluble guanylate cyclase; NOS, Nitric oxide synthase; and NO, Nitric Oxide.

1.8 Aim Of Thesis:

The aim of the thesis is to characterize and compare and contrast the effects of clinically used pharmacological agents on small human pulmonary arteries.

In recent years human pulmonary artery rings has become commonly used to have an insight of pulmonary vascular resistance that leads to pulmonary hypertension. Acute pulmonary hypertension following cardiac surgery can have a significant effect on postoperative morbidity and mortality. However little data is available on the systematic characterisation of the basic physiological pulmonary vascular reactivity. To bridge this knowledge gap these studies were performed. The purpose was not only to explore the factors that can contribute to pulmonary hypertension but also to compare the efficacy and potency of agents available to treat pulmonary hypertension.

The series of experiments I performed aimed to (i) determine the optimum resting tension (ORT) for *in vitro* human pulmonary artery ring preparations (ii) characterize the pharmacological effects of clinically used vasopressors on the human pulmonary vasculature (iii) characterize the pharmacological effects of clinically used prostacyclin analogues (Epoprostenol and Iloprost) on the human pulmonary arteries in comparison to phophodiesterase 3 & 5 inhibitors (Milrinone and Sildenafil respectively) and Nitric Oxide.

Chapter 2

Materials And Methods

2.1 General Discussion:

Pulmonary hypertension (PH) itself is not a diagnosis but it is caused by range of differing diseases, each with its specific management and prognosis (224). PH is relatively common and associated with high mortality but usually unrecognised for ages (225). Although over the past few decades considerable progress has been made to understand the pathophysiology of PH at both macroscopic and microscopic level but still it is not completely understood (177). Most of our knowledge has been gained by performing in vivo and in vitro experiments on animals, with little evidence available from humans. Rat and mice with hypoxic induced pulmonary hypertension are the most common used animal models for pulmonary hypertension, for example both in vivo and in vitro studies on animals show that vasopressin causes PA vasodilation (226, 227). It is also worthwhile to mention here that patients with pulmonary hypertension and animal models of pulmonary hypertension differ in hemodynamic changes, pulmonary vasculature histological changes and pharmacological responses (179). For example, hypoxia induced pulmonary hypertension is mice shows minimal vascular remodeling whereas in humans it results in marked pulmonary vascular remodeling with intimal and medial thickening (228). Also in humans adventitial thickening and fibrosis mainly involved the distal pulmonary arteries whereas in mouse these changes are more marked in proximal pulmonary arteries (229). Experimental animal tissues are generally obtained from healthy, young single sex animals with a pure genetic lineage, which have been raised in a clean and controlled environment, and as such results obtained from animal studies are unlikely to be truly representative of effects in a diverse human population. In addition to this there are significant interspecies

differences in the responses to hypoxia with some species contradict HPV completely (230). We need to adapt new methodologies to build upon the existing valuable human data to have an insight of pathophysiology of pulmonary artery hypertension. But this is not an easy task as there is always a scarcity of human tissue and only few centres have the cutting-edge technology to utilise human tissue to understand this phenomenon.

I used isolated pulmonary artery (PA) rings for evaluation of drugs and pharmacological agents on the human pulmonary artery at a tissue level. For a number of years isolated pulmonary artery rings have been used to study both human and animal models of diseases (231-233). Experiments on human pulmonary vasculature are difficult due to the presence and potential influence of clinical factors. On the other hand in-vitro experiments performed in a controlled setting, which might not reflect the true physiological environment. Like In this study we used $21_{\%}$ O_2 and $5_{\%}$ CO_2; however, we are aware that in vivo pulmonary arteries are exposed to hypoxic and hypercapnic blood.

All tissue samples were anonymised as a condition of the ethical approval and this prevents analysis of patient characteristics as factors affecting the vasoactivity of isolated arteries. For experiments the samples were collected from resected lungs that were removed from patients with lung disease mainly bronchial carcinomas. For this reason results can be debated as to whether they are true representative of normal physiology despite the fact that the tissue was resected from areas of tumour-free zones. However previous studies proved it as a validated experimental method that has yielded interesting results (234, 235).

The general methods for the relevant chapters are explained here. Individual experimental protocol related to the pertinent studies will be discussed in detail in the relevant chapter.

2.2 Isolated Human Pulmonary Artery Ring – In Vitro Study:

2.2.1 Sample collection:

Human pulmonary arteries were obtained from lungs or lobes immediately following resection for cancer or benign disease. Lengths of healthy vessel were exposed and resected by the operating surgeon, taking care to avoid the tumour margins, and placed in oxygenated (5% CO_2, 21% O_2) Krebs-Henseleit solution (containing: (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.4 and glucose 11 before being transferred to the laboratory.

2.2.2 Sample preparation:

Pulmonary arteries were dissected free of lung and connective tissues cut into 2mm long rings. The Internal diameter of vessels ranged between 2-4 mm. During the dissection excessive manipulation of tissue was avoided to prevent damage to vascular endothelium.

2.2.3 Sample storage:

All the collected sample was used within 24 hours as preliminary experiments confirmed that when stored in oxygenated Krebs-Henseleit solution at 5^oC pulmonary vessels remain viable for at least 24 hours.

2.2.4 Mounting of pulmonary artery (PA) ring:

A multiwire myograph system (DMT 620M) and an organ bath system (Radnotti) were used for mounting of PA rings. These are commonly used for studying physiology and pharmacology of isolated arteries. Organ bath is a traditional way to investigate vascular reactivity and functional responses and with appropriate transducers isometric measurements can be made. Myograph is an advanced version of organ bath that is utilised to examine vascular reactivity of smaller resistance vessels. Both organ bath and multiwire myograph system were used for first series of experiments (measurement of optimal resting tension). Myograph results are more precise and sensitive in comparison to organ bath and for the rest of experiments we used myograph as standard.

Multi-wire Myograph: Multi wire myograph system consists of 4 individual myograph units. Each unit is made of aluminium and has a centrally located 8 ml stainless steel chamber [Fig 5]. The tissue support pins were positioned in the chamber, with one side attached to a force transducer and the other side attached to a micrometre. Pulmonary artery rings were mounted between the pins. Each unit has individually controlled gas inflow and suction. Heating and connections for vacuum and gassing are in the myograph interface, permitting the preparations in all four chambers to be kept under physiological conditions (37°C, and bubbled with 21% O_2, 5% CO_2) [Fig 6]**.** The myograph system was connected to a PC via an amplifier (Power Lab 8/35, AD Instruments) for continuous measurement of isometric tension using data acquisition software (Lab Chart Pro Version 8.0).

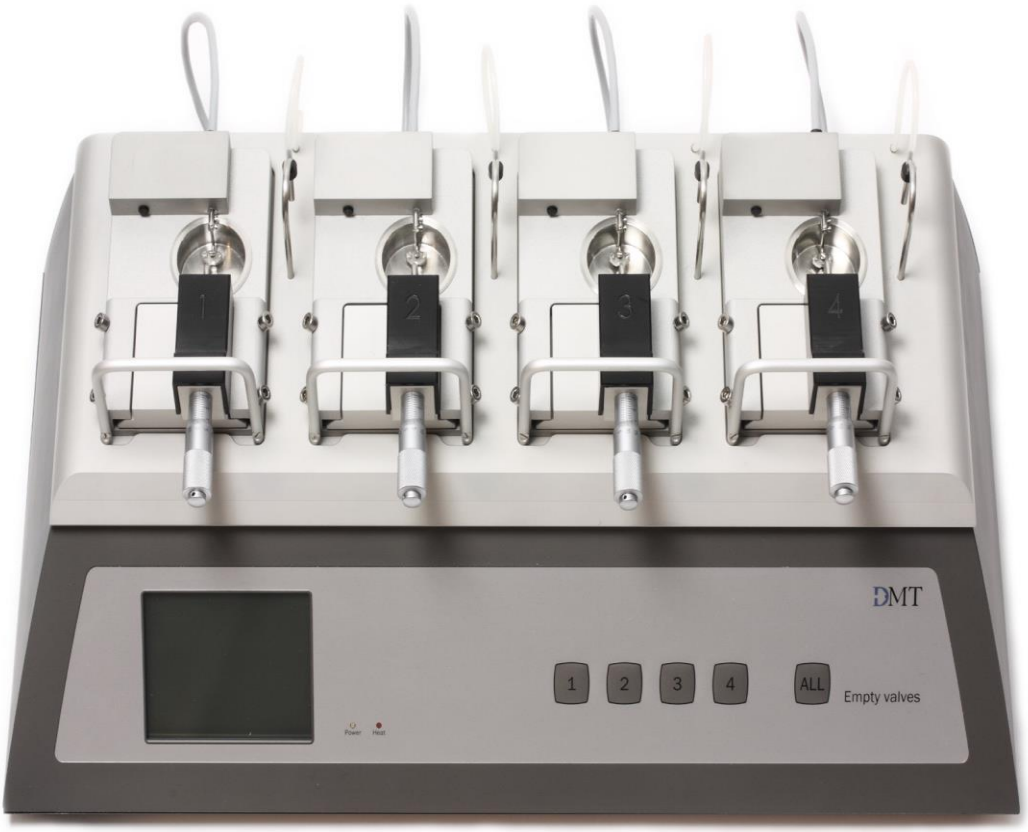

Figure 5: Multiwire Myograph System [DMT 620 M].

Figure 5a: Multiwire Myograph System [DMT 620 M] in function.

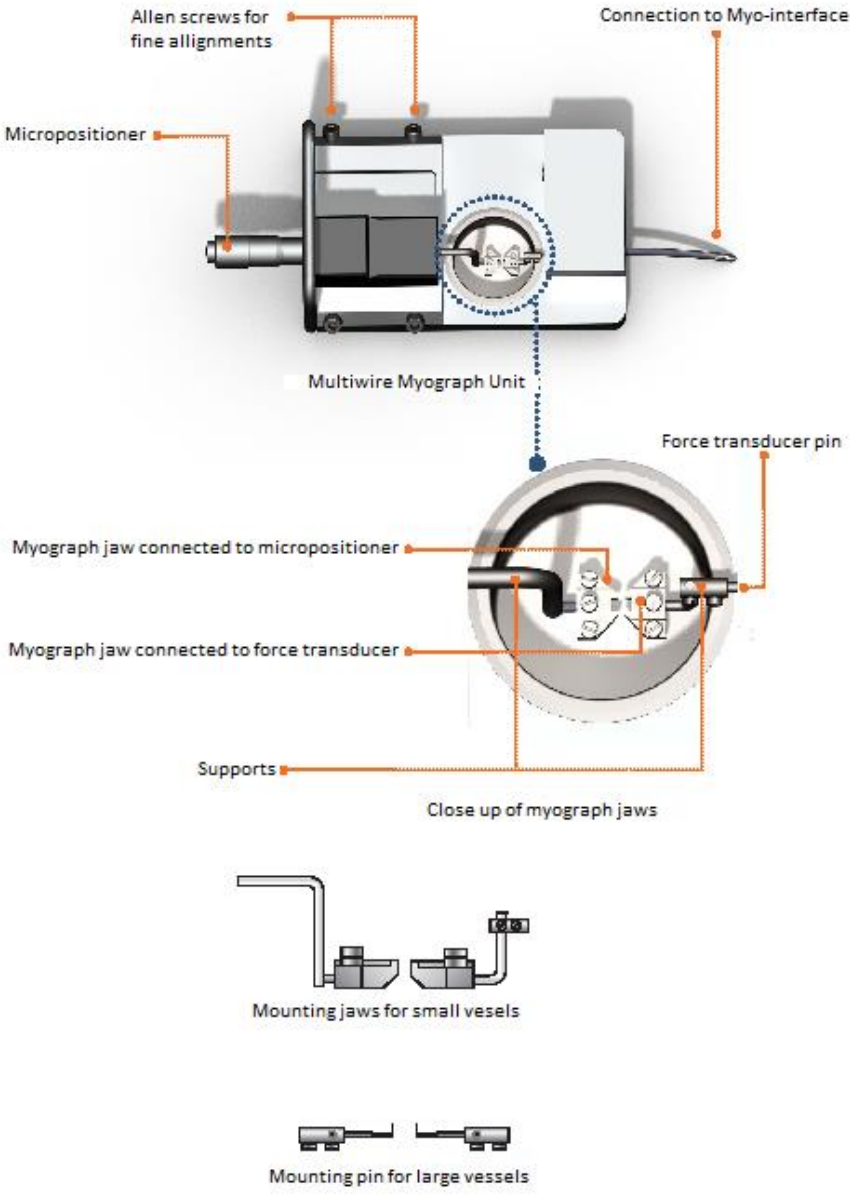

Figure 6: Multiwire Myograph Unit.

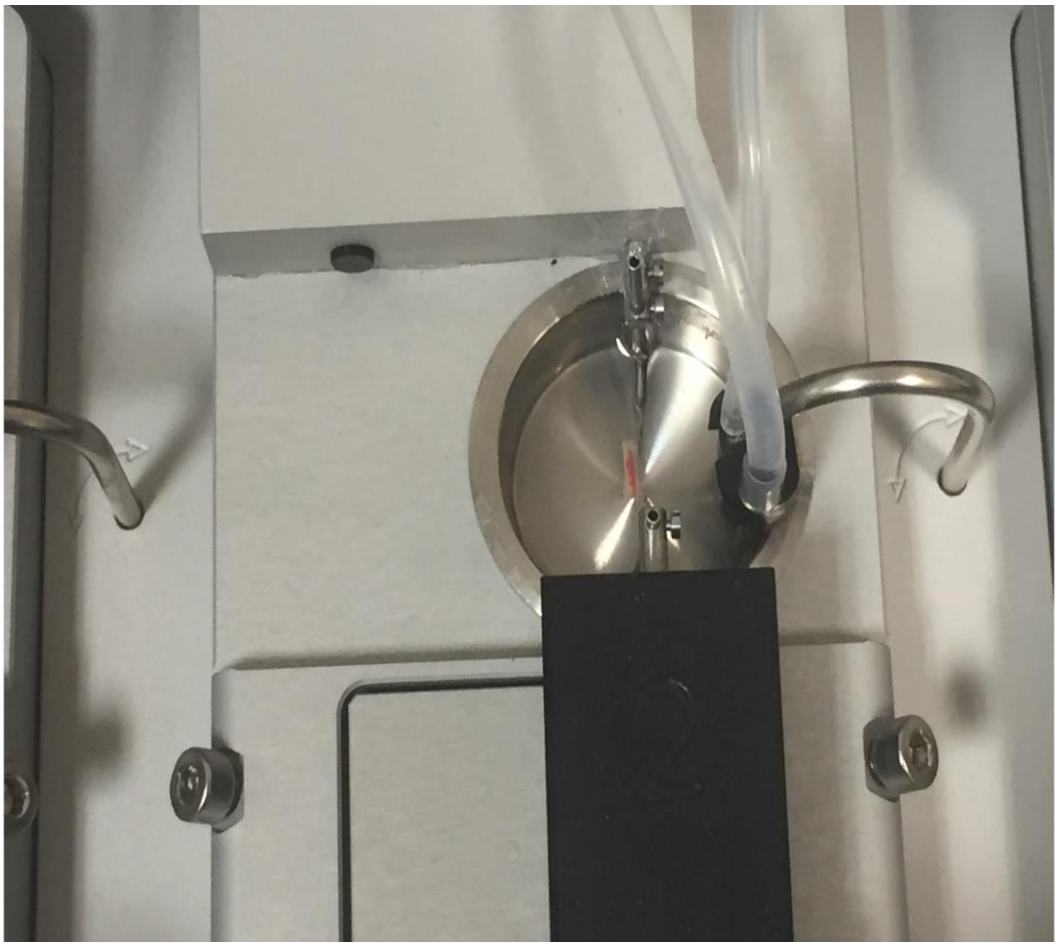

Figure 6a: Individual myograph unit of Multiwire Myograph System.

Organ Baths: The organ bath system consists of 4 organ baths connected to a gas inlet where gas mixtures can be bubbled through [Fig 7]. Surrounding each bath is a heat exchanger that recreated the physiological temperatures of the human body. Each organ bath contained Krebs's solution and the pulmonary artery rings were mounted between two hooks. One hook was fixed and the other attached to a force transducer (Harvard UF1) that was connected to the PC for continuous measurement of isometric tension [Fig 8].

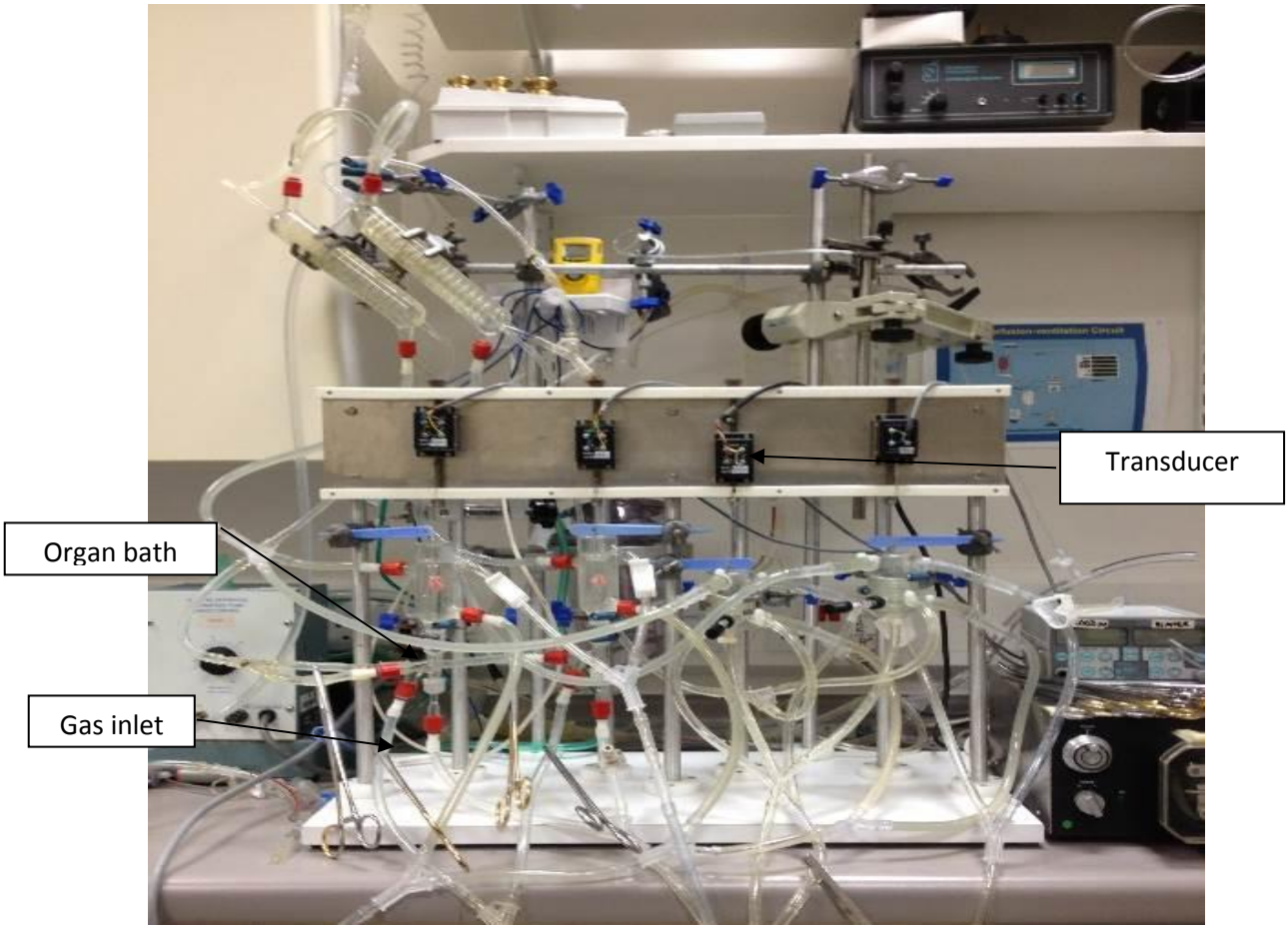

Figure 7: Radnotti Organ Bath System.

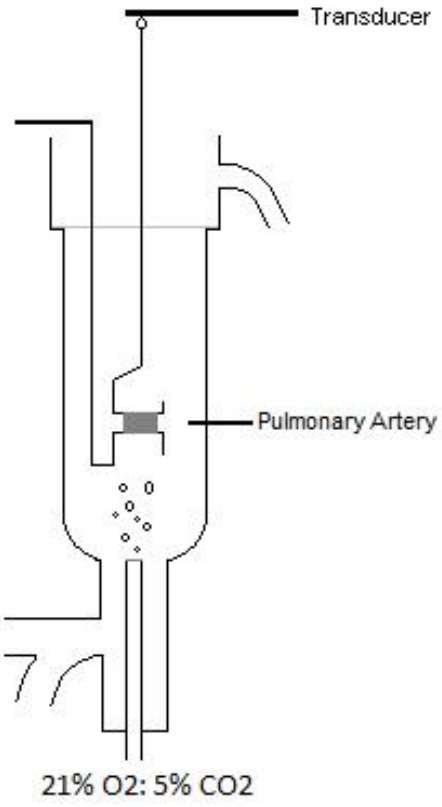

Figure 8: Schematic of Organ Bath system.

2.2.5 Isometric tension:

Increase in isometric tension due to contraction of vascular smooth muscle cells was quantified as active tension in gram force (gf) or % increase in baseline resting tension. The gram-force is a metric unit of force (*gf*). The gram-force is equal to a mass of one gram multiplied by the standard acceleration due to gravity on Earth, which is defined as exactly 9.80665 meter per second. Then one (1) gram-force is equal to 0.001 kg × 9.80665 meter per second = 0.00980665 kilogram × meter per second^2 = 0.00980665 newton (1N). Active tension was calculated as: isometric tension- resting tension.

Relaxation of vascular or smooth muscle caused a decrease in isometric tension and was quantified as absolute decrease in resting tension (gf) or % decrease in resting tension. Decrease in active tension (gf) or % reversal of maximum active tension demonstrated the Relaxation from active tension to contractile agonists.

2.2.6 Calibration:

2.2.6.1 Calibration of myograph:

The transducers of the myograph were calibrated before each experiment by acclimatizing the six steps methods as specified in the user manual. (http://www.dmt.dk/uploads/6/5/6/8/65689239/620m-manual.pdf)

2.2.6.2 Calibration of organ bath:

The transducers were calibrated against a 20 g weight before each experiment using the software calibration procedure.

2.3 Experimental Protocols:

2.3.1 Measurement of optimal resting tension:

After mounting of PA rings sample was left to equilibrate under 21% O2 and at 37^{o}C temperature. Experiments start with baseline tension of 1 gf. and 40 mM KCl added, active resting tension recorded after 30 min once the contractile response to KCl reached to a plateau. Specimen washed for 30 minutes and repeated again with 40 mM KCl. At 1 gf. baseline tension the active tension responses was repeated 3 times to check the reproducibility. Baseline tension then was increased to 1.2 gf. and active tension recorded with the addition of 40 mM KCl. The experiment repeated and active resting recorded at 1.4gf, 1.6gf, 1.8gf and 2.0 gf.

2.3.2 Equilibration – resting tension of pulmonary artery rings:

A baseline tension of 1.61gf was applied and the vessels left to equilibrate under 21% O_2 : 5% CO_2 at 37^{o}C for 60 - 90 minutes. Vessels were frequently washed during calibration and the resting tension adjusted to 1.61 gf. At the start of experiments the functional integrity of endothelium was confirmed by the addition of 1μM acetylcholine and then the vessels were then washed for 30 minutes by rapidly replacing the Krebs solution in the chambers with fresh solution three times every five minutes. Preliminary experiments determined that 1 μM ACh caused maximum relaxation in human pulmonary arteries. At the end of each experiment the contractility of vessels was confirmed by addition of 37.6 μM KCl (EC_{80}) and the rings that did not contract to KCl were excluded from the study.

2.3.3 Contractile agonist responses:

A multiwire myograph system (DMT 620M) was used for mounting of PA rings and determination of contractile agonist responses. Contractile agonists were added directly to the myograph and stepwise increases in agonist concentration were used when a plateau response had been obtained to the preceding dose. The effects of contractile agonists expressed as active tension (gf) or % increase in resting tension, which was calculated as maximum tension at plateau (gf) – resting tension (gf). The maximum efficacy (E_{max}) for each agent was determined in gf and expressed as gf / mm internal diameter of each vessel to take into account the variability in PA ring diameter.

2.3.4 Relaxant responses:

PA rings were mounted in the myograph to determine the effect of vasodilators on human pulmonary vessels**.** After mounting of PA rings a resting tension of 1.6 gf was applied (optimum resting tension determined in preliminary experiments) and the vessels were left to equilibrate with 21% $O_{2:}$ 5% CO_2 at 37^{o}C for 60 minutes. PA rings were than pre constricted with 11.21µM PGF2α (EC_{80}). When a stable resting tension was achieved cumulative concentration response curves were constructed by stepwise increases in agonist concentration in the myograph chamber when a plateau response had been obtained to the preceding concentration. Active tension was calculated in gram force (gf) as maximum tension at plateau (gf) – resting tension (gf). Emax represents the maximum relaxation response to single concentration agonist.

2.3.5 Agonist potency and efficacy:

Potency of drug is defined as amount of drug that is needed to produce a given effect and efficacy represents the maximum effect that a drug can produce regardless of dose. Agonist EC_{50} concentrations represents concentration required to elicit 50% of the maximum agonist response while and pEC_{50} signifies the negative logarithm to base 10 of the molar EC_{50} concentration. Both EC_{50} and pEC_{50} were calculated by using the statistical analysis functions of GraphPad Prism version 7.00 for Windows.

2.3.6 Antagonist studies:

When a stable baseline tension was achieved antagonists were then added directly to the myograph. Repeat responses in the presence of the antagonist were then obtained after a minimum of 30 minutes and when the baseline tension was stable. The effects of antagonists on agonist responses were quantified by determining the effect on the maximum agonist effect (Emax) obtained to a single concentration of agonist and expressed as % change in Emax.

2.3.7 Vehicle control:

Most of the reagents were serially diluted in distilled water but some drugs were either dissolved in Dimethyl sulfoxide **(**DMSO) or ethanol as per manufacture guidelines. For experiments performed on these drugs, control responses to the vehicle were obtained by the addition of corresponding volumes of solvent without the active agent in parallel preparations.

2.3.8 Disposal of tissue

Surplus tissue and tissue used for experiments were immersed in disinfectant solution (3% Vircon solution, Antec International) and later disposed off by incineration.

2.4 Materials:

2.4.1 Physiological salt (Krebs-Henseleit) solutions:

For all of our experiments physiological salt solution, Krebs-Henseleit solution was used which contained: (mM) NaCl 118, KCl 4.7, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, CaCl2 2.4 and glucose 11.

A Krebs stock solution was prepared by dissolving 53.9 g KCl, 24.6 g KH2PO4 and 44.5 g MgSO4.7H2O in 1 litre of deionised water (Purite Select Deioniser) and stored at 4^{o}C. Fresh Krebs-Henseleit solution was made for every experiment and used on the same day. 5 litres of fresh Krebs-Henseleit solution was made as follows; 34.5 g NaCl, 10.5 g NaHCO3 and 10 g of glucose were dissolved in 2 litres of deionized and added to 2.9 litres of deionized water. 32.5 ml of Krebs stock solution and 13 ml of 1 Molar CaCl2 was added and the solution mixed and immediately bubbled with 21% O2: 5% CO2 for 15 minutes to prevent precipitation of calcium carbonate.

2.4.2 Reagents:

The agents used were (supplier in parentheses) Adrenaline (Martindale Pharmaceuticals, Buckinghamshire, UK), Acetylcholine (Sigma-Aldrich, St. Louis,

MO, USA), Arginine Vasopressin (Mercury Pharmaceuticals, part of Concordia International, London, UK), Endothelin-1 (American Peptide Company, part of Thermo Fisher Scientific, Waltham MA, USA), Noradrenaline (Aguettant Pharmaceuticals, Bristol, UK), Sildenafil (Tocris Bioscience), Milrinone (Stragen, UK), Sodium Nitroprusside (Sigma-Aldrich), Epoprostenol (GlaxoSmithKlone), Iloprost (Tocris Bioscience), Treprostinil (Tocris Bioscience) and PGF2 α (Tocris Bioscience, part of Bio-Techne, Abingdon, UK).

All other reagents were obtained from Thermo Fisher Scientific unless otherwise stated.

2.4.3 Gases:

All gases were supplied by the British Oxygen Company Ltd, Guildford, Surrey, UK.

2.4.4 Drugs:

Stock solutions of drugs were prepared using the solvents recommended by the suppliers, and control responses to solvents were obtained when necessary. Fresh serial dilutions were made, using the appropriate solvent, for each experiment and solutions were stored at the temperature recommended by the supplier and protected from light if necessary.

2.5 Statistical Analysis:

Data are presented as mean ± standard deviation (SD), and n represents the number of individual PA rings used in an experiment. Agonist EC_{50} concentrations (the

concentration required to elicit 50% of maximum response) were determined using nonlinear regressions to fit a standard slope model using the statistical analysis functions of GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA. More details can be found at http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm). Agonist potency is presented as pEC_{50} (the negative logarithm of the molar EC_{50} concentration). One-sample Kolmogorov-Smirnov test was used to test for normality of distribution. Box-whisker plots display median, the interquartile range (box) and maximum and minimum values (whiskers). Outliers (1.5 – 3 x the interquartile range) and extreme outliers (> 3 x the interquartile range) were automatically removed from the data analysis by the statistics software. The potency p EC50 and efficacy of each drug were compared between groups with one-way analysis of variance (ANOVA) and Bonferroni test using SPPS Statistics 22 software (IBM, Armonk, NY). For experiments assessing vascular reactivity, concentration–relaxation curves of vasodilator agents were analyzed by Repeated Measures ANOVA to determine change in tone over time. Significance was taken as $p < 0.05$.

Chapter 3

Characterization Of Optimal Resting Tension In Human Pulmonary Arteries

3.1 Introduction:

Hypoxic pulmonary vasoconstriction (HPV) is a fundamental physiological mechanism to redirect the blood from poorly to better-aerated areas of lungs to optimize the ventilation perfusion matching (173). Persistent hypoxia leads to increased pulmonary vascular opposition and right ventricular afterload that leads to hypoxic pulmonary hypertension (174). HPV initially thought to be caused by alveolar hypoxia by means of local lung mechanism but recent advances suggest that PA smooth muscle cells constitute both the sensor and the transducer of the hypoxic signal as well as its contractile effector (175).

The pulmonary circulation carries deoxygenated blood from right section of heart towards lungs, under which it is subject to oxygenation while carbon dioxide is filtered, thereafter returning the clean blood onto the left section of the heart prepared for dissemination (2) . The pulmonary circulation is in series and reliant not only on the systemic blood flowing to right section of the heart, rather also the outflow from the left section (12). Therefore, in case of an increment under the left atrium pressure or increase in afterload like in aortic stenosis, greater pressure will be observed in the PA (176).

A series of experiments performed to explain the phenomenon on macroscopic and microscopic level has been reported although the underlying mechanism is not clear (48, 177, 178). However, vast majority of experiments performed in animals with little data available from humans. Experiments performed on animals are generally inapplicable on humans so we need to adapt new methodologies for use in human to understand the human disease biology.

The objective of this experiment is to identify the optimal tension to be used in human PA rings to facilitate future experiments and also to provide a methodology of isolation of PA and their use in studies in the form of arterial rings.

3.2 Methods And Materials:

Patients undergoing a lung lobectomy by a Consultant Cardiothoracic Surgeon at Castle Hill Hospital were consented for resected lung tissue to be included in this study prior to their operation at the time of their consent for surgery. Patients under the age of 18 and who cannot give informed consent were excluded from the study. Local research ethics committee and local research and development department approval was obtained for the use of human tissue for this study.

3.2.1 Isolation of PA rings:

Tissue samples were collected from patients undergoing surgical lung resection for cancer and immediately moved to the laboratory in Krebs-Henseleit solution. Pulmonary arteries were dissected from disease free areas of lung resection and after careful removal of adipose and connective tissues cut into 2mm long rings. The Internal diameter of vessels ranged between 2 - 4 mm.

3.2.2 Mounting of PA rings:

A multiwire myograph system (DMT 620M) and an organ bath system (Radnotti) were used for mounting of PA rings and measurement of ORT. The Pulmonary artery rings were mounted between the pins. All units were subject to administered gas inflow and suction. Connections for vacuum and gassing, as well as heating are provided in the myograph interface, allowing for all chambers to be smoothly

maintained under physiological settings (37°C, and bubbled with 21% O_2: 5% CO_2). The myograph system was connected to a PC via an amplifier (Power Lab 8/35, AD Instruments) for continuous measurement of isometric tension using data acquisition software (Lab Chart Pro Version 8.0).

The organ bath system consists of 4 organ baths connected to a gas inlet where gas mixtures can be bubbled through. Surrounding each bath is a heat exchanger that recreated the physiological temperatures of the human body. Each organ bath contained Krebs's solution and the pulmonary artery rings were mounted between two hooks. One hook was fixed and the other connected with a force transducer (Harvard UF1), which was linked to a PC for continuous measurement of isometric tension.

3.2.3 Determination of optimal resting tension:

After mounting of PA rings the resting tension was set at 1 gf and the vessels left to equilibrate under 21% O_2: 5% CO_2 at 37^{o}C for 60 minutes. When a stable resting tension was achieved the vessels were exposed to 40 mM KCl by direct addition to the organ bath. The maximum contraction to KCl was recorded when the contractile response reached a plateau. Active tension was calculated as maximum tension at plateau (gf) – resting tension (gf). Vessels were then washed for 30 minutes by rapidly replacing the Krebs solution in the chambers with fresh solution three times every five minutes. When a stable resting tension was achieved a repeat reaction with 40 mM KCl was obtained and the vessels again washed before obtaining a third reaction to 40 mM KCl for the purpose of confirming reproducibility in the response. When a reproducible response was obtained the maximum contraction to

40 mM KCl had been established from increasing resting tensions of 1.2, 1.4, 1.6, 1.8 and 2.0 gf with the vessels being washed for 30 minutes between responses.

At the end of each experiment the integrity of endothelium was confirmed by the addition of 1uM acetylcholine. Rings that did not contract to KCl were excluded from the study.

3.2.4 Chemicals and reagents:

5% Carbon dioxide / balance air (10 lt cylinders) was sourced from BOC Limited. All reagents were obtained from Fischer Scientific and acetylcholine from Sigma Aldrich.

3.2.5 Statistics:

Data are presented as mean ± SD (standard deviation) and n represents the number of PA rings used.

3.3 Results:

Twenty-four PA rings (internal diameter 2-4 mm) were obtained from 5 patients. Four PA rings from a patient were discarded due to non-contractility to KCl. Results showed that in human PA rings increasing the basal tension from 1.0 to 1.6 gf significantly augmented the 40 mmol/L KCl induced active tension. Increasing the active tension from 1.6 to 2.0 g mitigate the 40 mmol/L KCl induced response. Total 12 PA rings from four patients were used to perform the experiment using multi-wire myograph system. Increasing the resting tension from 1.0 gf to 1.6 gf significantly augmented the 40 mmol/L KCl induced active tension. Increasing the

active tension from 1.6 to 2.0 gf initially plateaued off than decreased the 40 mmol/L KCl induced response (Figure 9A). Total 8 PA rings from four patients were used to perform the experiment using organ bath system. Increasing the resting tension from 1.0 to 1.6 gf significantly augmented the 40 mmol/L KCl induced active tension. Increasing the active tension from 1.6 to 2.0 gf either decreased or plateaued off the 40-mmol/L KCl induced response (Figure 9B).

Both the multi-wire myograph and organ bath system have demonstrated identical conclusions (Figure 10), and confirmed that the most efficient resting tension for human PA rings is 1.61 gf (Figure 11).

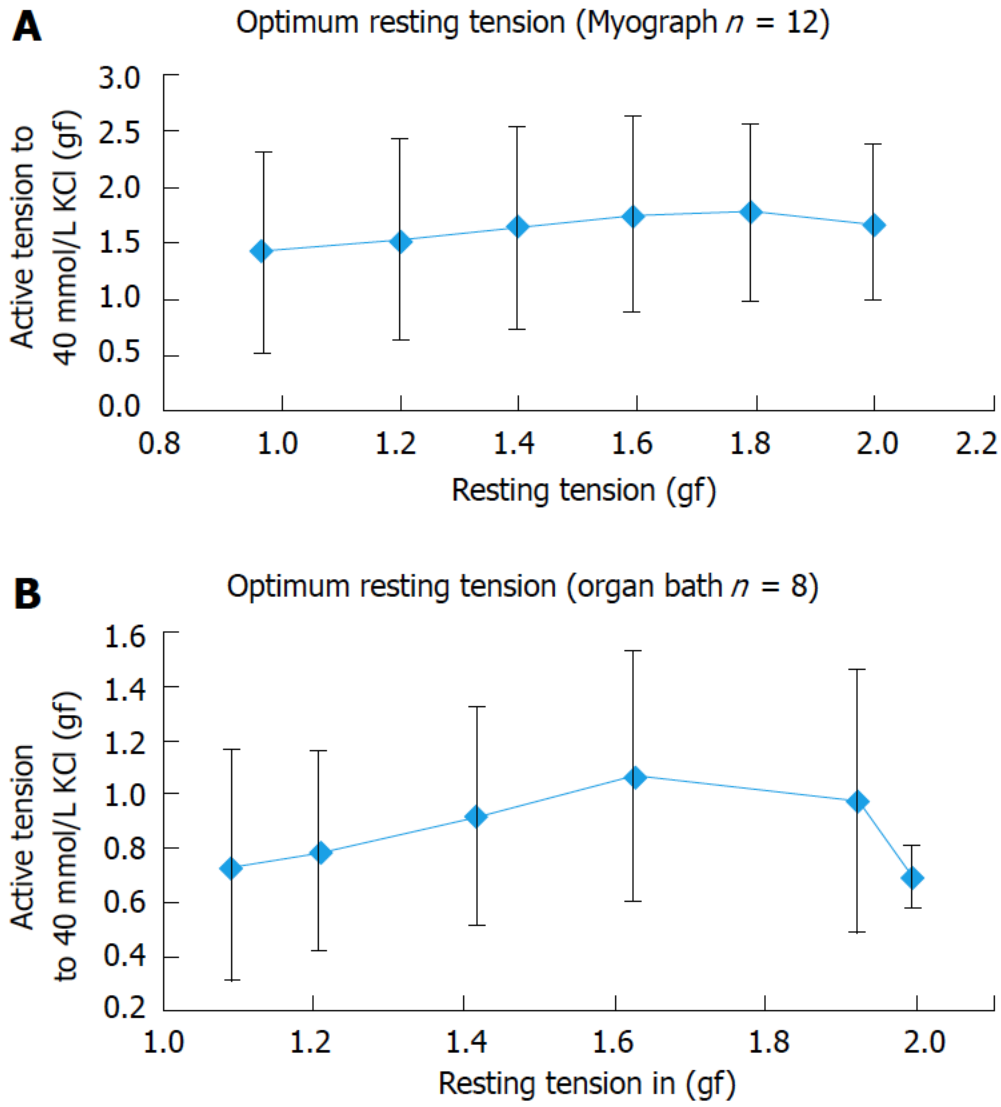

Figure 9: Measurement of Optimal Resting Tension using Multi-wire Myograph (A) and Organ Bath system (B).

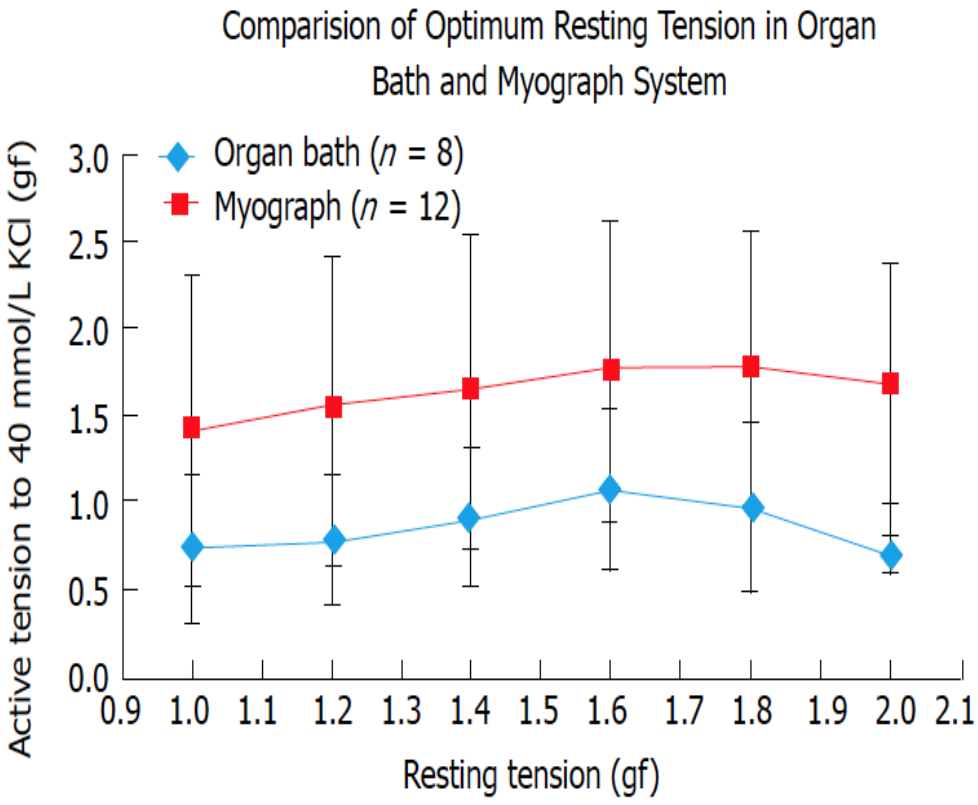

Figure 10: Comparison of Optimal Resting Tension measurement in Organ Bath and Myograph system.

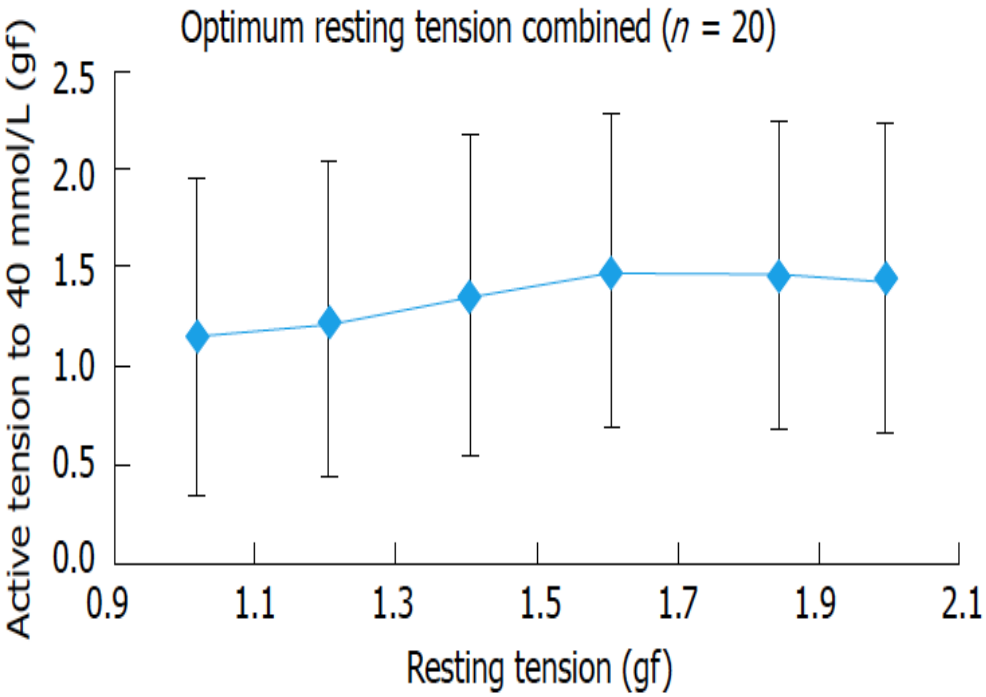

Figure 11: Combined result of optimal resting tension measurement.

3.4 Discussion:

PA vasoconstriction is an important physiological process to regulate blood flow in lungs but it also results in pathologies. Various models are utilized for assessing the baseline molecular and cellular functions of lung diseases, particularly pulmonary vascular condition. However, a great deal of researches is undertaken on animals with little similarity to humans. Few centers have the luxury to utilize human tissue to study this phenomenon. Isolation of human PA and measurement of pulmonary vascular tension are vital to understand the pathophysiology of human pulmonary vessels.

Various experiments were performed on both animals and humans by utilizing pulmonary artery rings. It is worth mentioning here that the resting tension used to perform these experiments shows variability with lack of evidence. Hoshino et al. used a resting tension of 2g to perform experiments on human pulmonary arteries (53), while Ariyaratnam et al. applied a resting tension of 1-2 g to perform hypoxic and hyperoxic experiments on human pulmonary artery rings (109). The vascular reactivity to a pharmacological agent highly dependent on the degree of resting tension so one needs to use optimal resting tension (ORT) for reliable results (179). Previous studies reported that endothelium dependent relaxation of vessels is highly dependent on initial stretch (180).

In this study we demonstrated the methodology of isolation of human pulmonary artery and measurement of ORT for small human PA. To characterise the ORT pulmonary artery rings is stretched to different tensions and a contractile agent like potassium chloride at each tension elicits a contraction. Optimal tension is the tension, which demonstrated the maximum contractile response.

Chapter 4

The Differential Effects Of Systemic Vasoconstrictors On Human Pulmonary Artery Tension

4.1 Introduction:

Pulmonary hypertension is an important prognostic factor in cardiac surgery and is associated with increased rates of morbidity and mortality. Patients are usually diagnosed with pulmonary hypertension prior to surgery but cardiac surgery itself can precipitate it. The mechanism of the underlying pathophysiology is complex, and pulmonary hypertension may exist prior to surgery or might develop during or after surgery (35).

Vasopressor drugs are commonly used perioperatively and postoperatively to treat low systemic arterial blood pressure and maintain organ perfusion. The choice of vasopressor and inotropes in patients having cardiac surgery should take into account their effects on pulmonary vascular resistance because these drugs could trigger pulmonary vasoconstriction, which, if persistent, could progress to pulmonary hypertension (47). Adrenaline (AD), Noradrenaline (NA) and Arginine Vasopressin (AVP) are frequently used vasopressors in cardiac surgery patients. AD and NA are sympathomimetic catecholamines whose vasopressor effect is predominantly mediated by the activation of vascular smooth muscle α1-adrenoceptors, whereas vasoconstriction by AVP is mediated via activation of vascular smooth muscle V1 receptors (113).

Ideally, cardiac surgical patients need a vasopressor drug that can selectively cause systemic vasoconstriction without triggering pulmonary vasoconstriction. The effects of commonly used vasopressor agents on pulmonary vascular tone have been investigated extensively in animal models. However, limited data are available about the effects of vasopressors on human pulmonary vascular tone. In recent

years, our group has used human PA rings to investigate the physiological and pharmacological effects on the human pulmonary vasculature. In this study, we used human PA rings to compare the efficacy and potency of clinically used vasopressors on human pulmonary vascular tone, which could lead to improved decision making in the perioperative and postoperative settings.

4.2 Materials And Methods:

Research ethics committee and local research and development department approval was obtained to use human tissue for this study. Patients gave written consent for the use of surplus tissue for research purposes. Patients under the age of 18 years and those who could not give informed consent were excluded from the study.

4.2.1 Isolation of pulmonary artery rings:

Human pulmonary arteries were obtained from lungs or lobes of patients immediately following resection for cancer. Lengths of healthy vessel were exposed and resected by the operating surgeon, who took care to avoid the tumour margins, and placed in oxygenated (5% CO2: 21% O2) Krebs –Henseleit solution (containing, in mM, NaCl 118, KCl 4.7, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, CaCl2 2.4 and glucose 11) before being transferred to the laboratory. After careful removal of adipose and connective tissue, the arteries were cut into 2-mm long rings with an internal diameter of 2–4 mm.

4.2.2 Mounting of pulmonary artery rings:

The multiwire myograph system (DMT 620M) described in the methodology

chapter was used for mounting PA rings and measuring isometric tension. All 4 individual myograph units were used with a PA ring mounted between the force transducer and the micrometer. The rings were equilibrated under physiological conditions (37^{o}C and bubbled with 21% O2: 5% CO2). The myograph system was connected to a PC via an amplifier (Power Lab 8/35, ADInstruments) for continuous measurement of isometric tension using data acquisition software (Lab Chart Pro Version 8, ADInstruments).

4.2.3 Effect of systemic vasopressors on pulmonary vascular tone:

After mounting of the PA rings, a resting tension of 1.6 gram force (gf) was applied (optimum resting tension determined in preliminary experiments), and the vessels were left to equilibrate with 21% O2: 5% CO2 at 37^{o}C for 60 min. When a stable resting tension was achieved, cumulative concentration response curves to the agonists [AD, NA, Endothelin-1 (ET-1), Prostaglandin F2α (PGF2α), KCl and AVP] were constructed by stepwise increases in agonist concentration in the myograph chamber when a plateau response had been obtained to the preceding concentration. Active tension was calculated as maximum tension at plateau (gf) – resting tension (gf).

The maximum efficacy (Emax) for each agent was determined in gf and expressed as gf/mm internal diameter of each vessel (to take into account the variability in the diameter of the PA ring) or percentage of the control response curve Emax KCl contraction. At the end of each experiment, the integrity of the endothelium was confirmed by the addition of 1 μM Acetylcholine. KCl was used to check the contractility of PA rings; rings that did not contract in response to KCl were

excluded from the study.

Agonist potency was compared by determining the agonist EC_{50} concentration (the concentration required to elicit 50% of a maximum response), which was presented as pEC_{50} (the negative logarithm of the molar EC_{50} concentration).

4.2.4 Effect of arginine vasopressin on active tension in response to prostaglandin F2α:

In another set of experiments, the potential vasodilator effect of AVP on pulmonary vascular tone was investigated. When a stable resting tension was achieved, vessels were preconstricted to 11.2 μM PGF2α (EC_{80}). When a stable plateau contraction was achieved, the effect of AVP on active tension was determined by cumulative addition to the myograph chambers.

4.3 Results:

A total of 67 human PA rings (mean internal diameter 2.8 ± 0.4 mm) were obtained from 22 patients. 10 rings that did not contract to KCl were excluded from the study.

4.3.1 Concentration-dependent effects of vasopressors on human pulmonary arteries:

AD, NA, ET-1, PGF2α and KCl caused the concentration dependent vasoconstriction of human pulmonary arteries, whereas AVP had no significant effect (Figs 12 - 23). The order of efficacy (expressed as gf, gf/mm internal diameter or percentage of KCl Emax) was KCl = PGF2α > A = NA > ET-1 and the order of

potency was ET-1 > A = NA > PGF2α > KCl. The effects of vasopressor agents on PA tone are summarized in Table 1 and Fig. 24.

Repeated results at different doses from each vasoconstrictor agent when compared with one-way analysis of variance (ANOVA) and Bonferroni test using SPPS Statistics shows that there were statistically differences in the mean % vasoconstriction made over time between vasoconstrictors group ($p < 0.05$). The mean % vasoconstriction for each vasoconstrictor in ascending order was; Endothelin-1: 8.74 (95% CI -5.45 – 22.95), KCl: 11.21 (95% CI 0.07 - 22.35), Noradrenaline: 26.83 (95% CI 15.23 – 38.42), Adrenaline: 34.75 (95% CI 20.55 – 48.95), and PGF2a: 40.13 (95% CI 25.93 – 54.33). Within subject analysis of all the vasodilator agents do display an increase in mean % vasodilation over time ($p<0.05$).

Emax (gf) for AD, NA, ET-1, PGF2α, KCl and vasopressin was 0.84 gf, 0.66 gf, 0.34 gf, 1.11 gf, 1.08 gf and 0.004 gf, respectively. Another useful indicator of the variation in the internal diameter of the PA rings is a comparison of the total active force of a 2mm long artery segment normalized for internal circumference (πD, The number **π** (/ˈpaɪ/) is a mathematical constant, the ratio of a circle's circumference to its diameter. It is approximately equal to 3.14159). Using this index, the values (Emax [gf/mm]) for AD, NA, ET-1, PGF2α, KCl and vasopressin were 0.08 gf/mm, 0.077 gf/mm, 0.035 gf/mm, 0.141 gf/mm, 0.121 gf/mm and 0.0004 gf/mm, respectively.

By taking the maximum response to KCl as 100%, the value of Emax (% KCl) to AD, NA, ET-1 and PGF2α ranges was 78%, 61%, 31% and 102%, respectively.

4.3.2 Concentration response curve of arginine vasopressin-induced pulmonary vasoreactivity:

To demonstrate the effect of AVP on PA rings, a concentration of AVP of 10 pM – 3 mM was used with no effect.

4.3.3 Concentration response curve of noradrenaline - induced pulmonary vasoconstriction:

All vessels constricted in response to NA. Increasing concentrations of NA from 3 nM to 30 mM were used on 12 PA rings. Maximal contraction was seen at 3 mM (log -5.5M); after that, the response of vessels to NA tailed off. The EC_{20}, EC_{50} and EC_{80} were 40.09 nM, 150nM and 563.55 nM, respectively. The hill slope was 1.049 ± 0.595.

4.3.4 Concentration response curve of ET-1-induced pulmonary vasoconstriction:

To evaluate the effect of ET-1 on pulmonary vessels, 8 PA rings and concentrations of ET-1 from 100 pM–30nM were used. As the concentration rose above 1 nM, vessels started constricting and the maximum contractile response was seen at 10nM (log -8 M). The EC_{20}, EC_{50} and EC_{80} were 0.95 nM, 1.46nM and 2.26 nM, respectively. The hill slope was 3.199 ± 2.154.

4.3.5 Concentration response curve of PGF2*α*-induced pulmonary vasoconstriction:

PGF2a at a concentration of 100 nM – 300 mM was used on 8 PA rings to

demonstrate its vasoconstriction effect. The EC_{20}, EC_{50} and EC_{80} were 3.6 mM, 6.35 mM and 11.21 mM, respectively. The hill slope was 2.441 ± 1.239.

4.3.6 Concentration response curve of KCl-induced pulmonary vasoconstriction:

A total of 13 PA rings were studied in this series. Increasing concentrations of KCl from 300 mM to 300 mM were used. Vessels started contracting as the concentration increased above 1 mM (log -3M); the maximal contraction was seen at 100 mM; after that, the response to KCl diminished. The EC_{20}, EC_{50} and EC_{80} were 7.98 mM, 17.29mM and 37.46 mM, respectively. The hill slope was 1.794.

4.3.7 Effect of arginine vasopressin on active tension in response to prostaglandin F2α:

AVP (0.01-3 mM) had no effect on active tension to PGF2α (n = 4 from 2 patients).

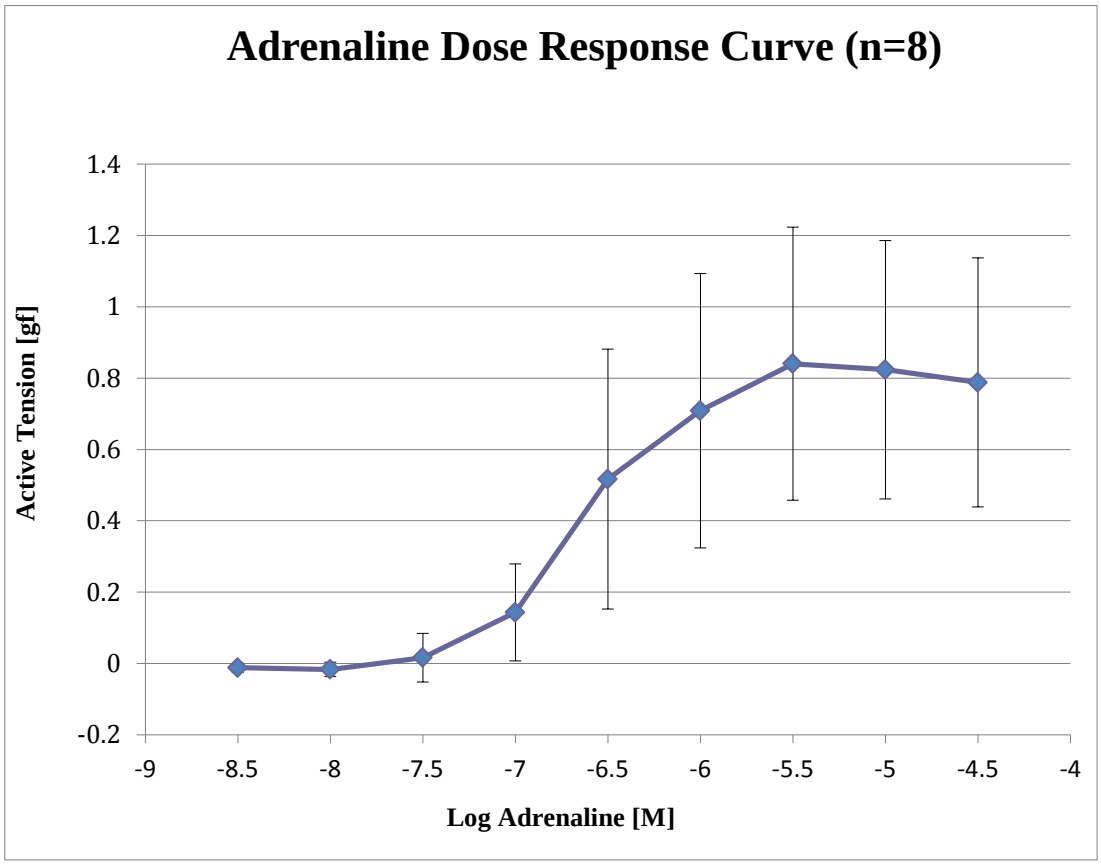

Figure 12: Concentration response curve to Adrenaline. A total of 8 pulmonary artery rings from 4 patients were used to determine the effect of AD on the pulmonary artery.

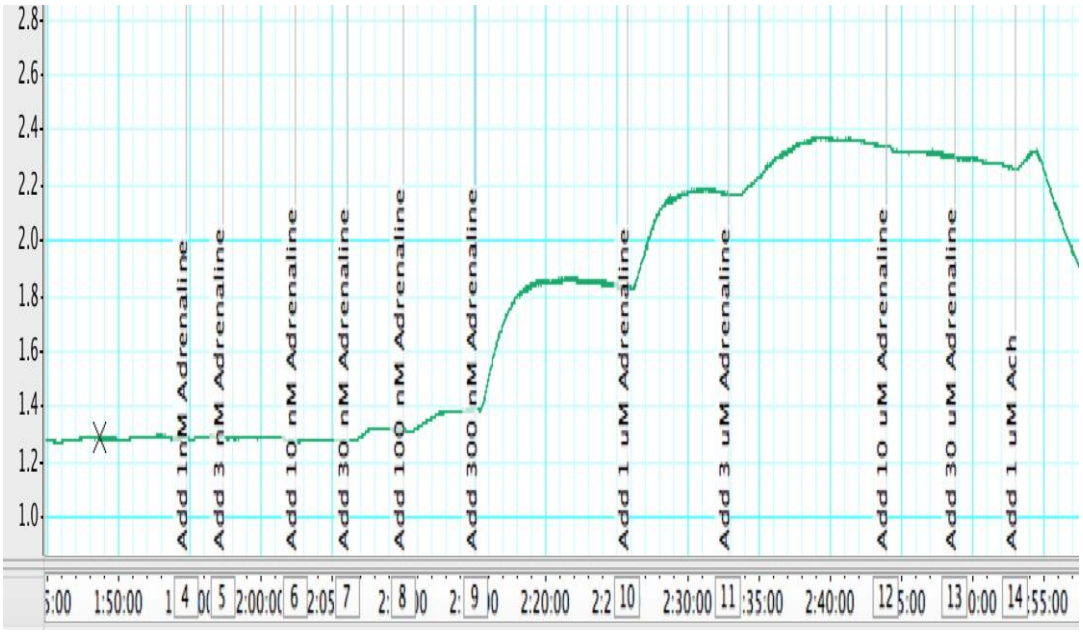

Figure 13: Experimental trace showing effect of Adrenaline on isolated human pulmonary artery ring.

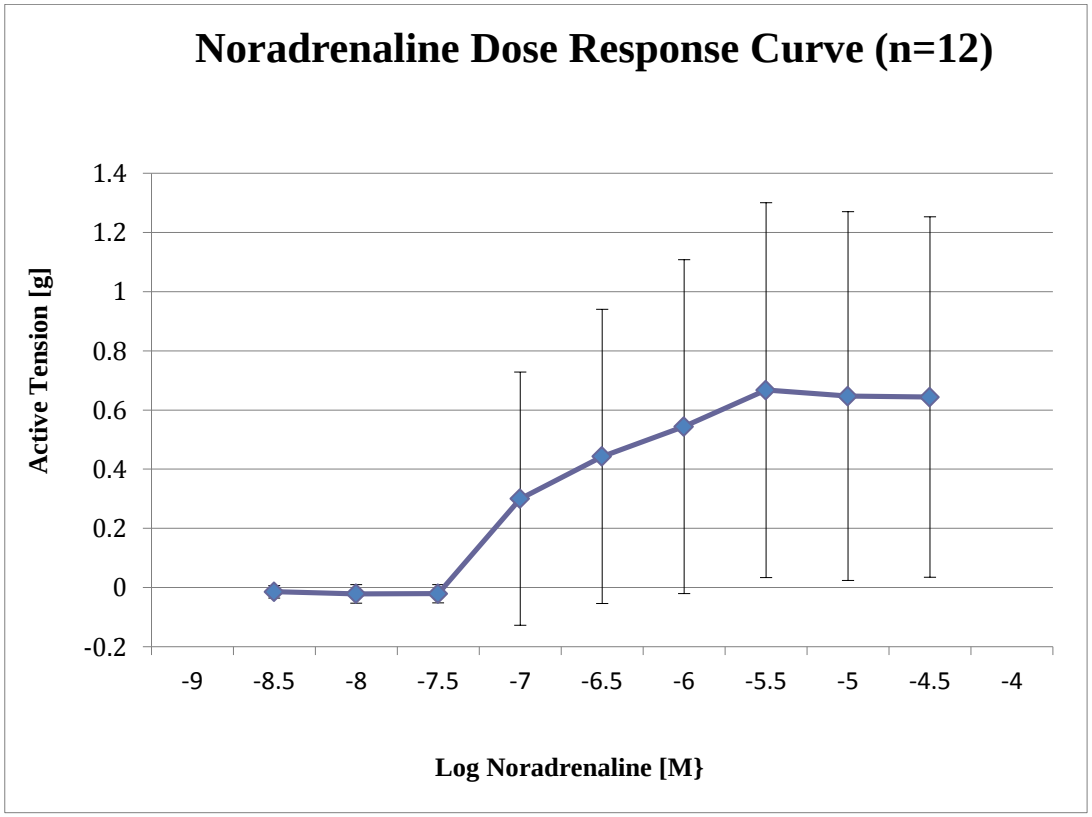

Figure 14: Concentration response curve to Noradrenaline (NA). All vessels constricted in response to NA.

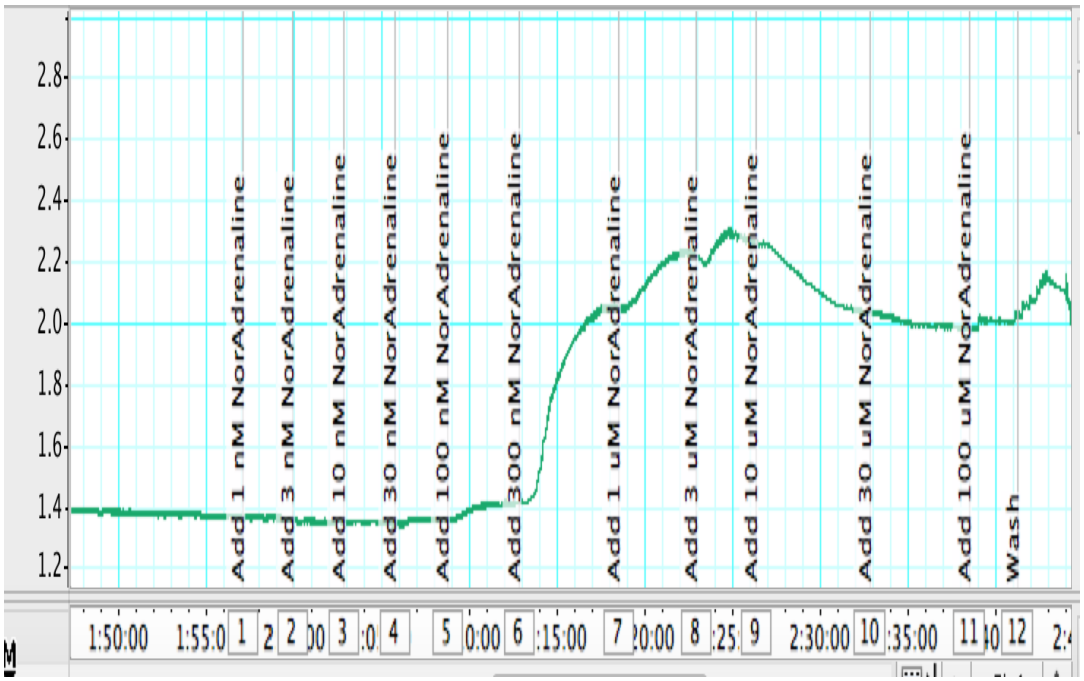

Figure 15: Experimental trace showing effect of Noradrenaline on isolated human pulmonary artery ring.

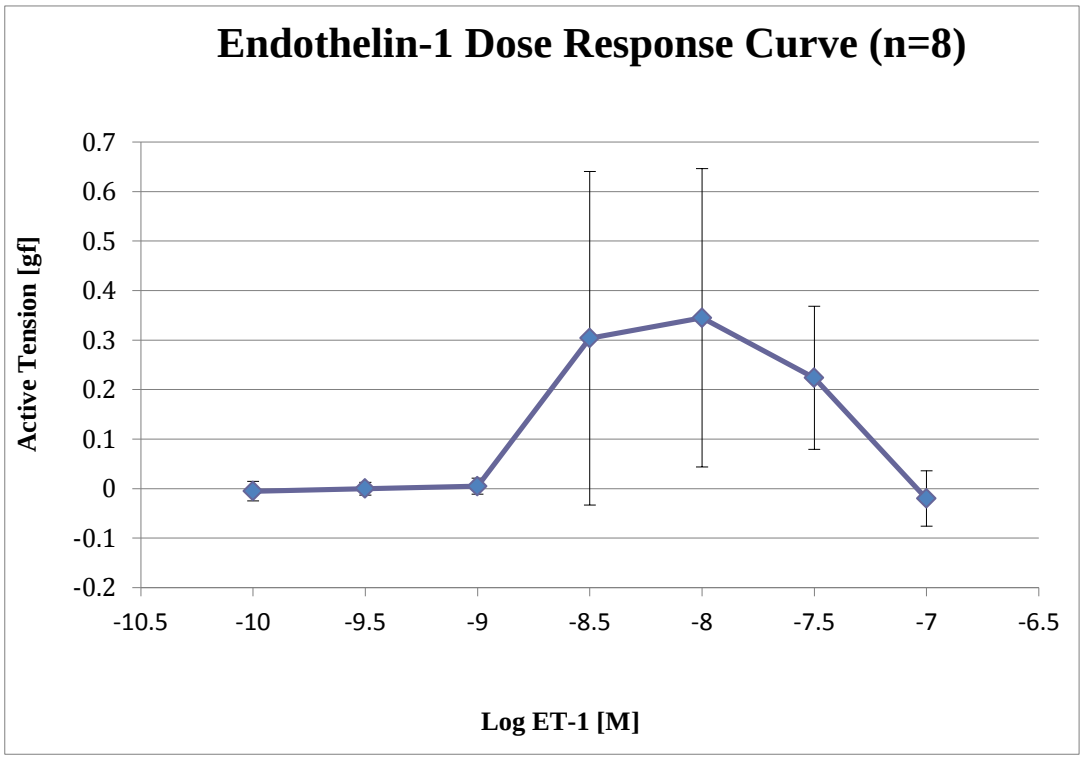

Figure 16: Concentration response curve to endothelin 1 (ET-1). Eight pulmonary artery rings from 2 different patients were prepared to test the effect of ET-1.

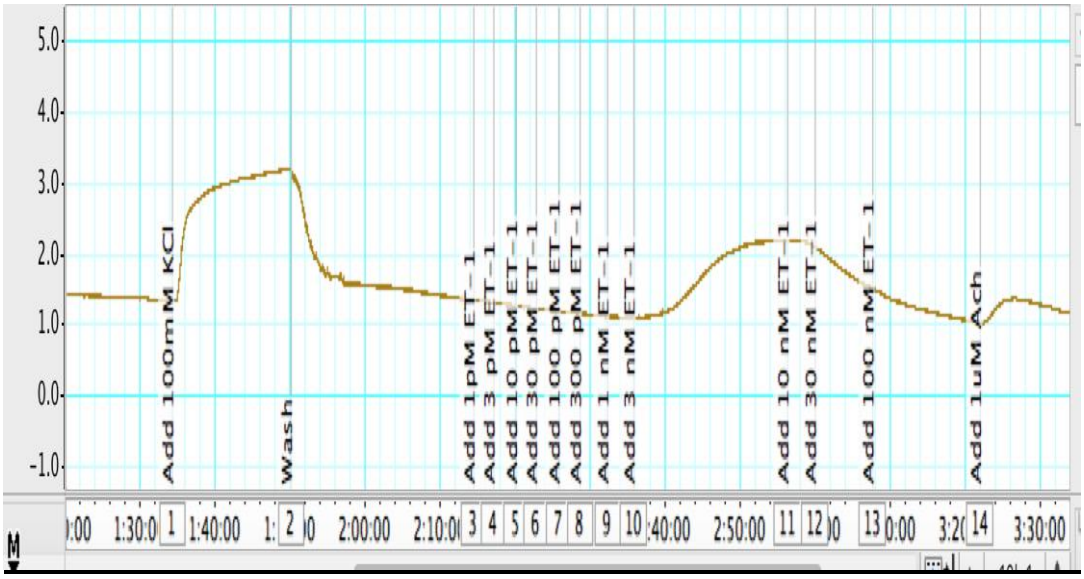

Figure 17: Experimental trace showing effect of Endothelin-1 on isolated human pulmonary artery ring.

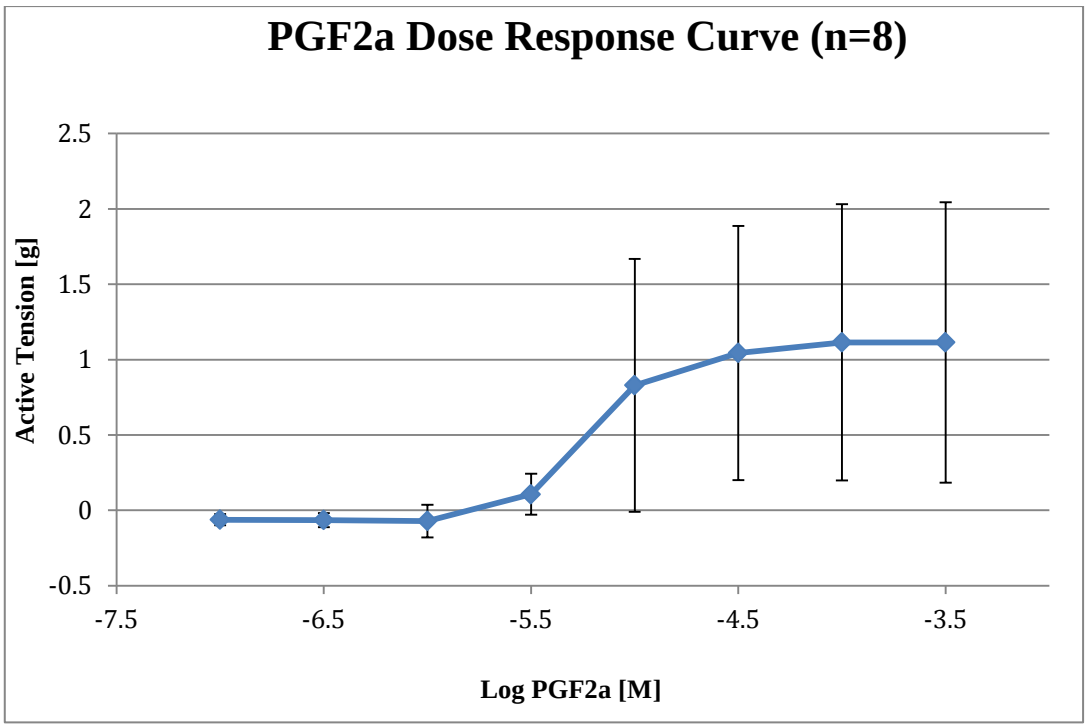

Figure 18: Concentration response curve to prostaglandin F2α (PGF2α). Concentrations of PGF2α from 100 nM to 300 mM (1E - 7 M–1E - 3.5 M) were used on 8 pulmonary artery rings from 3 different patients to demonstrate its vasoconstriction effects.

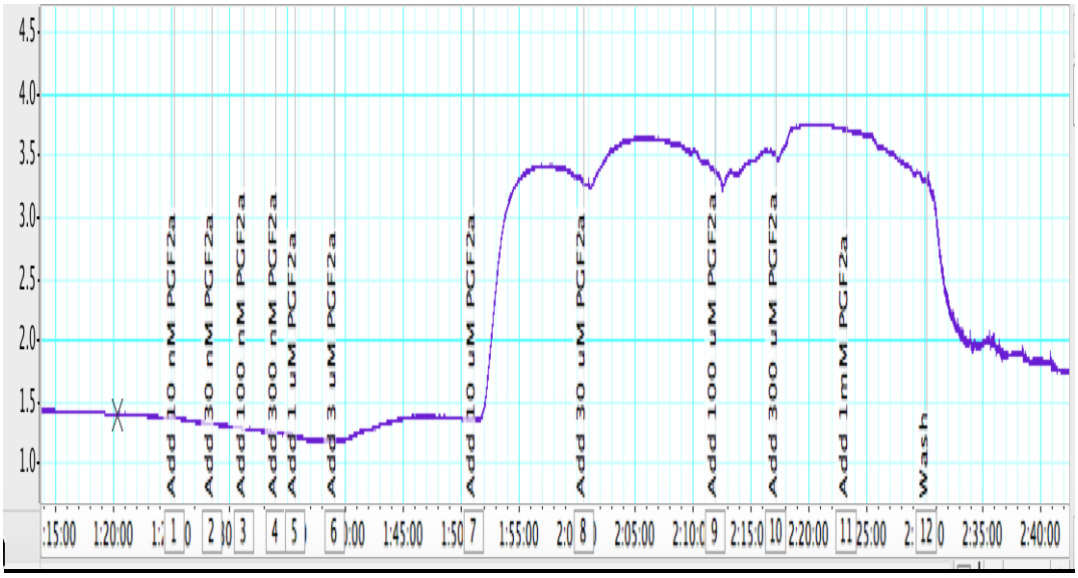

Figure 19: Experimental trace showing effect of Prostaglandin F2α on isolated human pulmonary artery ring.

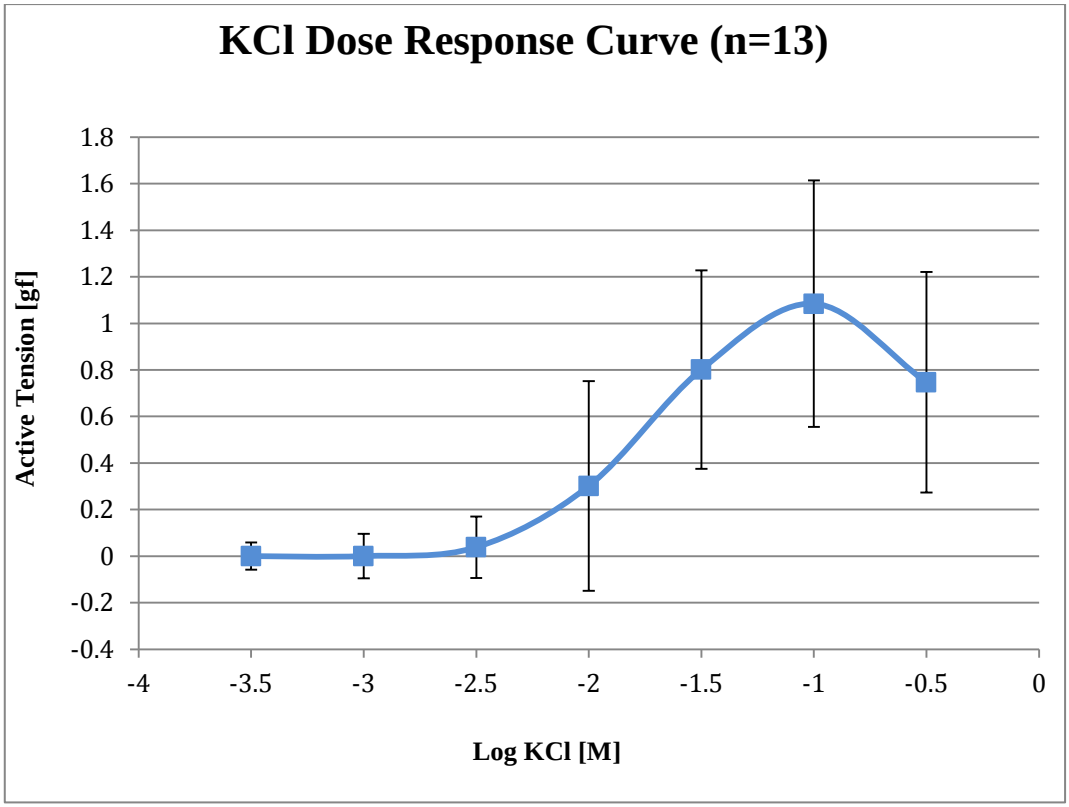

Figure 20: Concentration response curve to KCl. A total of 13 pulmonary artery rings from 4 different patients were studied in this series.

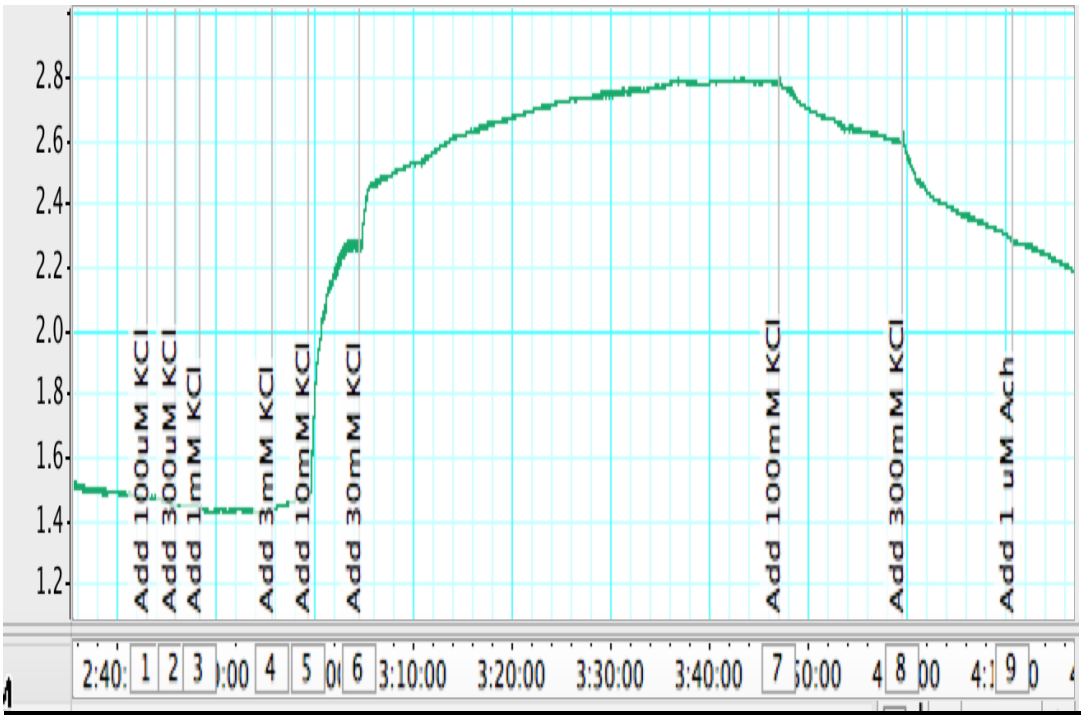

Figure 21: Experimental trace showing effect of KCl on isolated human pulmonary artery ring.

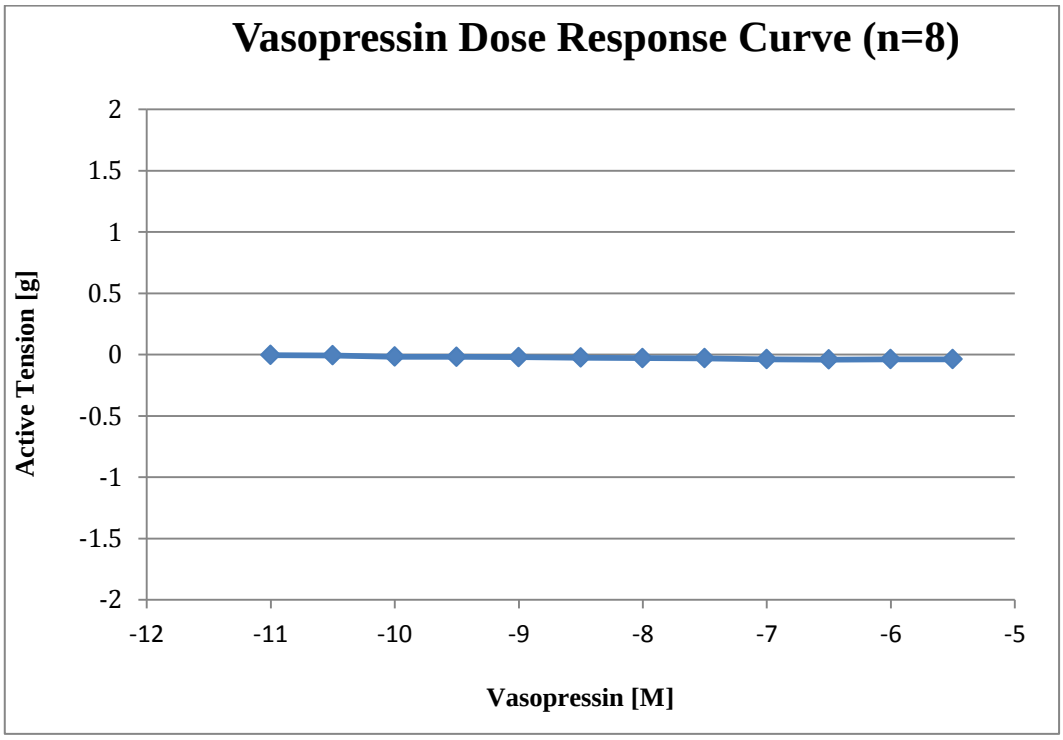

Figure 22: Concentration response curve to arginine vasopressin (AVP). Two different patient samples were used to prepare 8 pulmonary rings for AVP experiments.

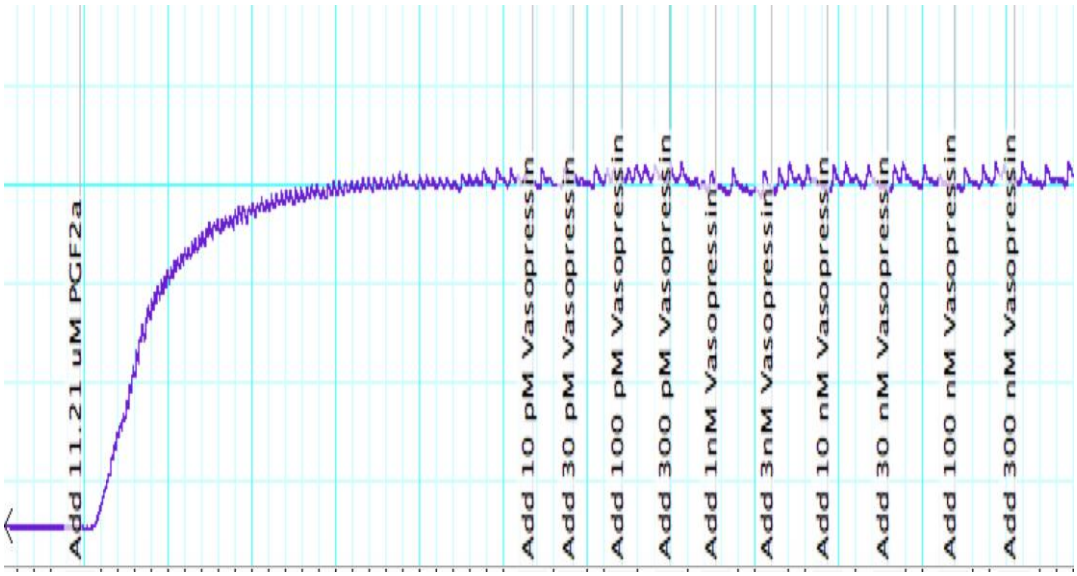

Figure 23: Experimental trace showing the effect of Arginine Vasopressin on resting tension in response to prostaglandin F2α.

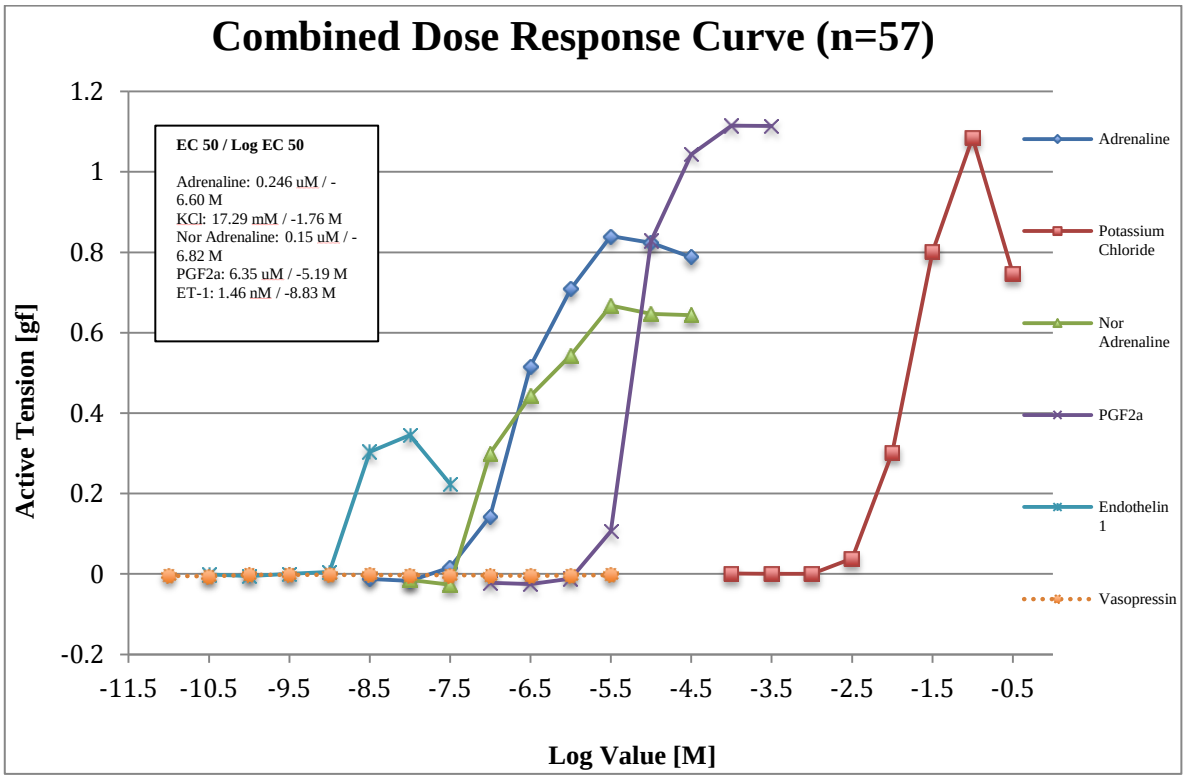

Figure 24: Cumulative concentration response curve to Adrenaline (AD) (n = 8), Noradrenaline (NA) (n = 12), Endothelin 1 (ET-1) (n = 8), Prostaglandin F2α (PGF2α) (n = 8), KCl (n = 13) and Vasopressin (n = 8). The findings show that PGF2α and KCl equally caused maximal constriction, whereas ET-1 had less effect and Vasopressin had no effect.

Table 1: Summary of vascular reactivity of pulmonary artery to KCl, Noradrenaline, Adrenaline, Vasopressin, Endothelin-1and Prostaglandin F2α to determine their effect on human pulmonary artery reactivity.

	Adrenaline	Noradrenaline	Endothelin 1	Prostaglandin F2a	KCl	Vasopressin
Number of pulmonary artery rings	08	12	08	08	13	08
Number of patients	04	04	02	03	04	02
Minimum concentration	3 nM	3 nM	100 pM	100 nM	300 µM	10 pM
Max concentration	30 µM	30 µM	30 nM	300 µM	300 mM	3 µM
Minimum log concentration	1E-8.5M	1E-8.5M	1E-10M	1E-7M	1E-3.5M	1E-11M
Diameter, mm	3.31 ± 0.96	2.72 ± 0.71	3.06 ± 1.01	2.5 ± 0.65	2.83 ± 0.34	2.87 ± 0.64
E_{max}, gf	0.84	0.66	0.34	1.11	1.08	0.004
E_{max}, gf/mm	0.08	0.077	0.035	0.141	0.121	0.0004
E_{max}, % KCl	78	61	31	102	100	0.18
pEC50	-6.608	-6.82	-8.83	-5.19	-1.76	
EC20 nM	97.6 nM	40.09 nM	0.95 nM	3.6 µM	7.98 mM	
EC50	246 nM	150 nM	1.46 nM	6.35 µM	17.29 mM	
EC80	622 nm	563.33 nM	2.26 nM	11.21 µM	37.46 mM	

Diameter: internal diameter of pulmonary artery rings; E_{max} (gf): maximum contractile response measured in gf; E_{max} (g/mm): maximum average response to the vasopressor agent as g force normalized per mm of averaged internal circumference; gf: gram force; pEC50: the negative logarithm of the molar concentration required to elicit 50% maximum response; EC50: molar concentration of the drug that gives half the maximal response.

4.4 Discussion:

This study is the first to demonstrate the differential in vitro effects of clinically used vasopressors and endogenous vasopressors on small human pulmonary vessels.

AD, NA and AVP are frequently used as systemic vasopressors in patients having cardiac surgery. ET-1 and PGF2α are endogenous vasoconstrictors that are thought to play an important role in maintaining and regulating endogenous vascular tone.

AD and NA are catecholamine sympathomimetic agents that act as agonists of the sympathetic nervous system via adrenoceptors. Adrenoceptors are 7 trans-membrane spanning, G-protein-coupled cell membrane receptors that are divided into 2 main groups, α and β, that are further subdivided into α1, α2, β1 β2 and β3 (181). NA acts predominantly on vascular smooth muscle α1 adrenoceptors, whereas AD acts on both α and β adrenergic receptors. Vascular smooth muscle has 2 types of alpha-adrenoceptors: alpha1 (α 1) and alpha2 (α 2), and both adrenoceptors mediate vasoconstriction and maintain basal vascular tone (182).

The α 1 adrenoceptors are located predominantly on vascular smooth muscle and cause vasoconstriction via the Gq-inositol triphosphate signal transduction pathway by increasing intracellular calcium, whereas α 2-adrenoceptors are found on the smooth muscle cells and are linked to Gi or Go proteins, which on activation increase the $Ca2^{+}$ through voltage gated $Ca2^{+}$ channels (183). This study illustrates that the constrictor potency of AD (pEC_{50} 6.6) is comparable to that of NA (pEC_{50} 6.8) and is likely due to their similar affinities for α 1 and α 2

adrenoceptors, as demonstrated by van Brummelen et al. (184). The distribution of human α1 adrenergic receptors varies with the vascular bed, and the PA α1 adrenoceptor density is reported to be 24 ± 5.2 fmol/mg total protein (185). Our study demonstrates that the pEC_{50} of NA was 6.82, and the reported pEC_{50} values in human coronary, internal mammary, inferior epigastric and gastroepiploic arteries ranged from 5.9 to 6.6 (186).

ET-1 is a 21-amino acid peptide, mainly secreted by vascular endothelial cells, lung fibroblasts and PA smooth muscle cells, that cause vascular smooth muscle contraction via stimulation of its receptors (187). ET-1 acts through ET_A receptors found in smooth muscle cells and cardiac myocytes and ET_B receptors found in smooth muscle and endothelial cells (115). Activation of smooth muscle cell endothelin receptors triggers phospholipase C, which causes vasoconstriction by increasing the levels of inositol triphosphate, diacylglycerol and intracellular calcium. Activation of endothelial ET_B receptors results in the release of Nitric Oxide (NO) and prostacyclin and mediates endothelial dependent vasodilation (188). In human lungs, ET_A receptors are present predominantly in the large pulmonary arteries, whereas ET_B receptors are present in airway smooth muscles, capillaries and the alveolar wall. The intravenous administration of ET-1 results in a biphasic response in humans with initial vasodilation followed by sustained vasoconstriction (189). The initial vasodilator response is due to NO release and potassium channel activation (189). Pulmonary vasoconstriction results from the activation of both ET_A and ET_B receptors, because blockade of both receptors is necessary to accomplish the maximum inhibition of ET-1-induced vasoconstriction (190). An ET-1 receptor antagonist, e.g. Bosentan (dual ET_A

and ET_B receptor antagonist) proved to be an effective treatment in pulmonary hypertension (149). Our study shows that ET-1 is more potent than other vasoconstrictors (the pEC_{50} for ET-1, NA, AD, PGF2α and KCl was 8.83, 6.82, 6.6, 5.19 and 1.76, respectively), and our calculated pEC_{50} of ET-1 (8.83) for human PA is comparable to that reported for human coronary (8.21) and mammary arteries (8.55) (191).

AVP is a neurohypophysial hormone that acts both as a vasopressor and antidiuretic hormone. It is released in response to decreased arterial pressure and increased plasma osmolality and mediates vasoconstriction, reabsorption of water and central nervous system effects through its V_1, V_2 and V_3 receptors (113). The activation of vasopressin V_1 receptors on vascular smooth muscles results in vasoconstriction via the activation of the Gq protein of phospholipase C and in an increase in intracellular $Ca2^+$ (192). V_2 receptors are found on renal distal tubules and collecting duct, and their stimulation results in reduced diuresis via activation of adenylate cyclase and an increase in cAMP (193). V_3 receptors are located in the pituitary gland and are involved in the release of adrenocorticotropic hormone via phospholipase C. Both in vivo and in vitro studies on animals show that vasopressin causes PA vasodilation (194). The authors also conclude that vasopressin mediates vasodilation through the release of NO because the vasodilatory response of Vasopressin was diminished when the NO synthesis inhibitor L-NAME was inserted (194, 195).

The intravenous administration of Vasopressin in normotensive and hypertensive patients causes no effect on PA pressure, as showed by Nelson et al. (196). On the other hand, an increase in PA pressure was found when intravenous

vasopressin was given to patients with advanced tuberculosis and liver cirrhosis (197, 198). Our study found that Vasopressin had no vasodilatory or vasoconstrictor effect on the PA. This result might be due to lack of V_1 receptors in the human PA or a lack of peptides involved in NO-vasopressin vasodilation. Therefore, AV may be safe to use for systemic vasoconstriction in patients with pulmonary hypertension (199).

PGF2α is an endogenous vasoconstrictor that causes vasoconstriction via activation of the prostaglandin F receptor, which is a G-protein-coupled receptor located on the pulmonary vascular smooth muscle. KCl grounds the depolarization of smooth muscles that leads to vasoconstriction via calcium entry through voltage gated calcium channels (200). This study shows that PGF2α (Emax 1.1 gf) and KCl (Emax 1.08 gf) cause maximal constriction equally.

Other commonly used perioperative vasoconstrictors, e.g. Phenylephrine and Metaraminol, were not investigated. These agents are predominantly α1-adrenoceptor agonists; it is agreed that their use as a systemic vasopressor is contraindicated in patients with pulmonary hypertension.

Having examined the differential effect of commonly used vasoconstrictors used in routine clinical practice on the human pulmonary arteries, the next phase of experiments will concentrate on the other group of vasoactive drugs involved in the management of established pulmonary hypertension. The main purpose of these studies is not only to identify the contributing factors to pulmonary hypertension but also to find out the best therapeutic agents.

Chapter 5

In Vitro Characterisation Of Pharmacological Effect Of Prostacyclin Analogues In Comparison To Phosphodiesterase Inhibitors On Small Human Pulmonary Vessels

5.1 Introduction:

Pulmonary hypertension (PH) is a life-threating disease, which if left untreated can lead to death from right heart failure. The median survival in untreated patients is 2 years from the time of diagnosis (201). Pathophysiology of pulmonary hypertension involves vasoconstriction that leads to intimal fibrosis, smooth muscle cells proliferation and medial hypertrophy. An imbalance in endogenously release vasodilators (NO and prostacyclin) and vasoconstrictors factors (Endothelin -1) induces vascular remodelling and therefore PH is more of a vasoproliferative rather than a vasoconstrictive disease (202). Treatment of PH depends on the aetiology of the disease and degree of functional impairment and the main aim is to improve patient's symptoms, quality of life and survival. Several therapeutics agents are currently in use for management of PH including prostanoids (Epoprostenol, Treprostinil, Iloprost), phosphodiesterase inhibitors (Sildenafil, Milrinone) and NO. Despite recent advances none of the current treatment regime is prosperous in repealing the remodelling of pulmonary vasculature and substantial reduction in mortality. This lead to recognition that still we are far behind to identify the novel therapeutic agent to treat PH (203).

Vasodilators drugs are commonly used peri-operatively to treat high pulmonary arterial blood pressure and to lower down the pulmonary vascular resistance. Sildenafil, Milrinone, Nitric Oxide and prostacyclin analogues are frequently used pulmonary vasodilators in cardiac surgery patients. Both Sildenafil and Milrinone are phosphodiesterase enzyme inhibitors (204) while NO is a guanylate cyclase stimulator (205). Sildenafil and NO mediate vasodilation by increasing the cGMP levels while Milrinone mediates cAMP interceded

vasodilation.

Prostacyclin (PGI_2) is potent endogenous vasodilators that synthesized from arachidonic acid and acts via G protein coupled receptors (IP) on endothelial cells and platelets (206). Activation of prostacyclin receptor produces cAMP via adenylyl cyclase, which in return inhibits platelet activation and reduces cytosolic calcium levels. Epoprostenol, Iloprost and Treprostinil are clinically proven prostacyclin analogues that are commonly used to treat PH. They exert their beneficial effect by reducing the platelet aggregation, pulmonary vascular resistance and ventricular afterload and also enhance cardiac output (207). Epoprostenol has a short half-life of 6 minutes due to rapid enzymatic hydrolyzation in blood, that necessitates continuous intravenous delivery ideally via placement of a central catheter (208). Iloprost requires frequent inhalations, 6-9 times per day due to half-life of only 20-30 minutes. Treprostinil is relatively stable at room temperature with a half-life of 4hr and is available as subcutaneous, intravenous and inhalation form (209).

The effects of commonly used vasodilators agents on pulmonary vascular tone have been extensively investigated in animal models. However, limited data is available about the effects of vasodilators on human pulmonary vascular tone. In this study human PA rings were used to demonstrate the differential in-vitro effects of clinically used prostacyclin analogues, phosphodiesterase inhibitors and SNP on small human pulmonary vessels. We also looked into the synergistic effect of Milrinone and Iloprost.

5.2 Methods And Materials:

Ethical approval from local ethics committee and local research and development department approval was obtained for the use of human tissue for this study. Patients undergoing lung resection gave written consent for the use of surplus tissue for research purposes. Patients under the age of 18 and who cannot give informed consent were excluded from the study.

5.2.1 Isolation and mounting of PA rings:

Human pulmonary arteries were obtained from human lungs or lobes immediately following resection for cancer and transferred to the laboratory in oxygenated (5% CO_2, 21% O_2) Krebs-Henseleit solution (containing: (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.4 and glucose 11). Pulmonary arteries were dissected from disease free areas of lung resection and after careful removal of adipose and connective tissues cut into 2mm long rings. A multiwire myograph system (DMT 620M) was used for mounting of PA rings and measurement of isometric tension. The myograph system was connected to a PC via an amplifier (Power Lab 8/35, AD Instruments) for continuous measurement of isometric tension using data acquisition software (Lab Chart Pro Version 8.0).

5.2.2 Determination of agonist induced relaxation:

After mounting of PA rings a resting tension of 1.6 gf was applied (optimum resting tension determined in preliminary experiments) and the vessels left to equilibrate with 21% O_2, 5% CO_2 at 37^{o}C for 60 minutes. PA rings were than pre

constricted with 11.21 μM PGF2α (EC_{80}), calculated earlier from previous experiments. When a stable resting tension was achieved cumulative concentration response curves were constructed to Sildenafil, Milrinone, Sodium Nitroprusside, Epoprostenol, Iloprost and Treprostinil by stepwise increases in agonist concentration in the myograph chamber when a plateau response had been obtained to the preceding concentration. Active tension was calculated in gram force (gf) as maximum tension at plateau (gf) – resting tension (gf).

The maximum efficacy (E_{max}) for each agent was determined in gf and also expressed as gf/mm internal diameter of each vessel (to take into account the variability in PA ring diameter). At the end of each experiment the integrity of the endothelium was confirmed by the addition of 1μM Acetylcholine. Potassium chloride (KCl) was used to check the contractility of PA rings and rings that did not contract to KCl were excluded from the study.

Agonist potency was compared by determining the agonist EC_{50} concentration (the concentration of required to elicit 50% of maximum response) which was presented as pEC_{50} (the negative logarithm of the molar EC_{50} concentration).

5.3 Results:

A total of 64 human pulmonary artery rings (mean internal diameter 2-4 mm) were obtained from 18 patients. 12 rings that did not contract to KCl were excluded from the study. The effects of vasodilator agents on pulmonary artery tone are summarised in table 2 and figure 37. Using small human pulmonary arteries, concentration response curve were constructed to agonist and study confirmed that prostacyclin analogues produce significantly reduction in

pulmonary artery tone induced by PGF2α.

The Sildenafil, Milrinone, SNP, Epoprostenol, Iloprost and Treprostinil caused dose-dependent vasodilation in the small human pulmonary arteries (pEC_{50}: 5.97 ± 0.22, 5.99 ± 0.12, 7.64 ± 0.08, 7.53 ± 0.14, 8.84 ± 0.15 and 9.48 ± 0.13 respectively, n = 8 to 12). The order of efficacy was Tp = Ip > Ep > Mil > SNP > Sd and the order of potency was Tp > Ip > SNP > Ep > Mil > Sd [Figure 25-36].

Repeated results at different doses from each vasodilator agent when compared with one-way analysis of variance (ANOVA) and Bonferroni test using SPPS Statistics shows that there were statistically differences in the mean % vasodilation made over time between vasodilators group ($p < 0.05$). The mean % vasodilation for each vasodilator in ascending order was; Sildenafil: 11.815 (95% CI 14.12 - 26.75), Milrinone: 15.28 (95% CI 8.96 - 21.59), Epoprostenol: 20.43 (95% CI 14.12 – 26.75), SNP: 24.85 (95% CI 18.54 – 31.16), Iloprost: 30.24 (95% CI 23.93 – 36.56) and Treprostinil: 41.74 (95% CI 35.42 - 48.05). Within subject analysis of all the vasodilator agents do display an increase in mean % vasodilation over time ($p<0.05$).

Emax(gf) for Sildenafil, Milrinone, SNP, Epoprostenol, Iloprost and Treprostinil was -1.078 gf, -1.749 gf, -1.533 gf, -1.82 gf, -2.001 gf and -2.03 gf respectively. Another useful indicator to take account of the variation in PA rings internal diameter is to compare the total active force of 2mm long artery segment normalised for internal diameter [(g/ πD), g= Emax (gf), π= mathematical constant, the ratio of a circle's circumference to its diameter, commonly approximated as 3.14159 and D = average internal diameter of vessels]. By using this index the value [Emax (gf/mm)] for Sildenafil, Sodium Nitroprusside,

Epoprostenol, Treprostenil and Iloprost was -0.11 gf/mm, -0.23 gf/mm, -0.17 gf/mm, -0.16 gf/mm, -0.24 gf/mm, and -0.17 gf/mm respectively.

5.3.1 Effect of sildenafil on active tension in response to prostaglandin F2*α*:

All vessels vasodilate in response to Sildenafil. Increasing concentrations of Sd from 100 pM to 100 µM were used on 12 PA rings. The EC_{20}, EC_{50} and EC_{80} were 37.32 nM, 1.06 µM and 30.33 µM, respectively. The hill slope was -0.414 ± 0.089.

5.3.2 Effect of milrinone on active tension in response to prostaglandin F2*α*:

To evaluate the effect of Milrinone on pulmonary vessels, 8 PA rings and concentrations of Milrinone from 3 nM – 100 µM were used. As the concentration rose above 10 nM, vessels started relaxing and the maximum response was seen at 30 µM The EC_{20}, EC_{50} and EC_{80} were 238 nM, 1.01µM and 4.3µM respectively. The hill slope was -0.959 ± 0.234.

5.3.3 Effect of sodium nitroprusside on active tension in response to prostaglandin F2*α*:

PGF2α at a concentration of 100 nM–300 mM was used on 8 PA rings to demonstrate its vasoconstriction effect. The EC_{20}, EC_{50} and EC_{80} were 6.1 nM, 22.6 nM and 83.95 nM, respectively. The hill slope was -1.058 ± 0.191.

5.3.4 Effect of epoprostenol on active tension in response to prostaglandin F2*α*:

A total of 08 PA rings from 03 patients were studied in this series. Increasing

concentrations of Epoprostenol from 100 pM to 3 µM were used. Vessels started to dilate as the concentration increased above 300 pM (log -9.5 M); the maximal relaxation was seen at 1 µM; after that, the response to Ep diminished. The EC_{20}, EC_{50} and EC_{80} were 10.25 nM, 29.4 nM and 84.56 nM, respectively. The hill slope was -1.314 ± 0.492.

5.3.5 Effect of iloprost on active tension in response to prostaglandin F2*α*:

To demonstrate the effect of Iloprost on 08 PA rings, a concentration of Ip of 10 pM–100 nM was used. All vessels were relaxed to Ip with maximal relation recorded at 30 nM. The EC_{20}, EC_{50} and EC_{80} were 381.91 pM, 1.43 nM and 5.395 nM, respectively. The hill slope was -1.047 ± 0.352.

5.3.6 Effect of treprostinil on active tension in response to prostaglandin F2*α*:

Treprostinil at a concentration of 1 pM–300 nM was used on 8 PA rings to demonstrate its vasodilator effect. The EC_{20}, EC_{50} and EC_{80} were 47.457 pM, 328 pM and 2.268 nM, respectively. The hill slope was -0.717 ± 0.142.

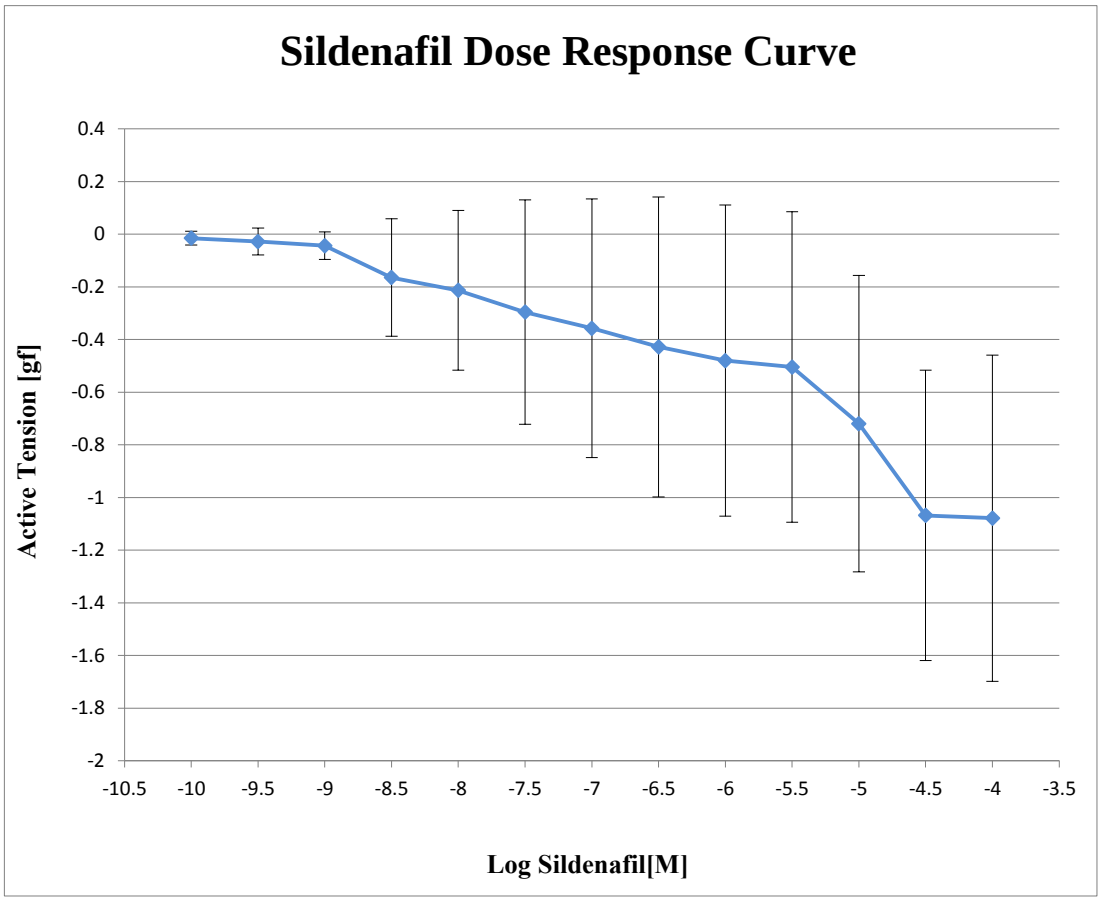

Figure 25: Concentration response curve to Sildenafil. Total 12 PA rings from four patients were used to perform the experiment of sildenafil effect on pulmonary artery. Concentration from 100pM – 100µM (1E-10M – 1E-4M) were used for these experiments. The EC_{50} was 1.06 µM.

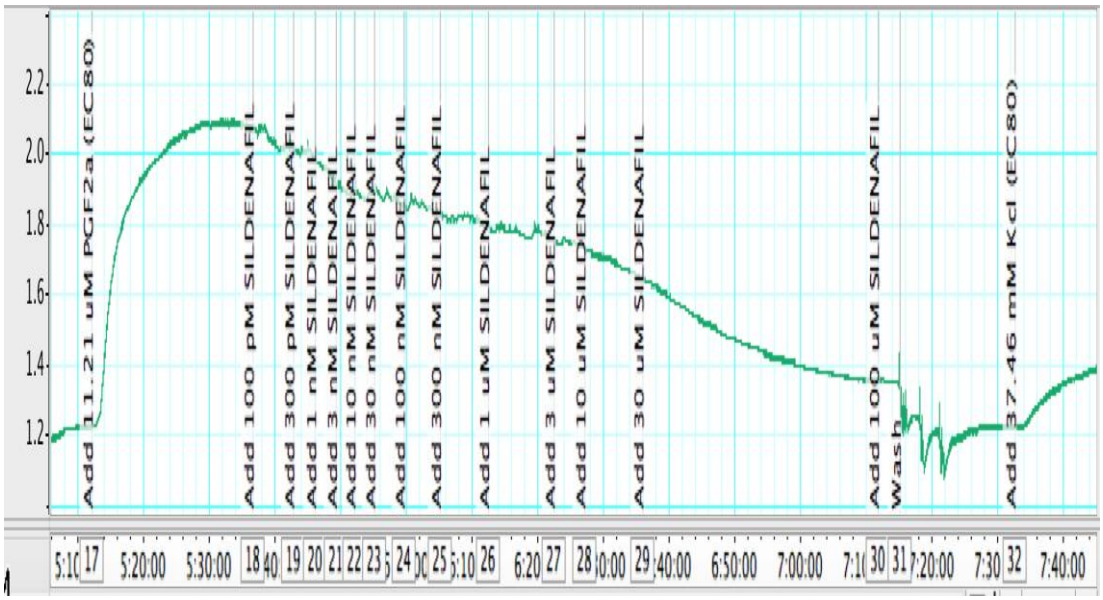

Figure 26: Experimental trace showing the effect of Sildenafil on resting tension in response to Prostaglandin F2α.

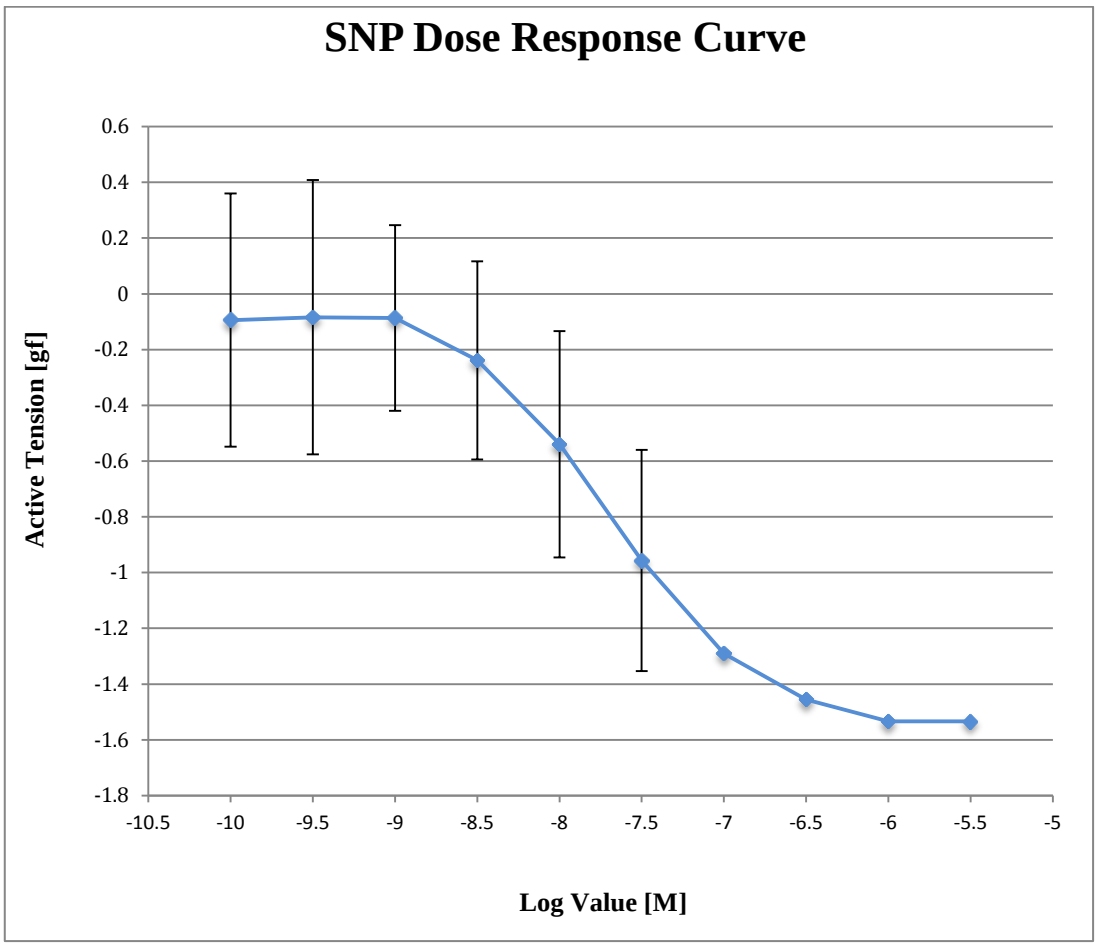

Figure 27: Concentration Response Curve to SNP. All vessels vasodilate in response to SNP. Increasing concentration of SNP from 100pM – 3µM were used on 8 PA rings from 2 different patients. The EC_{50} was 22.6nM.

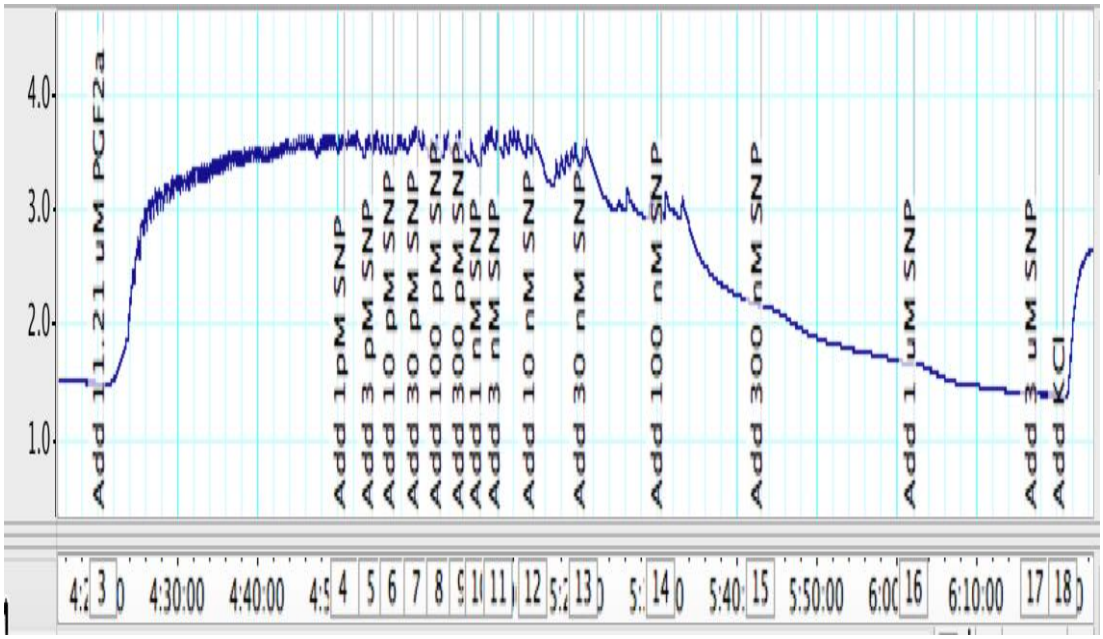

Figure 28: Experimental trace showing the effect of SNP on resting tension in response to Prostaglandin F2α.

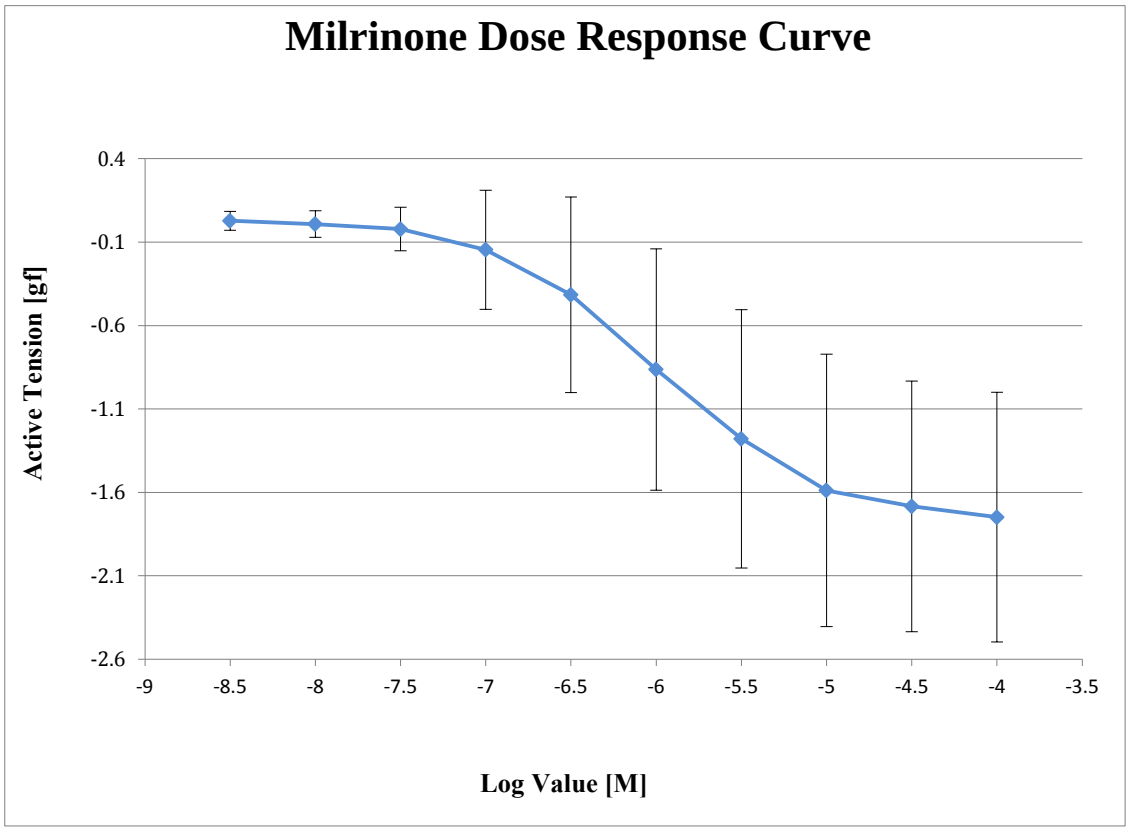

Figure 29: Concentration response curve to Milrinone. Total 08 PA rings from two patients were used to perform the experiment of Milrinone effect on pulmonary artery. Concentration from 10nM – 100µM (1E-8M – 1E-4M) were used for these experiments. The EC_{50} was 1.01 µM.

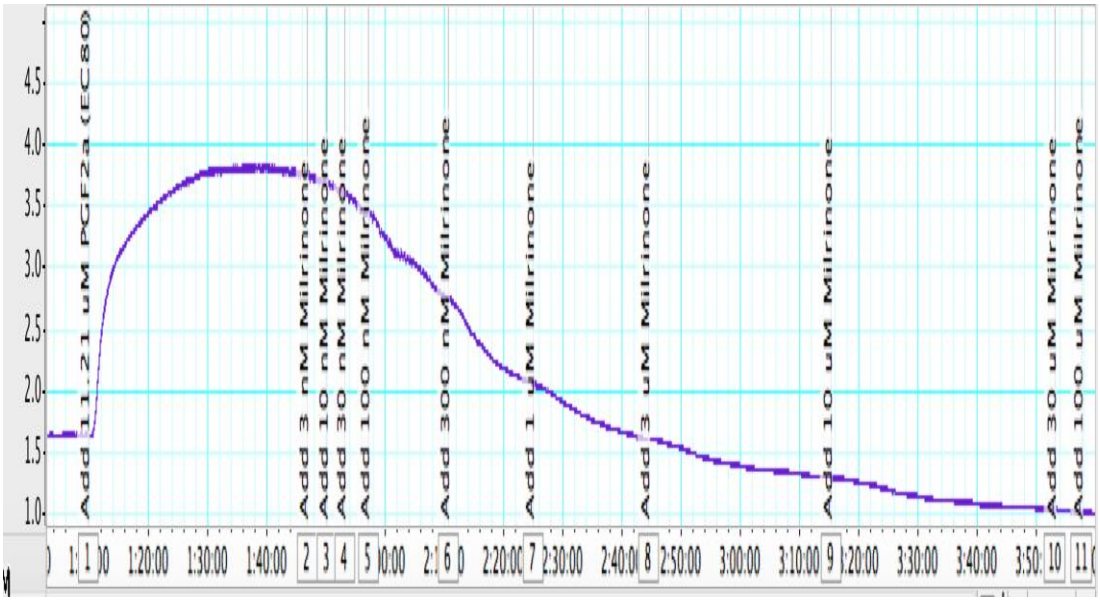

Figure 30: Experimental trace showing the effect of Milrinone on resting tension in response to Prostaglandin F2α.

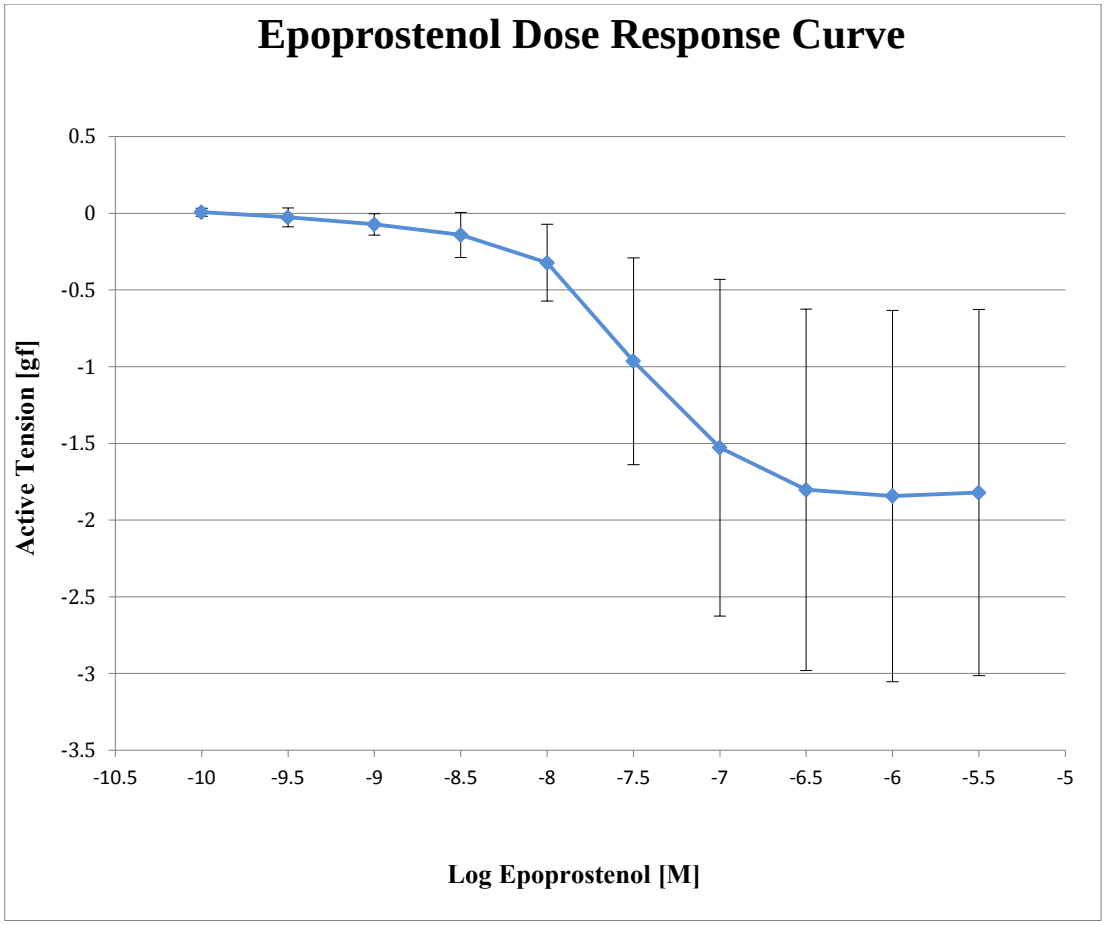

Figure 31: Concentration response curve to Epoprostenol. A total of 8 pulmonary artery rings from 3 patients were used to determine the effect of Epoprostenol on the pulmonary artery. Concentrations from 100 pM to 3 µM were used for these experiments. All vessels vasodilated in response to Ep. the maximal response was at 1µM (log -6.0 M). The EC_{50} was 29.4 nM.

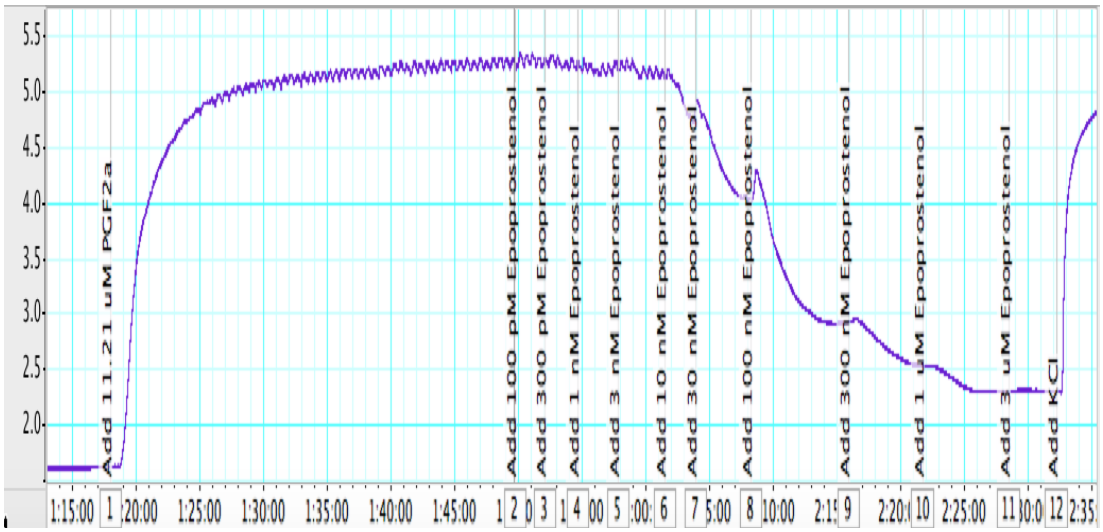

Figure 32: Experimental trace showing the effect of Epoprostenol on resting tension in response to Prostaglandin F2α.

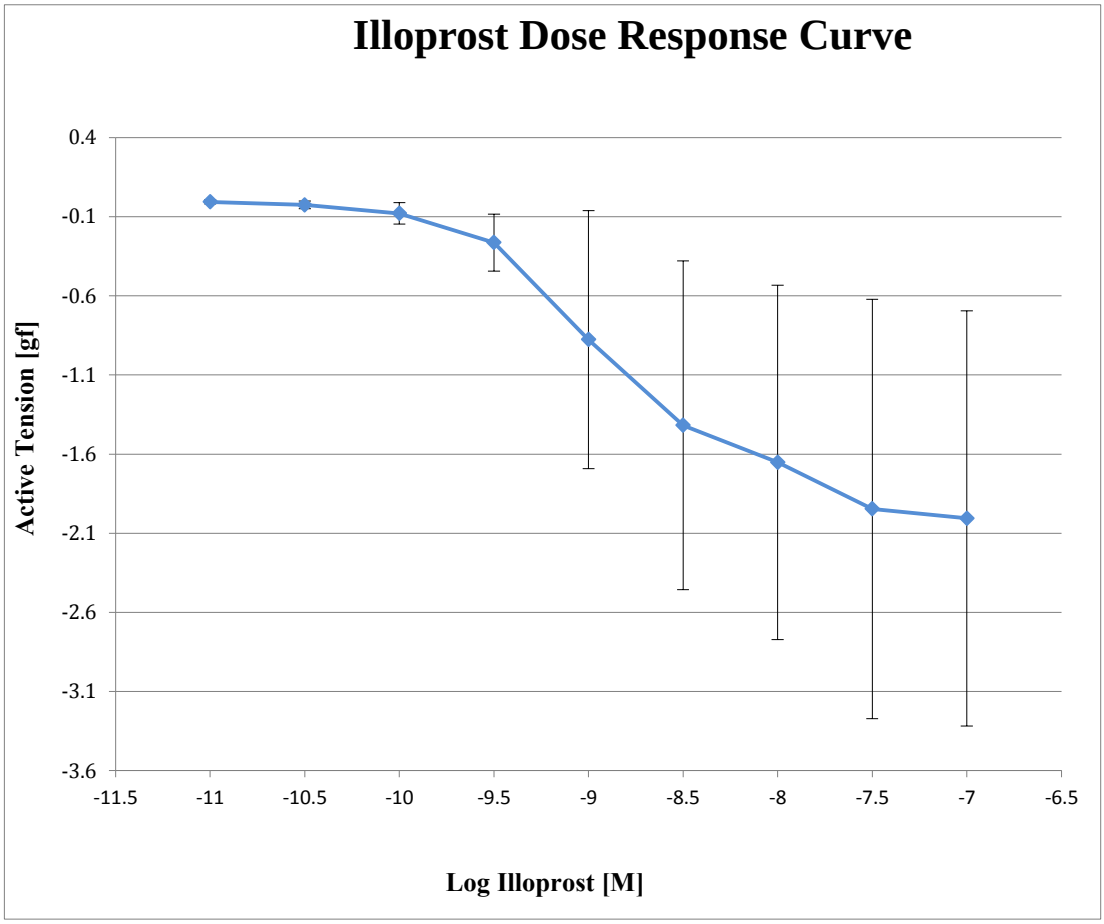

Figure 33: Concentration response curve to Iloprost (Ip). Eight pulmonary artery rings from 3 different patients were prepared to test the effect of Ip. Concentrations from 10pM to 100nM (1E-11 M–1E-7.0 M) were used. As the concentration increased above 30 pM, the vessels started relaxing; the EC_{50} was 1.43nM.

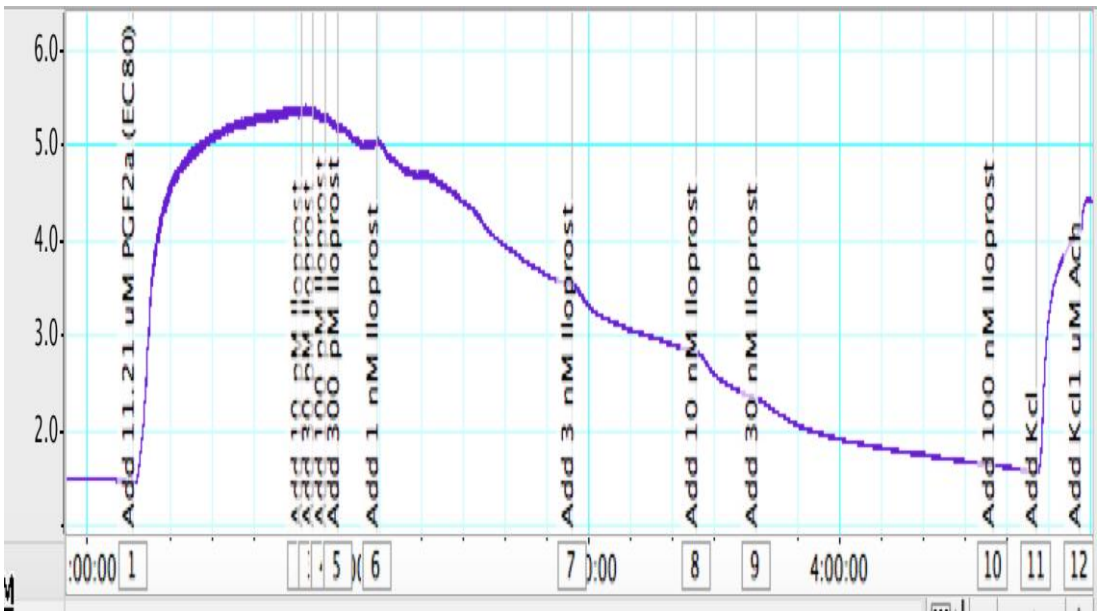

Figure 34: Experimental trace showing the effect of Iloprost on resting tension in response to Prostaglandin F2α.

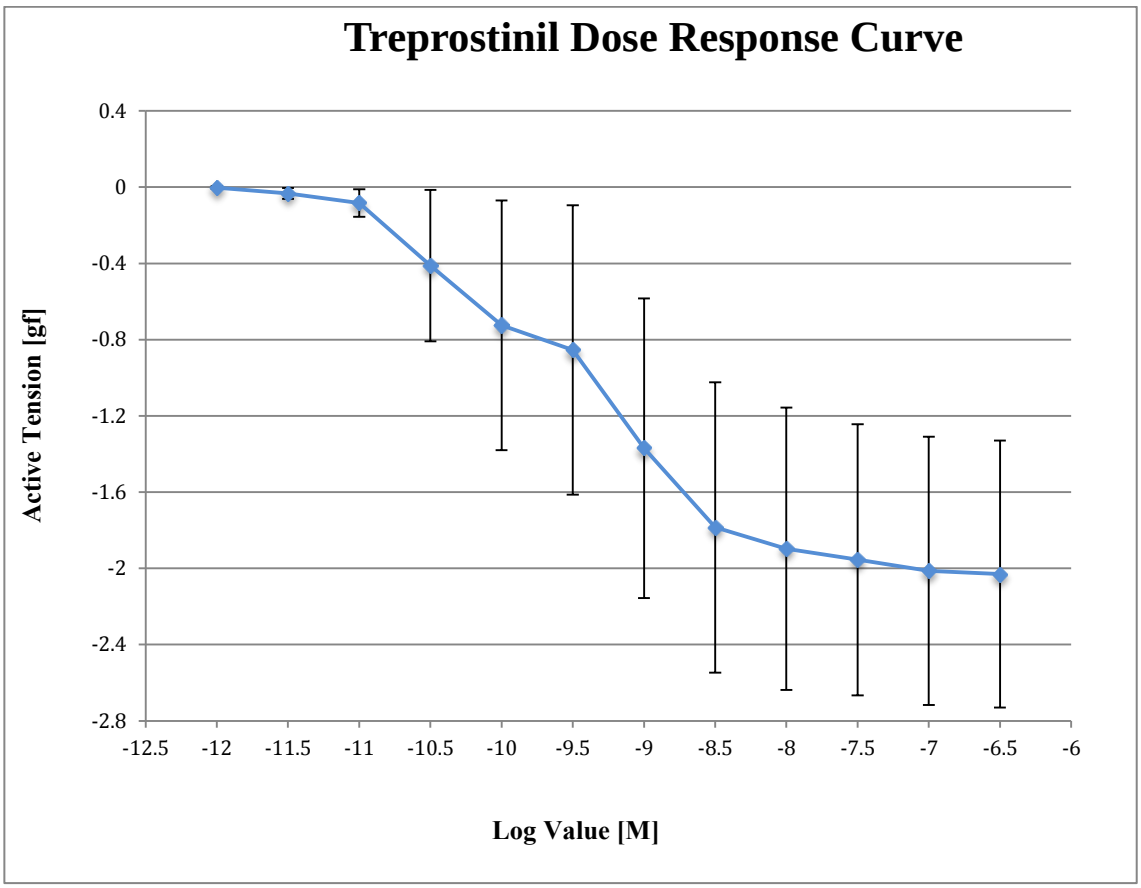

Figure 35: Concentration response curve to Treprostenil (Tp). A total of 08 pulmonary artery rings from 2 different patients were studied in this series. Increasing concentrations of Ep from 1 pM to 300nM (1E-12 M–1E-6.5 M) were used. Vessels started relaxing when the concentration was above 3pM and the EC_{50} was 328 pM.

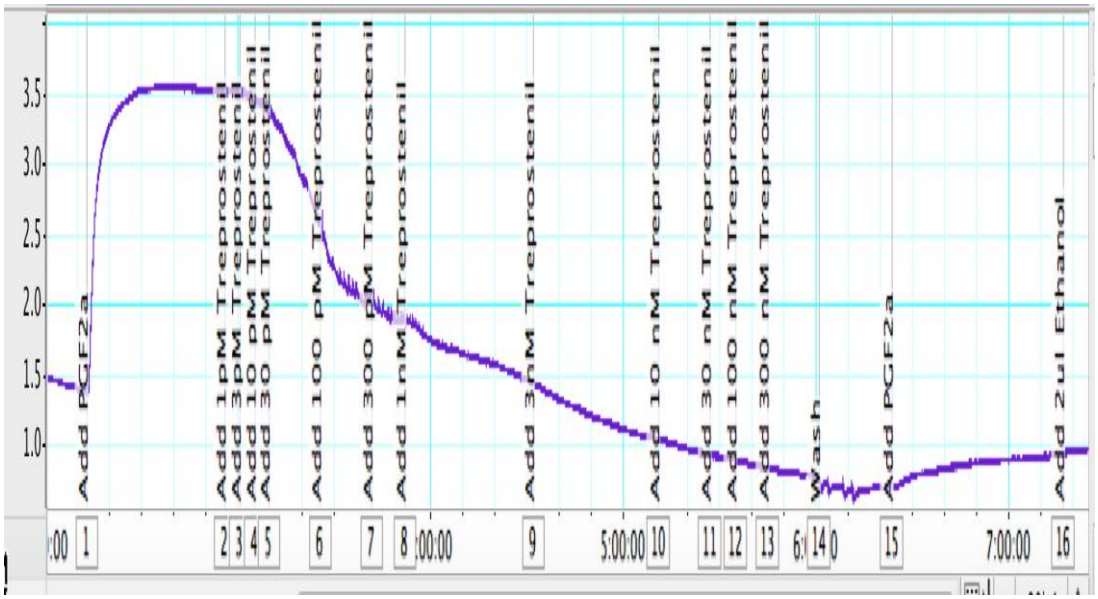

Figure 36: Experimental trace showing the effect of Treprostinil on resting tension in response to Prostaglandin F2α.

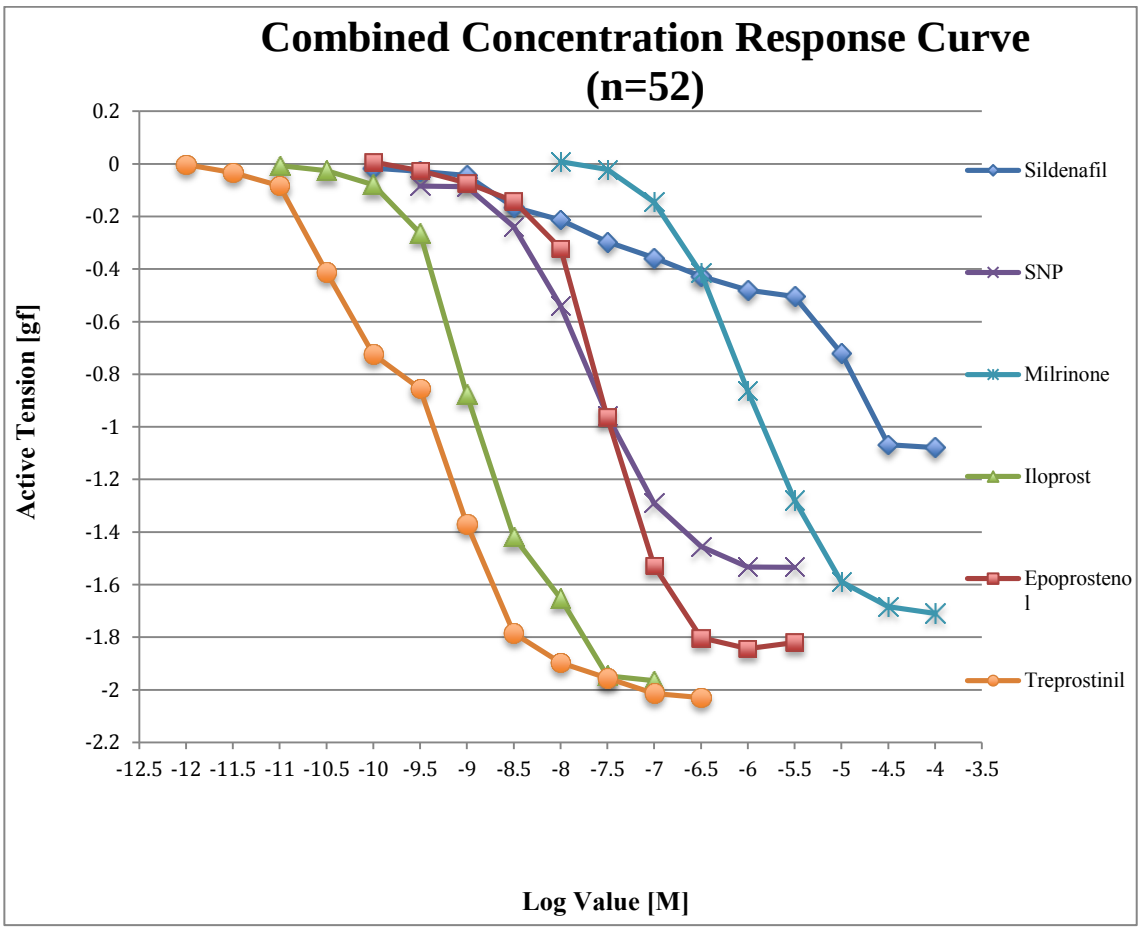

Figure 37: Cumulative concentration response curve to Sildenafil (n=12), Milrinone (n=8), SNP (n=8), Epoprostenol (n=8), Iloprost (n=8) and Treprostinil (n=8).

Table 2: Summary of number of PA rings (n) and concentration of Sildenafil, milrinone, SNP, Epoprostenol, Iloprost and Treprostinil used to determine their effect on human pulmonary artery reactivity.

	Sildenafil	Milrinone	SNP	Epoprostenol	Iloprost	Treprostinil
Number of PA rings	12	08	08	08	08	08
Number of patients	04	02	02	03	03	02
Min concentration	100pM	3nM	100pM	100pM	10pM	1pM
Max Concentration	100uM	100uM	3uM	3uM	100nM	300nM
Min log concentration	1E-10M	1E-8.5	1E-10M	1E-10M	1E-11M	1E-12M
Max log concentration	1E-4.0M	1E-4.0M	1E-5.5M	1E-5.5M	1E-7M	1E-6.5M
Diameter (mm)	2.79±0.39	2.43 ±0.67	2.93±0.62	3.5±0.53	2.62±0.44	3.75±0.26
Emax (gf)	-1.078	-1.749	-1.533	-1.82	-2.001	-2.03
Emax (gf/mm)	-0.11	-0.23	-0.17	-0.16	-0.24	-0.17
EC50	1.06uM	1.01uM	22.6nM	29.4nM	1.43nM	328pM

Emax (gf) =Maximum=maximum vasodilatory response measured in gf. Emax (g/mm) = maximum average response to the vasodilator agent as g force normalized per mm of averaged internal circumference. EC_{50} refers to molar concentration of the drug that gives half maximal response.

5.4 Discussion:

This study demonstrated the differential *in vitro* effects of clinically used vasodilators on small human pulmonary vessels. Sildenafil, Milrinone, Nitric oxide and prostacyclin analogues are frequently used as pulmonary vasodilators in cardiac surgery patients. Prostacyclin analogues provide better results in comparison to phosphodiesterase inhibitors and NO.

Nitric oxide synthase (NOS) produced NO by catalytic conversion of L-arginine to L-citrulline in the presence of oxygen (158). Nitric oxide either produced endogenously in pulmonary vascular endothelial cells or exogenously administered, stimulate synthesis of guanosine-3′, 5′-cyclic monophosphate (cGMP) by activating the soluble guanylate cyclase (GC) (159). cGMP activates different protein kinases and calcium gated K^+ channels and relax the vascular smooth muscle cells (160). Several different isoform of phosphodiesterase (PDE) especially PDE 1, 2, 3, 5, and 9 are present in lungs that can inactivate cGMP (161). Phosphodiesterase inhibitors have been proven to reduce pulmonary vascular resistance and now an approved treatment for pulmonary hypertension (162).

Sildenafil is a phosphodiesterase-5 (PDE-5) inhibitor that acts by reducing the cGMP breakdown, which results in increased sensitivity of pulmonary vascular smooth muscle cells to nitric oxide (NO) and subsequently pulmonary vasodilation (210). Milrinone is an inotropic and vasodilator agent that acts by inhibiting the phosphodiesterase-3 (PDE-3) enzyme. Inhibition of PDE-3 results in increase in cAMP level that enhances the relaxation of pulmonary vascular bed (211). Milrinone also acts as a positive inotropic agent and is proved to be

beneficial in right heart failure and PH (212). Sodium Nitroprusside is an inorganic compound that acts by releasing NO, which stimulates the guanylate cyclase in vascular smooth muscle cells to produce cGMP. This augmented cGMP than activates the protein kinase G that phosphorylates different proteins and sequentially caused vessel smooth muscle relaxation (213).

Prostacyclin (PGI_2) is a 20-carbon prostaglandin member of the eicosanoid family that is produced in smooth muscle and vascular endothelial cells by cyclooxygenase enzymes mediated oxidation of arachidonic acid. Epoprostenol, Treprostinil and Iloprost are FDA approved prostacyclin analogs for treatment of pulmonary hypertension. They exert their beneficial effect by promoting direct arterial vasodilation and inhibition of platelet aggregation which reduce pulmonary vascular resistance and ventricular afterload and increases cardiac output (214).

Several studies were performed on each class of agent that claimed to improve clinical symptoms and pulmonary haemodynamic however to date prostacyclin analogues validate the significant improvement in long-term survival (5year survival – 55%) (215). Prostacyclin synthase, the enzyme responsible for the formation of prostacyclin, may be deficient in the pulmonary endothelium of some patients with severe PH, resulting in excessive vasoconstriction and platelet aggregation (128). The prostacyclin and its analogues not only lower down pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) but also exhibit anti-inflammatory and anti-proliferative properties (216, 217).

This study confirmed that the Treprostinil not only had a significantly greater potency (pEC_{50} 9.48 ± 0.13) as compared to other agonists but also reduce the pulmonary vessels tone more efficaciously with an E_{max} value of -2.03 gf. The recorded potency of Iloprost and Treprostinil (pEC_{50} 8.84 ± 0.15 and 9.48 ± 0.13 respectively) in the current study is higher than recorded in animal studies (pEC_{50} 6.58 ± 0.08 for Treprostinil and 5.48 ± 0.16 for Iloprost) (218, 219). Such species differences in the potency of prostacyclin analogues on pulmonary vasculature is either due to the difference in the distribution or affinity of Ip receptors or depend on the agent used for preconstriction (220). Interestingly the differences in the potency of prostacyclin analogue on rat pulmonary artery found to be more active when pulmonary vessels were pre-constricted with PGF2α in comparison to phenylephrine (pEC_{50} 5.1 and 5.9, respectively) (221). On the other hand prostacyclin analogues found to activate Ip receptors with similar potency in both humans and animals (222, 223). Overall this study demonstrates a clear distinction in the pulmonary pharmacology of prostacyclin analogues, phosphodiesterase inhibitors and NO in human small pulmonary arteries.

There are several limitations to our study. First of all it's a laboratory-based research that might not reflect the true physiological environment that PA rings were exposed to. Secondly the concentration of drugs that used to perform these experiments could not correlate with the therapeutic doses and the strength of the drug that causing maximal relaxation might be beyond the safety net to use in humans. Thirdly, we only used PGF2α as a pre-constrictor but as explained earlier the potency of relaxing agent is also depended on preconstrictor used so results need to be verifying by using other pre-constrictor agents like Endothelin-1 and Phenylephrine. Fully blinded randomized control trials are needed to show

the potential bench to bed effect of this study.

Overall the current study is the only in vitro study that demonstrated the efficacy and potency of clinically used prostacyclin analogues, SNP and phosphodiesterase inhibitors on small human pulmonary vascular reactivity with Treprostinil and Iloprost causing maximal relaxation while Sildenafil and SNP had less effect. These effects may need to be taken into account in the clinical setting as prostacyclin analogues provide better results.

Chapter 6

Conclusions, Limitations And Future Direction

6.1 Conclusions:

The present studies have established that the radial optimal resting tension to perform experiments on human pulmonary artery rings is 1.61 gf (15.78 mN). Various models are utilized for assessing the baseline molecular and cellular functions of lung ailments, particularly pulmonary vascular affliction. However, a great deal of researches is undertaken on animals with little similarity to humans. Few centers have the luxury to utilize human tissue to study this phenomenon. Isolation of human PA and measurement of pulmonary vascular tension are vital to understand the pathophysiology of human pulmonary vessels. This is the first study that measured the ORT for size 2 - 4 mm vessels and also provides a methodology of isolation of PA and their use in studies in the form of arterial rings.

The present studies have also compare the efficacy and potency of clinically used vasopressors on human pulmonary vascular tone, which could lead to improved decision making in the perioperative and postoperative settings. The choice of vasopressor and inotropes in patients having cardiac surgery should take into account their effects on pulmonary vascular resistance because these drugs could trigger pulmonary vasoconstriction, which, if persistent, could progress to pulmonary hypertension. Results showed that Prostaglandin F2α and potassium chloride caused maximal amounts of constriction, whereas ET-1 had less effect and Vasopressin had no effect. The study highlighted that Vasopressin may be safe to use for systemic vasoconstriction inpatients with pulmonary hypertension.

The current study is also the only in vitro study that demonstrated the efficacy and potency of clinically used prostacyclin analogues; SNP and

phosphodiesterase inhibitors on human pulmonary vascular reactivity with Treprostinil and Iloprost causing maximal relaxation while Sildenafil and SNP had less effect. These effects may need to be taken into account in the clinical setting as prostacyclin analogues provide better results.

6.2 Limitations:

In this thesis ex vivo human lung tissue model was used to investigate the effect of clinically significant pharmacological agents on the human pulmonary vasculature. Limitations in the use of human tissue preparations should be considered when interpreting the results from studies in this thesis. Age, sex, smoking history, asthma status, medication, existing comorbidities can influence the responses of human lung tissue. The demographic and clinical data about the donor patients was not collected due to ethical approval restrictions; which could limit the clinical interpretation of the result.

There are a few limitations to our study. The model we used is an ex-vivo model that may not reflect the true physiological environment that pulmonary arteries are usually exposed to. In this study 21% O2 and 5% CO2 were used. However, we are aware that in vivo pulmonary arteries are exposed to hypoxic and hypercapnic blood.

In addition to this we have taken the samples from lung cancer patients and size and type was unknown. It is possible that the tumour which was present in all of the *ex vivo* lung preparations used could have released pharmacologically active agents and cytokines which altered vascular responses. However in our clinical experience the incidence of such effects are rare.

Also, the pulmonary arteries that we have studied have an internal diameter of 2 - 4 mm but not focused on smaller peripheral pulmonary arterioles in isolation. Dissection of smaller resistance arterioles is difficult in practice due to the close proximity of the tumour to these vessels. This would lead to an associated risk of inadvertently affecting subsequent tumour staging. We are in the phase of learning the lung slicing technique, which enables us to perform these experiments at arteriolar level.

The concentrations of drugs that we used in these experiments are based on the previous studies and also we construct the dose response curve to evaluate the potency and efficacy of drugs. Importantly the concentration of pharmacological agent that we used to elicit the vasoreactivity might not reflect the actual therapeutic dose that we routinely use in clinical practice. Clinical trials are needed to confirm the results of these experiments.

Although we used Prostaglandin F2α as a standard pre-constrictor agent, there are some studies suggesting that vasoconstrictive agents can affect the vasodilatory response of the vessel. These experiments need to be repeated with a range of different pre-constrictor agents (e.g. Endothelin-1) to confirm similar responses.

However, despite the above limitations, human studies on the pulmonary circulation are rare especially on isolated PA rings. Therefore, this study provides valuable information that not only bridges the knowledge gap about pulmonary hypertension but also provides a platform for further studies.

6.3 Future Directions:

While this thesis has demonstrated the differential *in vitro* effects of clinically used vasoactive drugs on small human arteries, many opportunities for extending the scope of this thesis remain.

In this study we mainly concentrated on the small human pulmonary artery, similar experiments need to be performing on pulmonary veins. Pharmacological agents that act as pulmonary artery dilator may result in pulmonary vein constriction, and beside to alleviate might aggravate the patient's symptoms. A therapeutic agent that resulted in both arterial and venous vasodilator could provide better results in pulmonary hypertension patients. Similarly it would have been interesting to determine if age, sex, systemic hypertension or antihypertensive medications had an effect on pulmonary vascular responses

There are new novel agents that prove beneficial in animals but still not tested in humans, for example recent studies in animals proves the beneficial role of angiotensin converting enzyme in reducing pulmonary vascular resistance. This study provides the strategical platform to test these agents on human pulmonary vessels to confirm their beneficial roles in humans.

This study also highlighted some key points (for example; vasopressin should be used as a first line systemic vasoconstriction in patients with pulmonary hypertension) that need to be adopted for better treatment of patients. Multi-centre randomised controlled trials need to be performed to authenticate these results and to see the true bench to bed effect of this study.

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Appendix I: Ethical Approval Document

Ethical approvals document No: 15/NW/0808

Health Research Authority

National Research Ethics Service
North West - Liverpool Central Research Ethics Committee
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8020

29 September 2015

Mr Azar Hussain
Clinical Research Fellow
Hull and East Yorkshire Hospital NHS Trust
Castle Hill Hospital
Castle Road
Cottingham
Hu16 5JQ

Dear Mr Hussain

Study title:	**The impact of oxygen on human pulmonary microvasculature and small airways**
REC reference:	**15/NW/0808**
IRAS project ID:	**190181**

The Proportionate Review Sub-committee of the North West - Liverpool Central Research Ethics Committee reviewed the above application on 29 September 2015.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mrs Carol Ebenezer, nrescommittee.northwest-liverpoolcentral@nhs.net . Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

There were no ethical issues raised.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Other [clarification]		24 September 2015
Participant consent form		
Participant information sheet (PIS)		
REC Application Form [REC_Form_24092015]		24 September 2015
Research protocol or project proposal		
Summary CV for Chief Investigator (CI)		

The attached document “After ethical review – guidance for researchers”

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With the Committee’s best wishes for the success of this project.

Regina Cader.

Enclosures:	*List of names and professions of members who took part in the review*
	"After ethical review – guidance for researchers"
Copy to:	*Mr James Illingworth, Research & Development Department*
	Mr Azar Hussain, Hull and East Yorkshire Hospital NHS Trust

North West - Liverpool Central Research Ethics Committee

Attendance at PRS Sub-Committee of the REC meeting on 29 September 2015

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Julie Brake	Specialist Diabetes Nurse / Chair	Yes	Expert in the Chair
Mrs Hannah Chambers	Lay Member	Yes	
Mr Fotios Polydoros	Statistician	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Regina Caden	REC Assistant

Appendix II: Local Research and Development Department Approval Document (Ref no: R1884 – 09/11/15)

Hull and East Yorkshire Hospitals NHS
NHS Trust

Research & Development Department
2nd Floor Daisy Build
Castle Hill Hospital
Castle Road
Cottingham
HU16 5JQ

09/11/2015

Mr Azar Hussain
Clinical Research Fellow
Cardiothoracic Surgery
Castle Hill Hospital
Cottingham
East Yorkshire
HU16 5JQ

Dear Mr Azar Hussain

Re: NHS Permission Granted for Research Application

Study Title	**The impact of oxygen on human pulmonary microvasculature and small airways**
HEY R&D ref	**R1884**
REC Ref	**15/NW/0808**
CSP Ref	**N/A**

I am pleased to notify you formally that the above titled study has been granted 'NHS Permission for Research' by Hull and East Yorkshire Hospitals NHS Trust and may proceed subject to the conditions outlined in the enclosed conditions of approval document.

Approved current documents are fully listed on the Research Ethics Committee Favourable Opinion Letter(s) and have been reviewed as part of the governance review process.

Core documents as follows:

Document	Version	Date
Study Protocol	**1.1**	**26/10/2015**
Participant Information Sheet	**1.1**	**06/10/2015**
Participant Consent Form	**1**	**Dec 2014**

Please ensure that the most recent REC approved documents are used. Please notify the R&D Office if the versions above are incorrect.

Please inform HEY R&D when you have recruited your first patient.

The target date for first patient recruited:	**8th January 2016**

Please note it is a condition of approval that you or your study team complete the Research Scorecard data tool to upload first patient recruitment data followed by all subsequent recruitment data on a monthly basis. Instructions on how to access the reporting tool will be sent to you separately.

NHS 'Permission for Research' is granted on the understanding that the study is conducted in accordance with the requirements of the Research Governance Framework[1], the NHS Intellectual Property Guidance and all other applicable regulations and associated Trust policies. In undertaking this study, you agree to comply with all reporting requirements, systems and duties of action put in place by the Trust to deliver research governance. In addition, you agree to accept the responsibilities associated with your roles which are outlined within the Research Governance Framework.

Please read and sign both copies of the conditions of approval document enclosed with this letter, keep one copy for your records along with this letter and return one signed copy of the conditions to the R&D Dept. ***Failure to do so may invalidate this NHS permission.***

I would like to wish you every success with this project

Yours sincerely

James Illingworth
Research & Development Manager

. [1] The Research Governance Framework for Health and Social Care (RGFHSC)(2nd Edition 2005) sets out the broad principles of good research governance

Re: R&D Ref - R1884 / REC Ref - 15/NW/0808 / CSP Ref – N/A

The impact of oxygen on human pulmonary microvasculature and small airways

Conditions of NHS Permission for Research (Non-IMP)

THIS DOCUMENT SETS OUT IMPORTANT GUIDANCE FOR INVESTIGATORS ON THE CONDUCT AND MANAGEMENT OF NON-IMP RESEARCH WITHIN THIS NHS TRUST.

1.0 Research Governance

1.1 The study should follow the REC approved protocol

1.2 All REC conditions of authorisation that pertain to Hull and East Yorkshire Hospitals NHS Trust must be adhered to prior to the first patient being recruited at this site.

1.3 All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant.

1.4 All HEY R&D conditions highlighted in the NHS Permission letter must be adhered to prior to the first patient being recruited at this site.

1.5 Any suspected misconduct by anyone involved in the study must be reported in accordance with HEY Trust policy.

1.6 All relevant support services and departments must be consulted on and authorise their involvement in the research prior to any patient being recruited into the trial.

1.7 The Sponsor, REC and HEY Trust R&D Office must be notified, as appropriate, of any serious breaches or incidents (at this site or any other participating sites) as soon as the Sponsor or site is made aware of them

1.8 Where applicable for Non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

2.0 Study Delivery and Monitoring

2.1 The site must co-operate with the monitoring schedule and monitoring plan put in place by the Sponsor to demonstrate compliance with all applicable legislation and institutional policies.

2.2 **Where HEY NHS Trust is the study Sponsor** – your study may be subject to an internal Audit conducted by the R&D office. Instructions for undertaking any audit will be communicated to you by an R&D Facilitator.

2.3 **Where applicable-** as Chief/Principal Investigator you will ensure that you understand and have acknowledged all obligations placed on you in any trial agreement and agree to adhere to these obligations

2.4 As Chief/Principal Investigator, you will ensure that any indirect or direct obligations imposed on the research team from any third party agreements/service level agreements will be complied with.

2.5 Copies of all amendments with related approvals, applications and documents need to be forwarded to the HEY Trust R&D Office. Amendments must be approved by the HEY Trust R&D Office prior to implementation.

2.6 **Where HEY NHS Trust is the study Sponsor** – all changes to the study must be notified to the HEY R&D office prior to submission to the REC. It is the decision of the Sponsor (HEY Trust R&D Office) whether the proposed changes are deemed substantial or non-substantial.

2.7 Copies of REC annual progress reports must be forwarded to the HEY Trust R&D Office.

2.8 Copies of the end of trial notification and summary reports must be forwarded to the HEY Trust R&D Office within the regulatory timelines.

2.9 The Research Scorecard data system must be utilised to upload all patient recruitment data on a monthly basis. Date of first patient recruitment should be uploaded at your earliest convenience.

3.0 Participant Safety

3.1 Participants should receive appropriate care while involved in the study. As a matter of courtesy, all relevant healthcare professionals should be informed of the study and the likely impact on day-to-day activities. Any activity should not be at the detriment of routine care.

3.2 Where applicable, appropriate counselling must be available to patients taking part in the study.

4.0 Informed Consent

4.1 All potential subjects should have enough information to make a free and informed decision about participation.

4.2 Only those staff authorised and documented on the delegation log obtain consent.

4.3 Where required, an appropriate consent process, approved by the Ethics Committee is implemented for participants lacking the capacity to consent prior to the study commencing at this Trust.

5.0 Training and Human Resources

5.1 No staff member should be added to the delegation log without appropriate training. The R&D office must be notified of any staffing issues that may prohibit the Trust from fulfilling its obligations during the course of the trial.

5.2 All staff participating in the research at HEY must hold substantial or honorary contracts/letters of access with this Trust. Any staff not holding an appropriate contract of employment (or honorary contract) will not be indemnified by HEY Trust

5.3 All Health and Safety legislation (including Trust and University policies) must be adhered to during the conduct of the research.

5.4 Where applicable, the relevant Trust and University lone working policies must be adhered whilst conducting the research.

6.0 Data Protection

6.1 No patient identifiable data should be sent outside of the research team at Hull and East Yorkshire Hospitals NHS Trust (except where covered by the patient consent form)

6.2 Before a patient is recruited into the trial that the security of data transfer must be in accordance with the Trust policy on encryption and data access controls should be in place (individual user accounts and passwords).

6.3 No patient identifiable data should be held on laptop computers (except where encrypted as per Trust policy). No laptops should be loaned from commercial companies for the storage and transfer of research data. The R&D Office must be notified of any computer equipment loaned or given to the Trust for the purposes of the study prior to implementation. Laptops or home computers that have not been Trust encrypted must not be used to collect or transfer data as part of this study.

6.4 An appropriate mechanism must be in place in line with the Sponsors instructions, the study protocol and Trust policy to check for any patient deaths prior to sending follow-up questionnaires or contacting patients for follow up.

6.5 The integrity and confidentiality of clinical and other records and data generated by the study must be maintained in accordance with the Data Protection Act (1998) and associated HEY Trust Information Governance policies

7.0 Studies Involving Tissue

7.1 Any central labs used in the research must hold the necessary license/accreditation and local Pathology authorisation and agreement (where required) for any processing, storage and handling of tissue (including archived tumour blocks) and bloods must be in place prior to recruiting the first patient at this site. (Appropriate finance must be available to cover the costs of storage and handling).

7.2 You must be aware of, and adhere to, all obligations placed on you as Chief/Principal Investigator with regards to ensuring all guidelines and regulations are adhered to for the storage and transfer of tissue and blood samples outside of HEY as part of the study protocol. Where required, a Material Transfer Agreement must be in place for the transfer and storage of human tissue. You must adhere to the Sponsor's written instructions and SOPs regarding tissue and blood handling)

7.3 There must be an appropriate custodian of the samples supplied to the lead site by this Trust and this custodian should be duly authorised by the Sponsor.

7.4 Appropriate consent must be obtained for all translational sub study and genetics work within the limits of the REC favourable opinion.

7.5 Please note that samples may be held after the declaration of the end of the trial, for analysis or verification of research data for up to one year. After this period legal authority to hold any human tissue under the ethical approval for this project will expire. To ensure that any continued storage is lawful, either the tissue must be held on premises with a storage licence from the Human Tissue Authority, or an application made for ethical approval of another project before the favourable ethical opinion of the existing project expires. Otherwise the tissue would need to be destroyed in accordance with the HTA Codes of Practice.

8.0 Use of equipment (including loans)

8.1 You should notify the Trust R&D Office of any equipment loaned or donated to the research team for the purposes of the research to ensure that the Medical Physics team can check that the appropriate indemnity arrangements are in place prior to using the equipment.

8.2 Where applicable, appropriate agreement must be in place (prior to the study commencing) to cover the cost and potential additional resources as part of this research.

8.3 Where applicable, all decontamination and sterilisation procedures required (as per Trust and national policy and regulations) for the duration of this research must be adhered to. The research team should be made aware of any obligations in this regard prior to commencing the research at this site.

8.4 It must be confirmed that all medical devices being used as part of the study are not within the remit of the Medical Devices Directive and therefore do not require authorisation from the MHRA.

9.0 Funding

9.1 Funds must be held by the department to cover all identified costs. It is the responsibility of the Chief/Principal Investigator to notify the HEY R&D Office if any funding issues are identified.

9.2 Where relevant, all eligible service support costs must be agreed with the Local Clinical Research Network prior to any patient being recruited into the trial.

9.3 Where applicable, all excess treatment costs should be agreed with the appropriate Clinical Commissioning Group (CCG) as per Department of Health guidance.

9.4 The research site must hold the necessary funds to cover the cost of the archiving period stipulated by the Sponsor. Where archiving responsibility is delegated to the participating sites, this should be clearly documented and agreed in the clinical trial agreements for each site.

10.0 IRMER / ARSAC

10.1 All necessary IRMER regulations must be adhered to at the HEY site during the course of the research and where appropriate, local radiology review (IRMER, ARSAC and service capacity assessments) must have been undertaken prior to the study commencing at this site.

11.0 Adverse Event Reporting

11.1 All serious adverse events (SAEs) that in the opinion of the Chief/Principal Investigator are related* to the research treatment/procedure and unexpected* require reporting to the Ethics Committee and R&D within 15 days of the CI/PI becoming aware of the event using the NRES report form available from:
http://www.nres.npsa.nhs.uk/applications/after-ethical-review/safetyreports/safety-reports-for-all-other-research/#safetynonCTIMPrepotingSAEs.
*Related i.e. the SAE resulted from administration of any of the research treatments or procedures; and Unexpected i.e. the SAE is not listed in the protocol as an expected occurrence.

11.2 All related and unexpected serious adverse events (SAEs) must be reported forthwith to the Sponsor REC and HEY Trust R&D Office (and other authorities specified in the protocol) as soon as you are made aware of them

Appendix III: Raw Data For Characterization Of Optimal Resting Tension In Human Pulmonary Arteries Experiments

Table 3: Measurement of Optimal Resting Tension using Multi-wire Myograph

Pulmonary Ring	RT at 1 gm	Active Tension at 1 gm.	RT at 1.2 gm	Active Tension at 1.2 gm	RT at 1.4 gm	Active Tension at 1.4 gm	RT at 1.6 gm	Active Tension at 1.6 gm	RT at 1.8 gm	Active Tension at 1.8 gm	RT at 2.0 gm	Active Tension at 2.0 gm
1	0.92	2.95	1.21	2.68	1.4	2.87	1.6	3.21	1.85	3.19		
2	0.84	2.05	1.18	1.87	1.39	1.89	1.59	2.07	1.77	1.85		
3	1.17	0.65	1.24	0.75	1.4	0.72	1.59	0.74	1.74	0.83		
4	1.05	2.92	1.2	2.62	1.39	2.68	1.59	2.88	1.79	2.46		
5	0.94	1.76	1.19	2.32	1.38	2.47	1.58	2.31	1.78	2.36	1.99	2.30
6	0.89	1.13	1.19	1.53	1.39	1.70	1.59	1.67	1.79	1.60	1.99	1.52

Measurement of Optimal Resting Tension using Multi-wire Myograph.contd…..

Pulmonary Ring	RT at 1 gm	Active Tension at 1 gm.	RT at 1.2 gm	Active Tension at 1.2 gm	RT at 1.4 gm	Active Tension at 1.4 gm	RT at 1.6 gm	Active Tension at 1.6 gm	RT at 1.8 gm	Active Tension at 1.8 gm	RT at 2.0 gm	Active Tension at 2.0 gm
7	0.71	1.89	1.17	2.76	1.38	2.89	1.59	2.69	1.79	2.81	2.00	2.93
8	1.14	0.28	1.21	0.51	1.40	0.51	1.59	0.78	1.78	0.78	2.00	0.72
9	0.98	0.95	1.20	0.93	1.39	1.00	1.59	1.33	1.78	1.44	1.98	1.58
10	0.98	0.49	1.19	0.30	1.39	0.51	1.57	0.61	1.79	0.88	1.98	1.05
11	0.97	0.95	1.19	1.05	1.40	1.17	1.60	1.38	1.79	1.64	1.98	1.81
12	0.99	0.89	1.20	1.01	1.40	1.20	1.59	1.36	1.80	1.39	2.00	1.48

Table 4: Measurement of Optimal Resting Tension using Organ Bath system.

Pulmonary Ring	RT at 1 gm	Active Tension at 1 gm	RT at 1.2 gm	Active Tension at 1.2 gm	RT at 1.4 gm	Active Tension at 1.4 gm	RT at 1.6 gm	Active Tension at 1.6 gm	RT at 1.8 gm	Active Tension at 1.8 gm	RT at 2.0 gm	Active Tension at 2.0 gm
1	1.16	0.60	1.19	0.82	1.4	0.91	1.62	1.06	1.8	0.94		
2	1.03	0.7	1.17	0.89	1.42	1.13	1.64	1.24	1.77	1.22		
3	0.79	0.61	1.19	0.9	1.42	1.06	1.6	1.19	1.79	1.23		
4	1.11	1.53	1.22	1.28	1.45	1.42	1.62	1.64	2.23	1.59		
5	0.97	1.24	1.19	1.25	1.39	1.4	1.67	1.66	2.15	1.5		
6	1.22	0.41	1.24	0.31	1.43	0.43	1.62	0.6	2.02	0.52		
7	1.29	0.36	1.24	0.38	1.42	0.42	1.63	0.49	1.79	0.30	1.99	0.41
8	1.14	0.41	1.23	0.46	1.41	0.55	1.6	0.61	1.81	0.48	1.98	0.57

Table 5: Combined result of optimal resting tension measurement.

Pulmonary Ring	RT at 1 gm	Active Tension at 1 gm	RT at 1.2 gm	Active Tension at 1.2 gm	RT at 1.4 gm	Active Tension at 1.4 gm	RT at 1.6 gm	Active Tension at 1.6 gm	RT at 1.8 gm	Active Tension at 1.8 gm	RT at 2.0 gm	Active Tension at 2.0 gm
1	1.16	0.60	1.19	0.82	1.4	0.91	1.62	1.06	1.8	0.94		
2	1.03	0.7	1.17	0.89	1.42	1.13	1.64	1.24	1.77	1.22		
3	0.79	0.61	1.19	0.9	1.42	1.06	1.6	1.19	1.79	1.23		
4	1.11	1.53	1.22	1.28	1.45	1.42	1.62	1.64	2.23	1.59		
5	0.97	1.24	1.19	1.25	1.39	1.4	1.67	1.66	2.15	1.5		
6	1.22	0.41	1.24	0.31	1.43	0.43	1.62	0.6	2.02	0.52		
7	0.92	2.95	1.21	2.68	1.4	2.87	1.6	3.21	1.85	3.19		

Combined result of optimal resting tension measurement. Contd……

Pulmonary Ring	RT at 1 gm	Active Tension at 1 gm	RT at 1.2 gm	Active Tension at 1.2 gm	RT at 1.4 gm	Active Tension at 1.4 gm	RT at 1.6 gm	Active Tension at 1.6 gm	RT at 1.8 gm	Active Tension at 1.8 gm	RT at 2.0 gm	Active Tension at 2.0 gm
8	0.84	2.05	1.18	1.87	1.39	1.89	1.59	2.07	1.77	1.85		
9	1.17	0.65	1.24	0.75	1.4	0.72	1.59	0.74	1.74	0.83		
10	1.05	2.92	1.2	2.62	1.39	2.68	1.59	2.88	1.79	2.46		
11	1.29	0.36	1.24	0.38	1.42	0.42	1.63	0.49	1.79	0.30	1.99	0.41
12	1.14	0.41	1.23	0.46	1.41	0.55	1.6	0.61	1.81	0.48	1.98	0.57
13	0.94	1.76	1.19	2.32	1.38	2.47	1.58	2.31	1.78	2.36	1.99	2.30
14	0.89	1.13	1.19	1.53	1.39	1.70	1.59	1.67	1.79	1.60	1.99	1.52

Combined result of optimal resting tension measurement. Contd…

Pulmonary Ring	RT at 1 gm	Active Tension at 1 gm	RT at 1.2 gm	Active Tension at 1.2 gm	RT at 1.4 gm	Active Tension at 1.4 gm	RT at 1.6 gm	Active Tension at 1.6 gm	RT at 1.8 gm	Active Tension at 1.8 gm	RT at 2.0 gm	Active Tension at 2.0 gm
15	0.713	1.89	1.17	2.76	1.38	2.89	1.59	2.69	1.79	2.81	2.00	2.93
16	1.14	0.28	1.21	0.51	1.40	0.51	1.59	0.78	1.78	0.78	2.00	0.72
17	0.98	0.95	1.20	0.93	1.39	1.00	1.59	1.33	1.78	1.44	1.98	1.58
18	0.98	0.49	1.19	0.30	1.39	0.51	1.57	0.61	1.79	0.88	1.98	1.05
19	0.971	0.95	1.19	1.01	1.40	1.17	1.60	1.38	1.79	1.64	1.98	1.81
20	0.99	0.89	1.20	1.01	1.40	1.20	1.59	1.36	1.80	1.39	2.00	1.48

Appendix IV: Raw Data For The Differential Effects Of Systemic Vasoconstrictors On Human Pulmonary Artery Tension Experiment

Table 6: Effect of Adrenaline on Pulmonary Artery Ring.

Pulmonary Ring	Baseline Tension	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100nM	Active Tension at 100nM	Tension at 300nM	Active Tension at 300nM	Tension at 1uM	Active Tension at 1uM	Tension at 3uM	Active Tension at 3uM	Tension at 10uM	Active Tension at 10uM	Tension at 30uM	Active Tension at 30uM
1	1.59	1.60	0.01	1.59	0.00	1.64	0.04	1.68	0.09	2.66	1.07	2.61	1.01	2.68	1.09	2.66	1.07	2.62	1.03
2	1.29	1.29	0.00	1.28	-0.01	1.32	0.03	1.38	0.09	1.84	0.55	2.18	0.89	2.35	1.06	2.31	1.02	2.27	0.98
3	1.12	1.09	-0.02	1.10	-0.01	1.11	-0.01	1.13	0.01	1.14	0.02	1.30	0.18	1.50	0.39	1.50	0.38	1.49	0.37
4	1.25	1.22	-0.03	1.21	-0.03	1.22	-0.03	1.22	-0.02	1.28	0.03	1.37	0.13	1.47	0.23	1.47	0.22	1.47	0.23

Effect of Adrenaline on Pulmonary Artery Ring. Contd…

Pulmonary Ring	Baseline Tension	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100nM	Active Tension at 100nM	Tension at 300nM	Active Tension at 300nM	Tension at 1uM	Active Tension at 1uM	Tension at 3uM	Active Tension at 3uM	Tension at 10uM	Active Tension at 10uM	Tension at 30uM	Active Tension at 30uM
5	1.58	1.56	-0.02	1.56	-0.02	1.57	-0.01	1.71	0.13	1.96	0.38	2.14	0.56	2.36	0.78	2.42	0.84	2.37	0.79
6	1.60	1.59	-0.01	1.59	-0.01	1.62	0.03	1.99	0.39	2.16	0.56	2.36	0.76	2.41	0.81	2.45	0.85	2.45	0.85
7	1.58	1.58	0.00	1.58	0.01	1.73	0.16	1.79	0.22	2.36	0.79	2.72	1.15	2.97	1.40	2.91	1.33	2.88	1.30
8	1.45	1.42	-0.02	1.39	-0.06	1.37	-0.08	1.69	0.25	2.19	0.74	2.44	0.99	2.42	0.97	2.32	0.87	2.19	0.75

Table 7: Effect of Noradrenaline on Pulmonary Artery Ring

Pulmonary Ring	Baseline Tension	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100nM NA	Active Tension at 100nM NA	Tension at 300nM NA	Active Tension at 300nM NA	Tension at 1uM NA	Active Tension at 1uM NA	Tension at 3uM NA	Active Tension at 3uM NA	Tension at 10uM NA	Active Tension at 10uM NA	Tension at 30uM NA	Active Tension at 30uM NA
1	1.63							2.05	0.42	2.26	0.63	2.30	0.67	2.30	0.66	2.35	0.72	2.51	0.88
2	1.67							2.10	0.43	2.22	0.55	2.32	0.65	2.38	0.71	2.34	0.67	2.37	0.70
3	1.69							2.99	1.30	3.36	1.67	3.58	1.89	3.72	2.03	3.71	2.02	3.67	1.98
4	1.60							1.96	0.35	2.15	0.55	2.21	0.61	2.32	0.72	2.32	0.71	2.29	0.68
5	1.45							1.47	0.02	1.48	0.03	1.48	0.03	1.49	0.04	1.52	0.07	1.47	0.02
6	1.61							2.50	0.89	2.39	0.78	2.58	0.97	3.09	1.48	3.08	1.47	3.02	1.41

Effect of Noradrenaline on Pulmonary Artery Ring. Contd….

Pulmonary Ring	Baseline Tension	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100nM NA	Active Tension at 100nM NA	Tension at 300nM NA	Active Tension at 300nM NA	Tension at 1uM NA	Active Tension at 1uM NA	Tension at 3uM NA	Active Tension at 3uM NA	Tension at 10uM NA	Active Tension at 10uM NA	Tension at 30uM NA	Active Tension at 30uM NA
7	1.56	1.57	0.01	1.57	0.00	1.57	0.01	1.57	0.01	1.90	0.34	2.03	0.46	2.27	0.71	2.56	0.99	2.57	1.00
8	1.60	1.59	-0.01	1.59	-0.01	1.58	-0.02	1.58	-0.01	1.59	-0.01	1.70	0.11	1.81	0.21	1.99	0.39	1.91	0.31
9	1.67	1.67	0.00	1.68	0.01	1.67	0.00	1.67	0.00	1.67	0.00	1.71	0.05	1.74	0.07	1.76	0.09	1.78	0.11
10	1.37	1.36	-0.01	1.35	-0.02	1.35	-0.02	1.36	-0.01	1.41	0.04	2.04	0.67	2.22	0.85	2.28	0.91	2.05	0.68
11	1.42	1.41	-0.01	1.40	-0.02	1.40	-0.02	1.39	-0.03	1.37	-0.04	1.38	-0.04	1.39	-0.03	1.51	0.09	1.48	0.06
12	1.02	0.99	-0.03	0.97	-0.05	0.94	-0.08	0.95	-0.08	0.89	-0.13	0.89	-0.13	0.91	-0.11	0.93	-0.10	0.96	-0.06

Table 8: Effect of ET-1 on Pulmonary Artery Ring.

Pulmonary Ring	Baseline Tension	Tension at 100pM	Active Tension at 100 pM	Tension at 300pM	Active Tension at 300pM	Tension at 1nM	Active Tension at 1nM	Tension at 3nM	Active Tension at 3nM	Tension at 10nM	Active Tension at 10nM	Tension at 30nM	Active Tension at 30nM	Tension at 100nM	Active Tension at 100 nM
1	1.61	1.57	-0.04	1.55	-0.06	1.48	-0.13	1.48	-0.13	1.51	-0.1	1.55	-0.06		
2	1.58	1.48	-0.1	1.47	-0.11	1.48	-0.1	1.78	0.2	1.84	0.26	1.81	0.23		
3	1.59	1.5	-0.09	1.49	-0.1	1.47	-0.12	1.61	0.02	1.66	0.07	1.65	0.06		
4	1.6	1.4	-0.2	1.38	-0.22	1.35	-0.25	1.6	0	1.67	0.07	1.6	0		

Effect of ET-1 on Pulmonary Artery Ring. Contd…..

Pulmonary Ring	Baseline Tension	Tension at 100pM	Active Tension at 100 pM	Tension at 300pM	Active Tension at 300pM	Tension at 1nM	Active Tension at 1nM	Tension at 3nM	Active Tension at 3nM	Tension at 10nM	Active Tension at 10nM	Tension at 30nM	Active Tension at 30nM	Tension at 100nM	Active Tension at 100 nM
5	1.26	1.14	-0.12	1.12	-0.14	1.1	-0.16	1.11	-0.15	1.1	-0.16	1.11	-0.15	1.09	-0.17
6	1.58	1.4	-0.18	1.34	-0.24	1.31	-0.27	1.58	0	1.57	-0.01	1.45	-0.13	1.22	-0.36
7	1.37	1.18	-0.19	1.14	-0.23	1.11	-0.26	2.2	0.83	2.16	0.79	1.58	0.21	1.06	-0.31
8	1.26	1.02	-0.24	0.96	-0.3	0.92	-0.34	1.25	-0.01	1.26	0	1.22	-0.04	0.96	-0.3

Table 9: Effect of PGF2a on Pulmonary Artery Rings.

Pulmonary Ring	Baseline Tension	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM	Tension at 10 uM	Active Tension at 10 uM	Tension at 30 uM	Active Tension at 30 uM	Tension at 100 uM	Active Tension at 100 uM	Tension at 300 uM	Active Tension at 300 uM
1	1.45	1.39	-0.05	1.39	-0.06	1.38	-0.07	1.37	-0.08	1.43	-0.02	2.00	0.56	2.31	0.86	2.28	0.84
2	1.30	1.23	-0.07	1.28	-0.02	1.13	-0.16	1.49	0.20	2.88	1.59	3.10	1.80	3.28	1.99	3.15	1.85
3	1.29	1.18	-0.10	1.16	-0.13	1.15	-0.14	1.54	0.25	3.12	1.83	3.47	2.19	3.69	2.40	3.64	2.35
4	1.38	1.26	-0.12	1.24	-0.14	1.19	-0.19	1.37	-0.01	3.36	1.99	3.48	2.10	3.50	2.13	3.73	2.35

Effect of PGF2α on Pulmonary Artery rings. Contd…

Pulmonary Ring	Baseline Tension	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM	Tension at 10 uM	Active Tension at 10 uM	Tension at 30 uM	Active Tension at 30 uM	Tension at 100 uM	Active Tension at 100 uM	Tension at 300 uM	Active Tension at 300 uM
5	1.57	1.57	0.00	1.57	0.00	1.56	-0.01	1.56	-0.01	1.53	-0.04	1.58	0.01	1.65	0.08	1.79	0.22
6	1.89	1.89	0.00	1.88	-0.01	1.86	-0.03	1.85	-0.04	1.85	-0.04	2.16	0.27	2.17	0.28	2.35	0.46
7	1.59	1.57	-0.02	1.53	-0.06	1.51	-0.08	1.50	-0.09	1.49	-0.10	1.60	0.01	2.29	0.71	2.40	0.81
8	2.25	2.24	-0.01	2.23	-0.02	2.21	-0.04	2.20	-0.05	2.42	0.16	2.45	0.19	2.44	0.19	2.47	0.21

Table 10: Effect of KCl on Pulmonary artery rings.

Pulmonary Ring	Baseline Tension	Tension at 300uM KCl	Active Tension at 300uM KCl	Tension at 1mM KCl	Active Tension at 1mM KCl	Tension at 3mM KCl	Active Tension at 3mM KCl	Tension at 10mM KCl	Active Tension at 10mM KCl	Tension at 30mM KCl	Active Tension at 30mM KCl	Tension at 100mM KCl	Active Tension at 100mM KCl	Tension at 300mM KCl	Active Tension at 300mM KCl
1	1.59	1.58	-0.01	1.59	0	1.68	0.09	2.67	1.08	2.88	1.29	3.18	1.59		
2	1.58	1.52	-0.06	1.57	-0.01	1.73	0.15	2.6	1.02	2.89	1.31	3.08	1.5		
3	1.62	1.71	0.09	1.79	0.17	1.84	0.22	2.1	0.48	2.21	0.59	2.31	0.69		
4	1.6	1.7	0.1	1.77	0.17	1.79	0.19	2.13	0.53	2.24	0.64	2.41	0.81		
5	1.66	1.76	0.1	1.78	0.12	1.91	0.25	2.11	0.45	2.38	0.72	2.48	0.82		
6	1.66	1.66	0	1.65	-0.01	1.71	0.05	1.73	0.07	1.99	0.33	2.66	1		
7	1.59	1.57	-0.02	1.55	-0.04	1.58	-0.01	1.62	0.03	2.03	0.44	3.58	1.99		

Effect of KCl on Pulmonary artery rings. Contd…

Pulmonary Ring	Baseline Tension	Tension at 300uM KCl	Active Tension at 300uM KCl	Tension at 1mM KCl	Active Tension at 1mM KCl	Tension at 3mM KCl	Active Tension at 3mM KCl	Tension at 10mM KCl	Active Tension at 10mM KCl	Tension at 30mM KCl	Active Tension at 30mM KCl	Tension at 100mM KCl	Active Tension at 100mM KCl	Tension at 300mM KCl	Active Tension at 300mM KCl
8	1.6	1.6	0	1.6	0	1.6	0	1.62	0.02	1.85	0.25	1.85	0.25	1.81	0.21
9	1.58	1.52	-0.06	1.51	-0.07	1.5	-0.08	1.5	-0.08	1.82	0.24	1.83	0.25	1.78	0.2
10	1.42	1.38	-0.04	1.30	-0.12	1.28	-0.14	1.27	-0.15	2.79	1.36	3.13	1.70	2.86	1.44
11	1.47	1.44	-0.02	1.43	-0.04	1.46	-0.01	2.26	0.78	2.78	1.31	2.60	1.12	2.31	0.83
12	1.53	1.49	-0.03	1.43	-0.10	1.41	-0.12	1.36	-0.17	2.44	0.91	2.66	1.13	2.41	0.88
13	1.52	1.5	-0.02	1.47	-0.05	1.44	-0.08	1.39	-0.13	2.55	1.02	2.76	1.23	2.44	0.91

Table 11: Effect of Vasopressin on Pulmonary Artery Ring.

Pulmonary Ring	Baseline Tension	Tension at 100pM	Active Tension at 100 pM	Tension at 300pM	Active Tension at 300pM	Tension at 1nM	Active Tension at 1nM	Tension at 3nM	Active Tension at 3nM	Tension at 10nM	Active Tension at 10nM	Tension at 30nM	Active Tension at 30nM	Tension at 100nM	Active Tension at 100 nM	Tension at 300nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active tension at 3 uM
1	1.47	1.43	-0.04	1.43	-0.04	1.42	-0.05	1.42	-0.05	1.41	-0.06	1.40	-0.07	1.40	-0.07	1.39	-0.08	1.39	-0.08	1.39	-0.08
2	1.43	1.38	-0.05	1.37	-0.06	1.36	-0.07	1.35	-0.08	1.35	-0.09	1.34	-0.10	1.33	-0.10	1.32	-0.11	1.32	-0.11	1.31	-0.12
3	1.47	1.44	-0.03	1.43	-0.03	1.42	-0.04	1.42	-0.04	1.41	-0.05	1.41	-0.05	1.41	-0.06	1.41	-0.06	1.40	-0.07	1.39	-0.07
4	1.43	1.39	-0.04	1.38	-0.05	1.37	-0.06	1.36	-0.07	1.36	-0.07	1.35	-0.08	1.35	-0.08	1.34	-0.09	1.34	-0.09	1.32	-0.11

Effect of Vasopressin on Pulmonary Artery Ring. Contd…

Pulmonary Ring	Baseline Tension	Tension at 100pM	Active Tension at 100 pM	Tension at 300pM	Active Tension at 300pM	Tension at 1nM	Active Tension at 1nM	Tension at 3nM	Active Tension at 3nM	Tension at 10nM	Active Tension at 10nM	Tension at 30nM	Active Tension at 30nM	Tension at 100nM	Active Tension at 100 nM	Tension at 300nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active tension at 3 uM
5	1.59	1.60	0.01	1.60	0.01	1.60	0.02	1.60	0.02	1.60	0.02	1.61	0.03	1.60	0.01	1.60	0.02	1.60	0.02	1.61	0.03
6	1.65	1.66	0.00	1.66	0.01	1.66	0.01	1.66	0.01	1.66	0.00	1.66	0.01	1.65	-0.01	1.66	0.00	1.65	0.00	1.67	0.01
7	1.52	1.52	0.00	1.52	0.00	1.52	0.01	1.52	0.01	1.52	0.00	1.53	0.01	1.52	0.00	1.52	0.01	1.53	0.01	1.54	0.03
8	1.54	1.56	0.01	1.56	0.02	1.56	0.02	1.56	0.02	1.56	0.02	1.56	0.02	1.55	0.01	1.52	-0.02	1.55	0.01	1.56	0.02

Appendix V: Raw Data For In Vitro Characterization Of Pharmacological Effect Of Prostacyclin Analogues In Comparison To Phosphodiesterase Inhibitors On Small Human Pulmonary Vessels Experiments

Table 12: Effect of Sildenafil on Pulmonary Artery Ring.

Pulmonary Ring	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM	Tension at 10 uM	Active Tension at 10 uM	Tension at 30 uM	Active Tension at 30 uM	Tension at 100 uM	Active Tension at 100 uM
1	1.44	1.83	1.86	0.03	1.87	0.04	1.87	0.05	1.88	0.05	1.87	0.05	1.87	0.04	1.86	0.04	1.85	0.03	1.84	0.01	1.83	0.00	1.81	-0.01	1.60	-0.23	1.58	-0.247
2	1.22	2.08	2.02	-0.06	2.01	-0.07	1.95	-0.13	1.89	-0.19	1.89	-0.20	1.87	-0.21	1.84	-0.24	1.80	-0.28	1.77	-0.31	1.74	-0.34	1.66	-0.43	1.36	-0.72	1.351	-0.73
3	1.30	2.46	2.43	-0.03	2.30	-0.16	2.37	-0.08	2.27	-0.19	2.28	-0.17	2.15	-0.31	2.10	-0.36	2.02	-0.44	1.93	-0.53	1.90	-0.55	1.84	-0.62	1.40	-1.06	1.376	-1.081
4	1.43	2.99	2.97	-0.02	3.01	0.02	3.01	0.02	3.02	0.03	3.02	0.03	3.02	0.03	2.97	-0.02	2.94	-0.04	2.90	-0.09	2.91	-0.07	2.71	-0.28	2.17	-0.82	2.126	-0.861
5	1.55	2.45	2.45	-0.01	2.43	-0.02	2.39	-0.06	2.39	-0.06	2.38	-0.08	2.41	-0.05	2.32	-0.13	2.31	-0.15	2.28	-0.18	2.20	-0.26	1.66	-0.80	1.34	-1.11		
6	1.15	2.69	2.69	0.00	2.68	-0.01	2.67	-0.02	2.65	-0.04	2.65	-0.04	2.64	-0.05	2.63	-0.06	2.60	-0.09	2.57	-0.12	2.53	-0.16	2.11	-0.58	1.06	-1.64		

Effect of Sildenafil on Pulmonary Artery Ring. Contd…

Pulmonary Ring	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM	Tension at 10 uM	Active Tension at 10 uM	Tension at 30 uM	Active Tension at 30 uM	Tension at 100 uM	Active Tension at 100 uM
7	1.71	2.38	2.37	-0.01	2.37	-0.01	2.35	-0.02	2.36	-0.02	2.34	-0.04	2.33	-0.05	2.31	-0.06	2.31	-0.07	2.25	-0.13	2.21	-0.17	1.81	-0.57	1.33	-1.05		
8	1.33	3.16	3.11	-0.06	3.11	-0.05	3.08	-0.08	3.04	-0.13	3.05	-0.12	2.96	-0.20	2.97	-0.19	2.91	-0.25	2.84	-0.32	2.83	-0.33	2.39	-0.77	1.65	-1.52		
9	1.53	2.50	2.48	-0.02	2.47	-0.03	2.44	-0.06	2.17	-0.33	2.06	-0.44	1.94	-0.56	1.84	-0.66	1.71	-0.79	1.63	-0.86	1.61	-0.89	1.52	-0.98	1.50	-1.00	1.17	-1.32
10	1.64	1.95	1.95	0.00	1.95	0.00	1.95	0.01	1.94	0.00	1.94	0.00	1.93	-0.01	1.93	-0.02	1.92	-0.03	1.90	-0.05	1.88	-0.07	1.82	-0.12	1.78	-0.16	1.38	-0.56
11	1.59	2.88	2.85	-0.04	2.83	-0.06	2.78	-0.10	2.49	-0.39	2.27	-0.61	2.04	-0.84	1.91	-0.97	1.70	-1.19	1.62	-1.27	1.60	-1.28	1.44	-1.45	1.41	-1.48	1.21	-1.67
12	1.71	3.54	3.55	0.01	3.55	0.01	3.50	-0.05	2.83	-0.72	2.60	-0.95	2.19	-1.36	1.94	-1.60	1.71	-1.84	1.62	-1.92	1.60	-1.95	1.52	-2.03	1.51	-2.04	1.39	-2.15

Table 13: Effect of SNP on Pulmonary Artery Ring.

Pulmonary Ring	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM
1	1.50	3.58	3.56	-0.02	3.46	-0.12	3.59	0.01	3.54	-0.04	3.44	-0.14	3.00	-0.58	2.20	-1.38	1.68	-1.90	1.42	-2.16	1.40	-2.18
2	1.46	2.58	2.62	0.04	2.60	0.02	2.51	-0.07	2.32	-0.26	2.09	-0.49	1.58	-1.00	1.35	-1.23	1.25	-1.33	1.21	-1.37	1.20	-1.38
3	1.55	2.65	2.71	0.06	2.68	0.03	2.69	0.04	2.49	-0.16	2.33	-0.32	1.87	-0.77	1.64	-1.01	1.49	-1.15	1.42	-1.22	1.41	-1.24
4	1.46	3.44	3.47	0.03	3.49	0.06	3.42	-0.02	3.00	-0.44	2.31	-1.13	1.86	-1.58	1.71	-1.72	1.63	-1.80	1.54	-1.90	1.52	-1.92

Effect of SNP on Pulmonary Artery Ring. Contd……

Pulmonary Ring	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM
5	1.34	2.77	2.58	-0.19	2.68	-0.09	2.583	-0.19	2.54	-0.23	2.21	-0.56	1.71	-1.06	1.55	-1.22	1.52	-1.25	1.42	-1.35	1.38	-1.39
6	1.15	3.19	3.01	-0.18	3.07	-0.12	3.07	-0.12	2.98	-0.21	2.74	-0.45	2.26	-0.93	1.66	-1.53	1.44	-1.75	1.43	-1.76	1.46	-1.73
7	0.94	2.05	1.96	-0.09	2.02	-0.03	2.03	-0.02	2.08	0.03	2.09	0.04	1.93	-0.12	1.37	-0.68	1.16	-0.89	1.16	-0.89	1.11	-0.94
8	1.42	3.24	2.84	-0.40	2.82	-0.42	2.92	-0.32	2.64	-0.60	1.97	-1.27	1.64	-1.60	1.7	-1.54	1.68	-1.56	1.64	-1.60	1.56	-1.68

Table 14: Effect of Milrinone on Pulmonary Artery Ring.

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM	Tension at 10 uM	Active Tension at 10 uM	Tension at 30 uM	Active Tension at 30 uM	Tension at 100 uM	Active Tension at 100 uM
1	1.22	2.36	2.38	0.02	2.41	0.05	2.44	0.08	2.45	0.09	2.36	0.00	1.90	-0.46	1.58	-0.79	1.25	-1.11	1.08	-1.28	1.02	-1.33
2	1.35	2.21	2.26	0.04	2.26	0.05	2.26	0.05	2.26	0.05	2.17	-0.04	2.00	-0.22	1.63	-0.59	1.32	-0.90	1.08	-1.13	1.01	-1.19
3	1.18	2.08	2.18	0.10	2.17	0.08	2.16	0.08	2.15	0.07	2.06	-0.03	1.93	-0.16	1.67	-0.41	1.44	-0.65	1.29	-0.80	1.17	-0.91
4	1.10	2.40	2.52	0.12	2.52	0.12	2.52	0.11	2.48	0.08	2.23	-0.18	1.71	-0.70	1.35	-1.06	1.16	-1.24	1.05	-1.35	1.00	-1.39

Effect of Milrinone on Pulmonary Artery Ring. Contd……

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM	Tension at 10 uM	Active Tension at 10 uM	Tension at 30 uM	Active Tension at 30 uM	Tension at 100 uM	Active Tension at 100 uM
5	1.87	3.08	3.07	0.00	3.04	-0.03	2.99	-0.09	2.98	-0.09	2.79	-0.29	2.24	-0.83	1.75	-1.32	1.34	-1.74	1.32	-1.76	1.26	-1.81
6	1.66	4.37	4.37	0.00	4.32	-0.05	4.29	-0.08	4.08	-0.29	3.46	-0.91	2.57	-1.80	1.96	-2.42	1.50	-2.88	1.48	-2.90	1.38	-2.99
7	1.63	2.72	2.72	0.00	2.70	-0.02	2.68	-0.04	2.63	-0.09	2.52	-0.20	2.11	-0.61	1.52	-1.20	1.22	-1.50	1.20	-1.52	1.14	-1.58
8	1.64	3.76	3.71	-0.05	3.63	-0.13	3.48	-0.28	2.79	-0.97	2.09	-1.67	1.63	-2.13	1.31	-2.45	1.05	-2.71	1.03	-2.73	1.00	-2.76

Table 15: Effect of Epoprostenol on Pulmonary Artery Ring.

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM
1	1.52	3.66	3.67	0.02	3.51	-0.14	3.48	-0.18	3.31	-0.35	3.06	-0.59	2.63	-1.03	2.40	-1.26	1.93	-1.73	1.58	-2.08	1.55	-2.11
2	1.55	3.20	3.16	-0.04	3.12	-0.08	3.05	-0.15	2.82	-0.39	2.50	-0.70	2.13	-1.07	1.96	-1.24	1.63	-1.58	1.41	-1.79	1.40	-1.81
3	1.69	2.57	2.57	0.00	2.58	0.01	2.58	0.01	2.57	0.00	2.54	-0.03	2.50	-0.07	2.35	-0.22	2.15	-0.42	2.04	-0.52	2.00	-0.57
4	1.53	1.48	1.48	0.00	1.47	-0.01	1.47	-0.01	1.47	-0.01	1.47	-0.01	1.52	0.04	1.67	0.18	1.70	0.22	1.95	0.46	1.98	0.49

Effect of Epoprostenol on Pulmonary Artery Ring. Contd…

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM	Tension at 1 uM	Active Tension at 1 uM	Tension at 3 uM	Active Tension at 3 uM
5	1.56	4.79	4.78	-0.01	4.74	-0.05	4.67	-0.12	4.63	-0.16	4.61	-0.18	3.60	-1.19	2.69	-2.10	2.39	-2.40	2.23	-2.56	2.18	-2.61
6	1.64	5.31	5.33	0.02	5.33	0.02	5.29	-0.01	5.24	-0.06	4.86	-0.44	3.23	-2.07	2.32	-2.98	2.23	-3.07	2.85	-2.46	3.06	-2.25
7	1.60	5.43	5.47	0.04	5.47	0.04	5.38	-0.05	5.36	-0.07	5.14	-0.29	4.27	-1.16	3.15	-2.28	2.69	-2.74	2.61	-2.82	2.67	-2.76
8	1.62	5.27	5.29	0.02	5.26	-0.01	5.20	-0.06	5.17	-0.09	4.94	-0.33	4.11	-1.16	2.94	-2.33	2.57	-2.70	2.30	-2.97	2.31	-2.96

Table 16: Effect of Iloprost on Pulmonary Artery Ring.

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 10 pM	Active Tension at 10 pM	Tension at 30 pM	Active Tension at 30 pM	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM
1	1.57	4.21	4.18	-0.04	4.17	-0.05	4.12	-0.09	3.88	-0.33	3.08	-1.14	2.05	-2.16	1.75	-2.47	1.27	-2.94	1.24	-2.97
2	1.59	4.44	4.43	0.00	4.37	-0.07	4.21	-0.22	3.80	-0.64	2.52	-1.92	1.84	-2.59	1.63	-2.81	1.27	-3.17	1.25	-3.19
3	1.44	3.98	3.97	-0.01	3.95	-0.03	3.91	-0.07	3.70	-0.28	2.50	-1.48	1.75	-2.23	1.55	-2.43	1.20	-2.78	1.17	-2.81
4	1.47	5.34	5.35	0.01	5.31	-0.04	5.22	-0.13	5.01	-0.34	3.55	-1.80	2.84	-2.51	2.36	-2.99	1.65	-3.70	1.57	-3.77

Effect of Iloprost on Pulmonary Artery Ring. Contd…

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 10 pM	Active Tension at 10 pM	Tension at 30 pM	Active Tension at 30 pM	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM
5	1.65	3.40	3.40	0.00	3.40	0.00	3.35	-0.05	3.22	-0.18	2.95	-0.44	2.62	-0.77	2.40	-0.99	2.25	-1.14	2.14	-1.26
6	1.69	2.38	2.38	0.00	2.37	-0.01	2.34	-0.04	2.27	-0.11	2.18	-0.20	1.93	-0.45	1.73	-0.65	1.55	-0.83	1.41	-0.97
7	1.66	2.38	2.37	-0.01	2.37	-0.01	2.35	-0.03	2.28	-0.10	2.22	-0.16	2.04	-0.34	1.87	-0.51	1.81	-0.57	1.76	-0.61
8	1.49	2.28	2.28	0.00	2.28	-0.01	2.27	-0.02	2.15	-0.14	2.14	-0.15	1.99	-0.30	1.91	-0.38	1.83	-0.45	1.82	-0.46

Table 17: Effect of Treprostinil on Pulmonary Artery Ring.

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 1pM	Active Tension at 1pM	Tension at 3pM	Active Tension at 3pM	Tension at 10pM	Active Tension at 10pM	Tension at 30pM	Active Tension at 30pM	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM
1	1.42	2.49	2.48	0.00	2.48	-0.01	2.47	-0.02	2.45	-0.03	2.43	-0.05	2.42	-0.06	2.34	-0.15	2.09	-0.40	1.91	-0.58	1.72	-0.77	1.58	-0.90	1.54	-0.95
2	1.59	2.98	2.98	-0.01	2.97	-0.01	2.98	-0.01	2.96	-0.02	2.92	-0.07	2.93	-0.05	2.01	-0.97	1.53	-1.46	1.42	-1.56	1.42	-1.57	1.37	-1.62	1.39	-1.60
3	1.61	2.84	2.83	-0.01	2.81	-0.02	2.77	-0.07	2.75	-0.09	2.61	-0.23	2.58	-0.26	2.15	-0.69	1.58	-1.26	1.47	-1.37	1.43	-1.41	1.43	-1.41	1.42	-1.42
4	1.45	2.73	2.73	-0.01	2.73	0.00	2.73	-0.01	2.69	-0.04	2.60	-0.14	2.52	-0.22	1.83	-0.90	1.18	-1.55	1.06	-1.67	1.02	-1.71	0.98	-1.75	0.90	-1.83

Effect of Treprostinil on Pulmonary Artery Ring.Contd…

Pulmonary Rings	Baseline Tension	Tension with 11.21 uM PGF2a	Tension at 1pM	Active Tension at 1pM	Tension at 3pM	Active Tension at 3pM	Tension at 10pM	Active Tension at 10pM	Tension at 30pM	Active Tension at 30pM	Tension at 100 pm	Active Tension at 100 pm	Tension at 300 pm	Active Tension at 300 pm	Tension at 1 nM	Active Tension at 1 nM	Tension at 3 nM	Active Tension at 3 nM	Tension at 10 nM	Active Tension at 10 nM	Tension at 30 nM	Active Tension at 30 nM	Tension at 100 nM	Active Tension at 100 nM	Tension at 300 nM	Active Tension at 300 nM
5	1.54	4.04	4.05	0.01	4.00	-0.04	3.89	-0.15	3.30	-0.74	2.92	-1.12	2.55	-1.49	1.77	-2.27	1.33	-2.71	1.22	-2.82	1.15	-2.89	1.07	-2.97	1.06	-2.98
6	1.46	3.44	3.43	0.00	3.40	-0.04	3.34	-0.09	2.80	-0.63	2.12	-1.32	1.91	-1.53	1.47	-1.97	1.16	-2.28	1.06	-2.37	1.01	-2.43	0.95	-2.49	0.96	-2.48
7	1.37	3.36	3.35	0.00	3.27	-0.09	3.15	-0.21	2.51	-0.85	1.95	-1.41	1.75	-1.61	1.41	-1.95	1.19	-2.17	1.14	-2.22	1.11	-2.25	1.08	-2.28	1.10	-2.26
8	1.41	3.53	3.52	0.00	3.48	-0.05	3.41	-0.12	2.65	-0.88	2.06	-1.47	1.90	-1.62	1.47	-2.06	1.06	-2.47	0.95	-2.58	0.91	-2.62	0.85	-2.67	0.80	-2.73

Appendix VI: Drugs Used

Drug stock solutions: DMSO; Dimethylsulfoxide.

Drug	**Pharmacological Action**	**Supplier**	**Molar Mass (g/mol)**	**Mass (mg)**	**Solvent**	**Solvent Volume (ml)**	**Stock solution [mM]**
Acetylcholine chloride (ACh)	Cholinergic agonist	Sigma-Aldrich	181.66	120	D/Water	6.61	100
Adrenaline	α and β adrenoceptor agonist	Pharma. Ltd.	183.2	1	D/Water	1	5.49
ANP	Natriuretic Peptide Receptor-A Agonist	Tocris Bioscience	3080.44	1	D/Water	3.25	0.1
Arginine Vasopressin	V_1, V_2 and V_3 receptors agonist	Mercury Pharmaceuticals	1084.23	0.4	D/Water	1	368.93

Drug stock solutions continued…

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
BNP	Natriuretic Peptide Receptor-A Agonist	Tocris Bioscience	3464.04	1	D/Water	2.88	0.1
Endothelin-1(ET-1) (human)	ETA and ET_B receptors agonist	American Peptide Company	2492.2	3	D/Water	12.04	0.1
Epoprostenol	Prostaglandin I_2 receptor (IP) agonist	GlaxoSmithKlime	374.45	0.5	D/Water	13.35	0.1
Iloprost	Prostaglandin I_2 receptor (IP) agonist	Tocris Bioscience	360.49	1mg	DMSO/Water	0.277	10

Drug stock solutions continued…

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Milrinone	Phosphodiesterase-3 (PDE-3) Inhibitor	Stragen	211.22	10	D/Water	10	4.73
Noradrenaline	Adrenoreceptor Agonist	Aguettant Ltd	169.18	1	D/Water	1.00	6
Potassium Chloride (KCl)	Membrane Depolarisation	Fisher Chemical	74.55	0.745	D/Water	5	2
Prostaglandin F2a	Prostaglandin F Receptor Agonist	Tocris Bioscience	475.62	10	D/Water	2.1	10

Drug stock solutions continued…

Drug	Pharmacological Action	Supplier	Molar Mass (g/mol)	Mass (mg)	Solvent	Solvent Volume (ml)	Stock solution [mM]
Sildenafil	Phosphodiesterase-5 (PDE-5) Inhibitor	Tocris Bioscience	671.2	10	DMSO/Water	1.48	10
Sodium Nitroprusside (SNP)	Nitric oxide donor	Rotta Pharma Plc	297.95	50	D/Water	4.2	40
Treprostenil	Prostaglandin I_2 receptor (IP) agonist	Tocris Bioscience	390.51	10	Ethanol/water	2.56	10

Appendix VII: Test Of Normality

Optimal Resting Tension Experiments

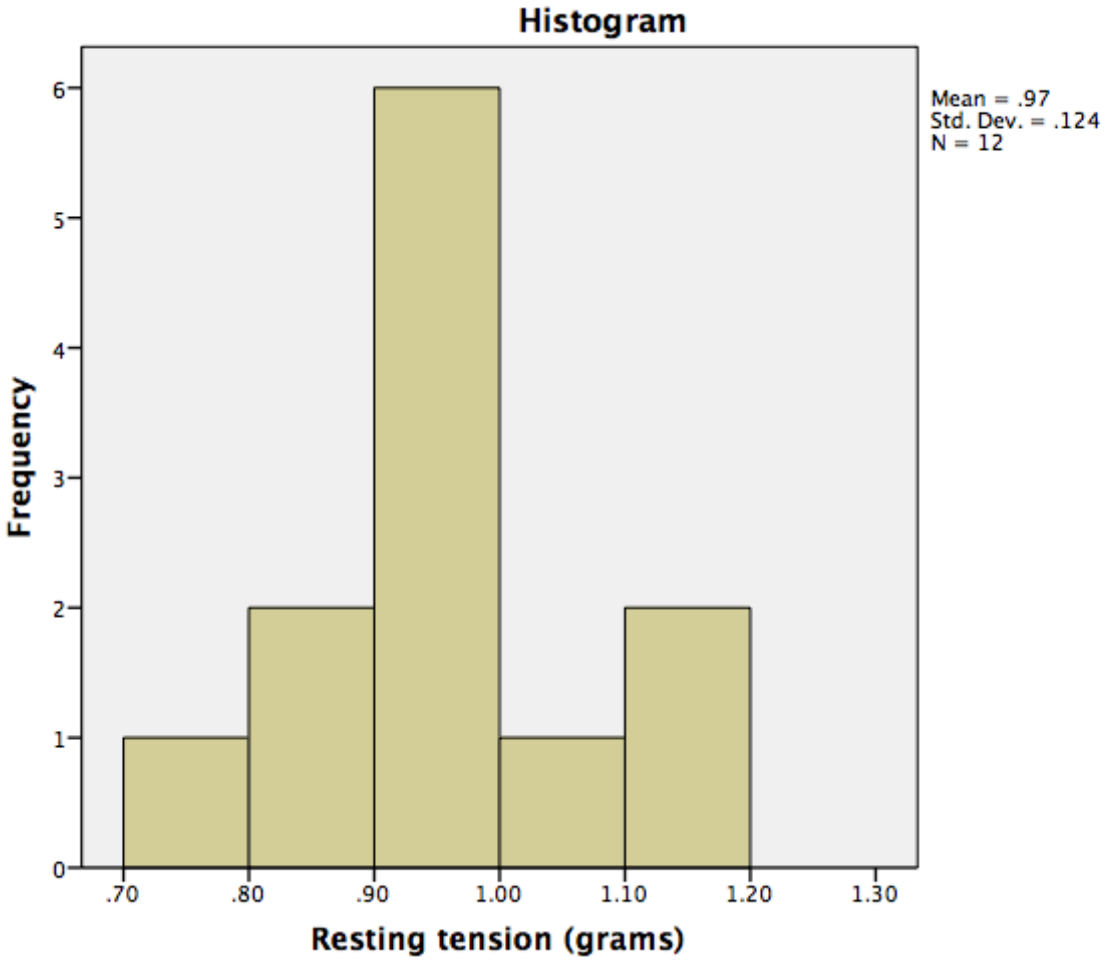
(1) Tension in arteries undergoing optimal resting tension measurement using multi-wire myograph system:

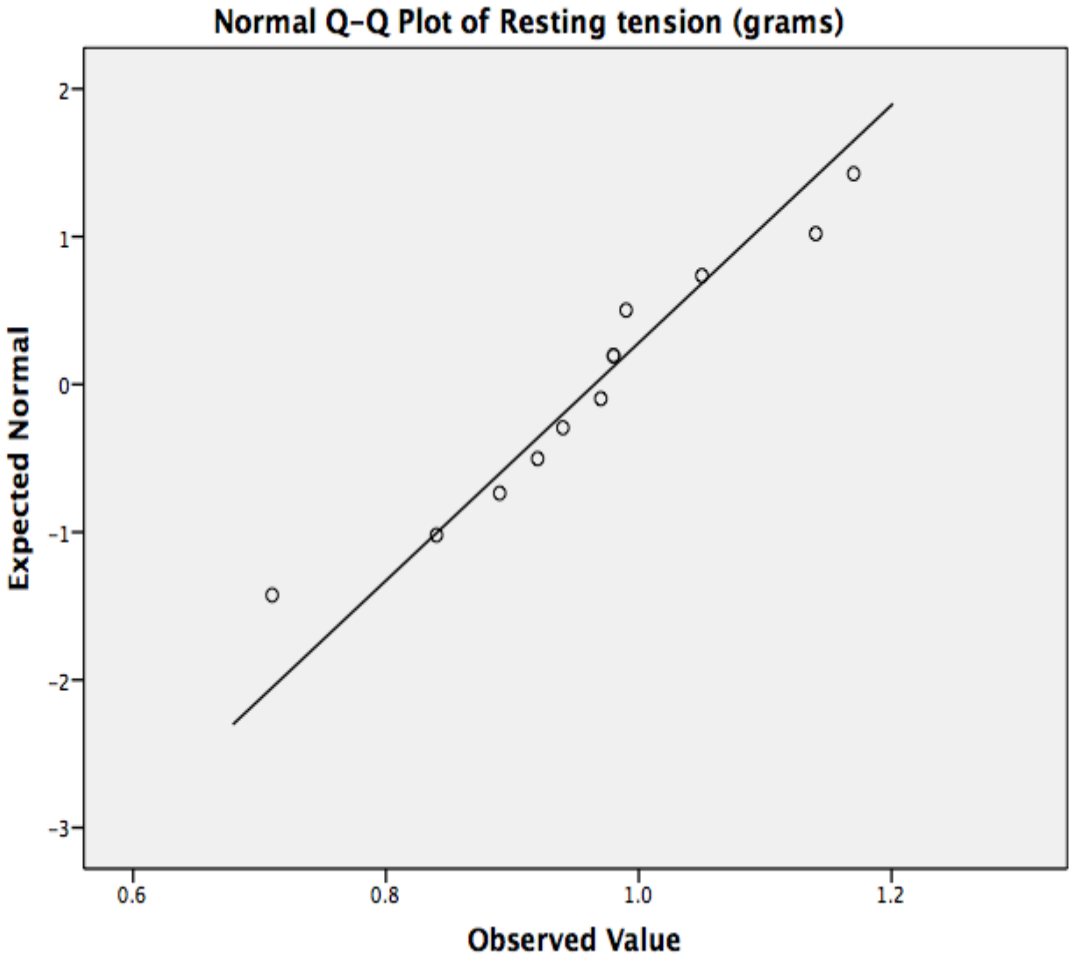
Tests of Normality

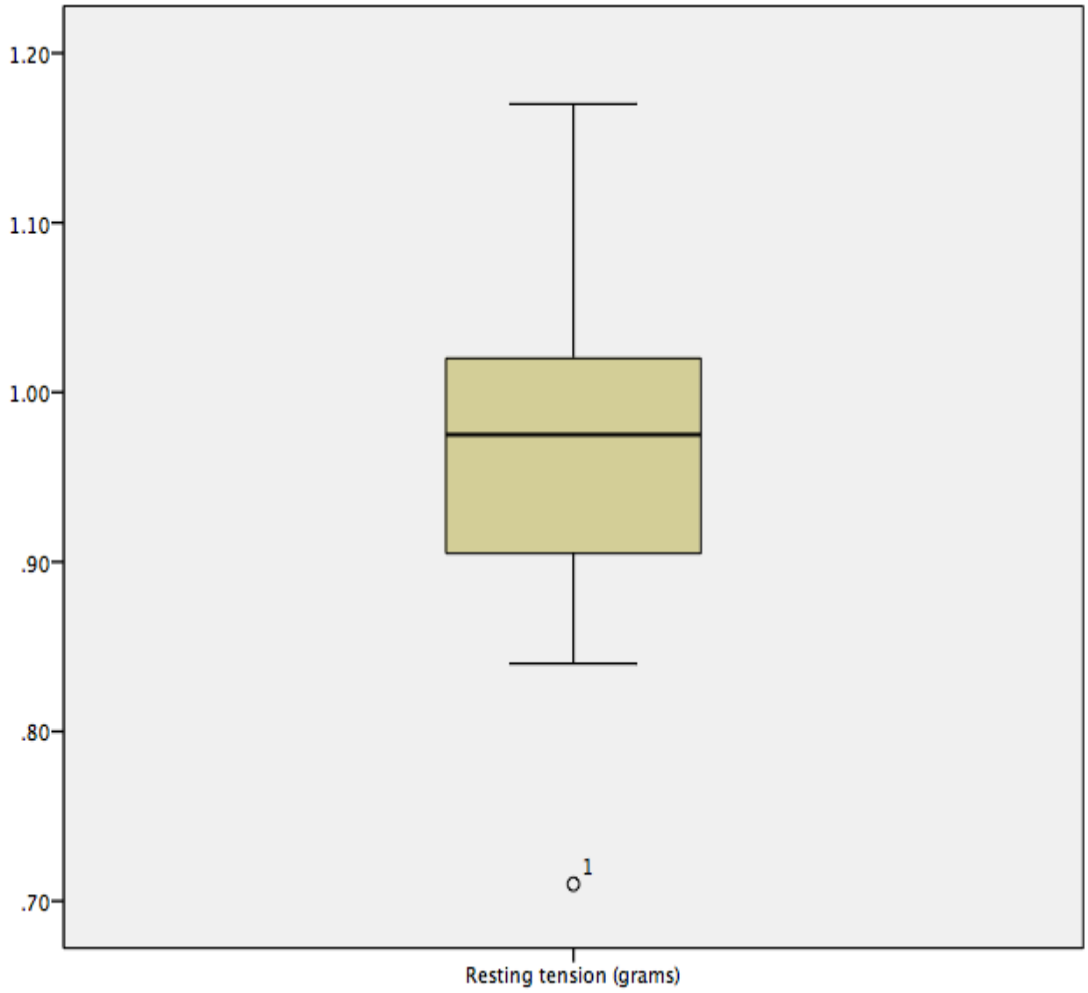
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.170	12	.200*	.962	12	.809

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = .97
Std. Dev. = .124
N = 12
Frequency
0
1
2
3
4
5
6
.70
.80
.90
1.00
1.10
1.20
1.30
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
-3
0.6
0.8
1.0
1.2
Observed Value


1.20
1.10
1.00
.90
.80
.70
1
Resting tension (grams)

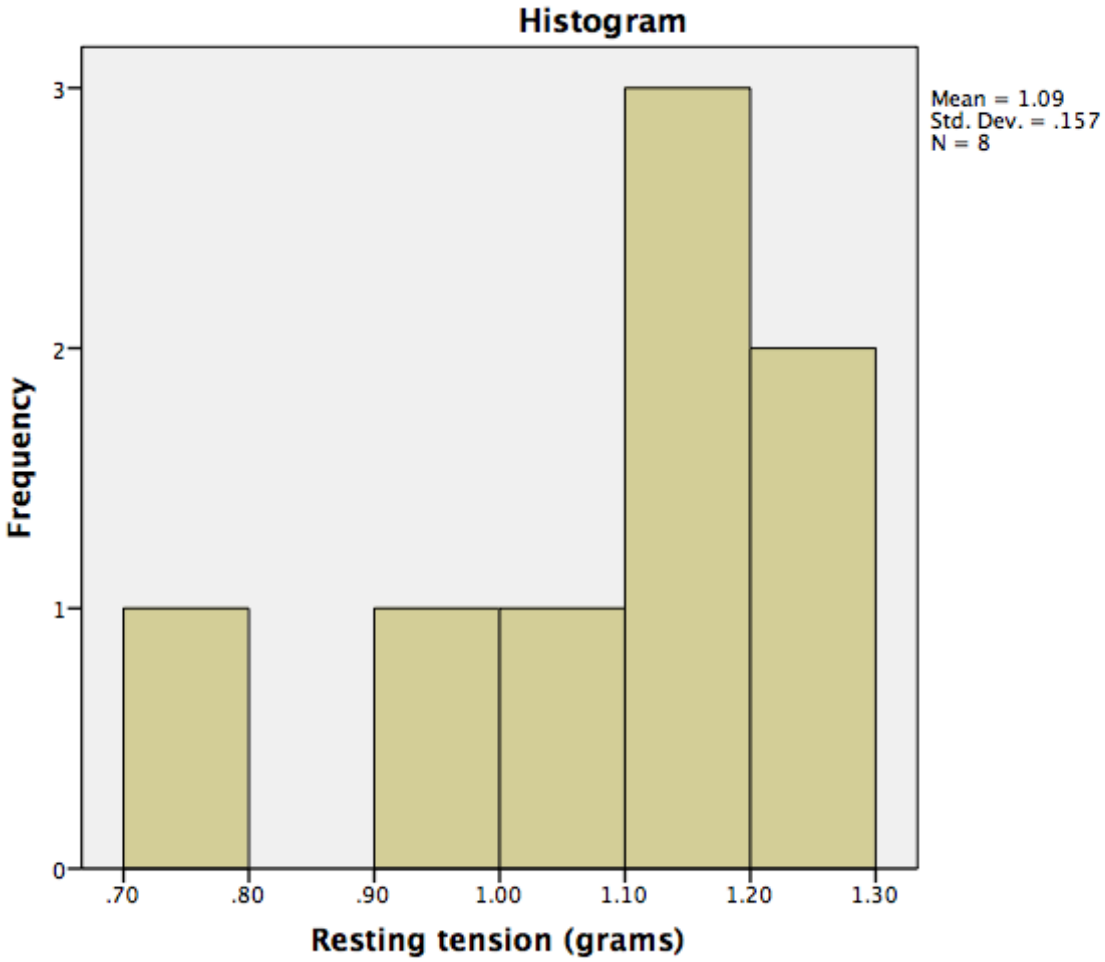
(2) Tension in arteries undergoing optimal resting tension measurement using organ bath system:

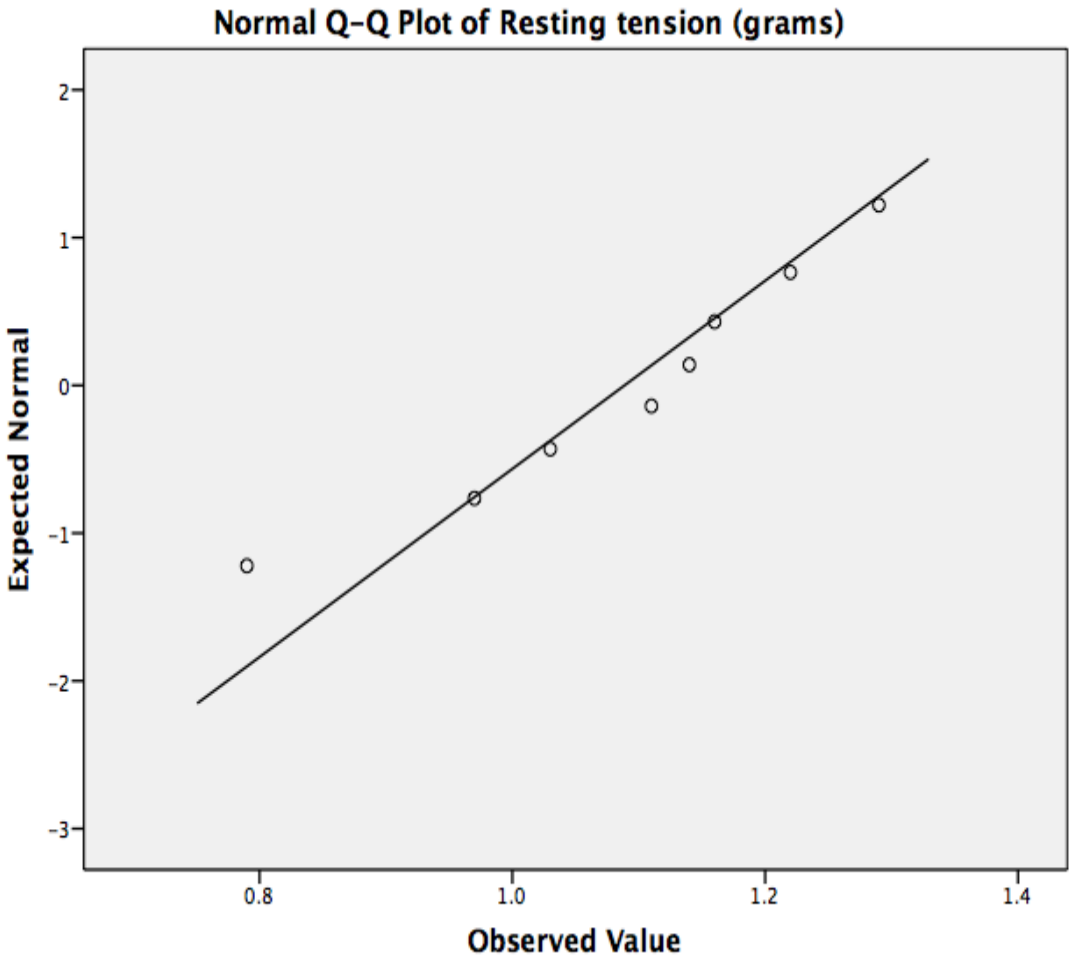
Tests of Normality

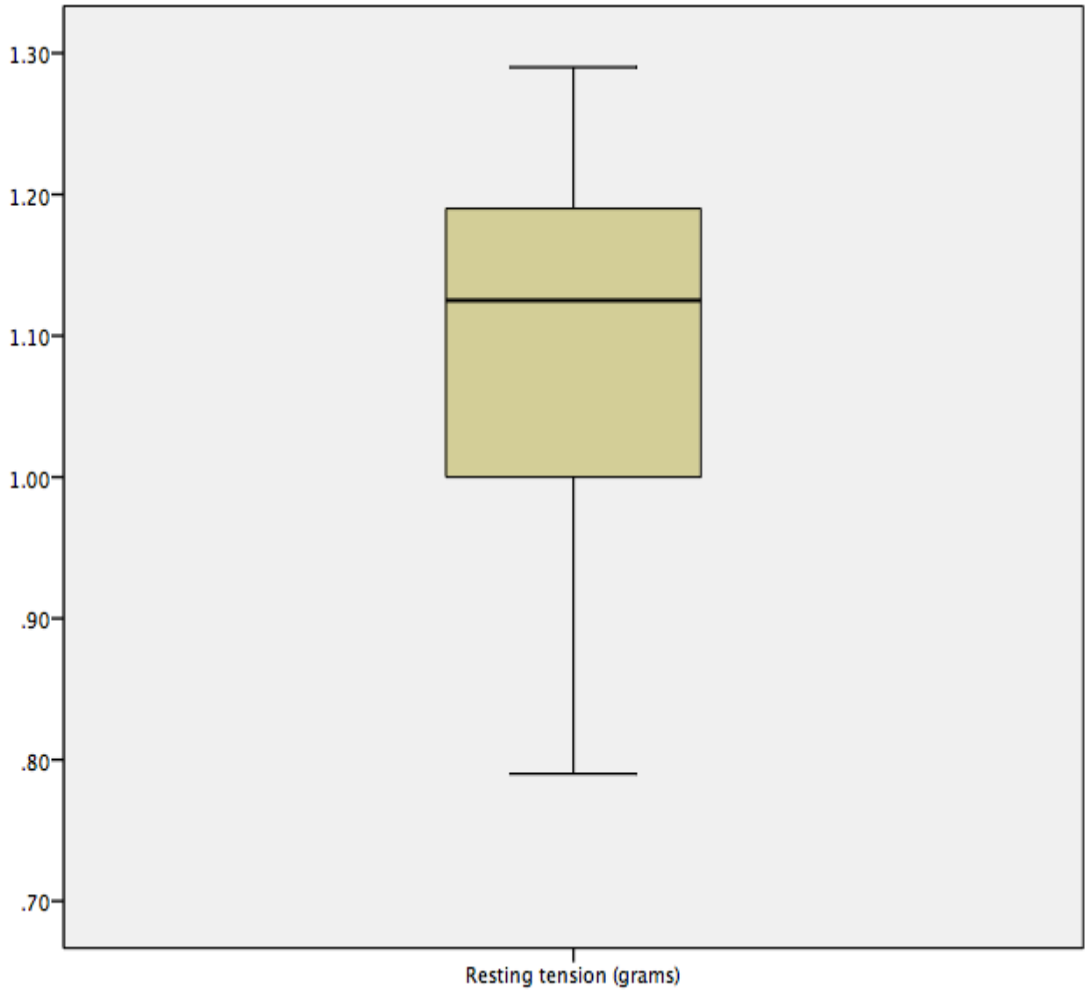
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.179	8	.200*	.955	8	.760

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.09
Std. Dev. = .157
N = 8
Frequency
0
1
2
3
.70
.80
.90
1.00
1.10
1.20
1.30
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
-3
0.8
1.0
1.2
1.4
Observed Value


1.30
1.20
1.10
1.00
.90
.80
.70
Resting tension (grams)

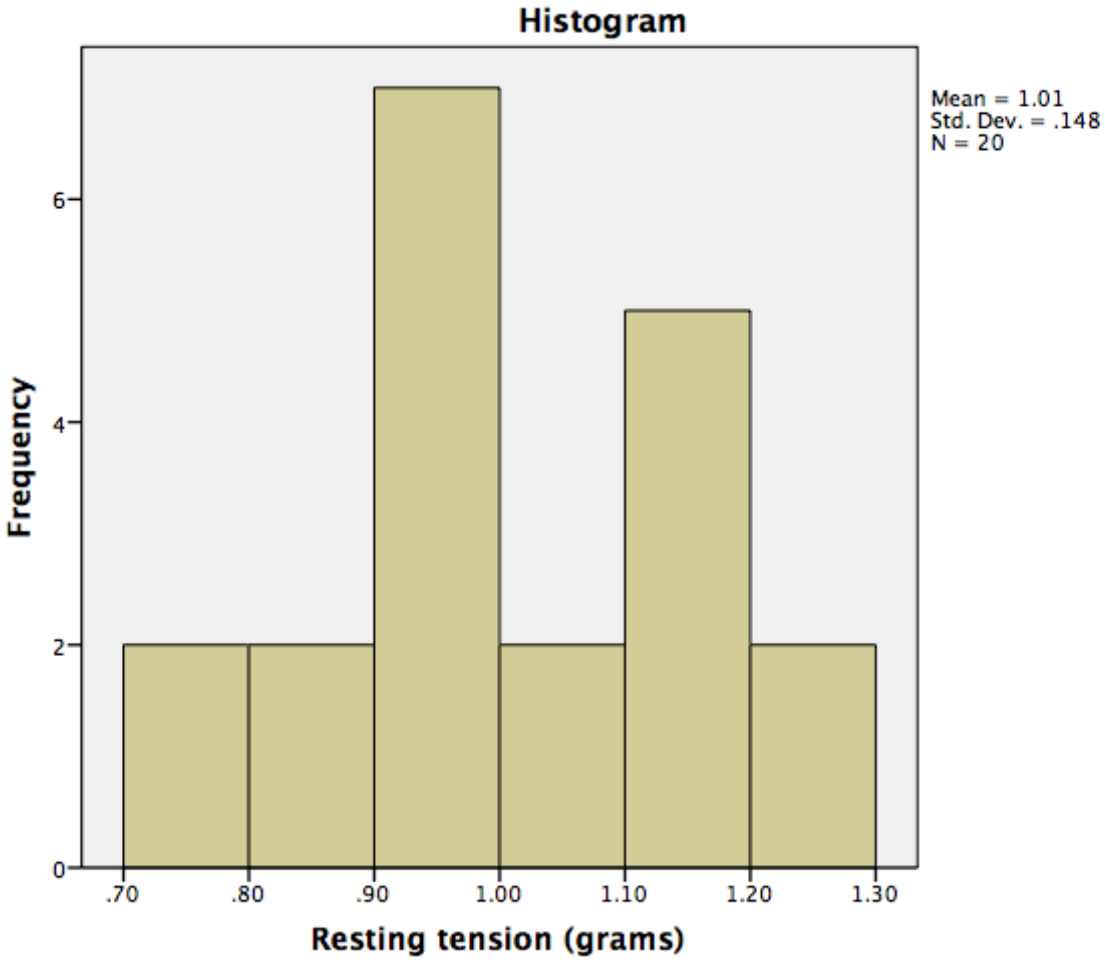
(3) Tension in arteries undergoing optimal resting tension measurement using both organ bath and multi-wire myograph system:

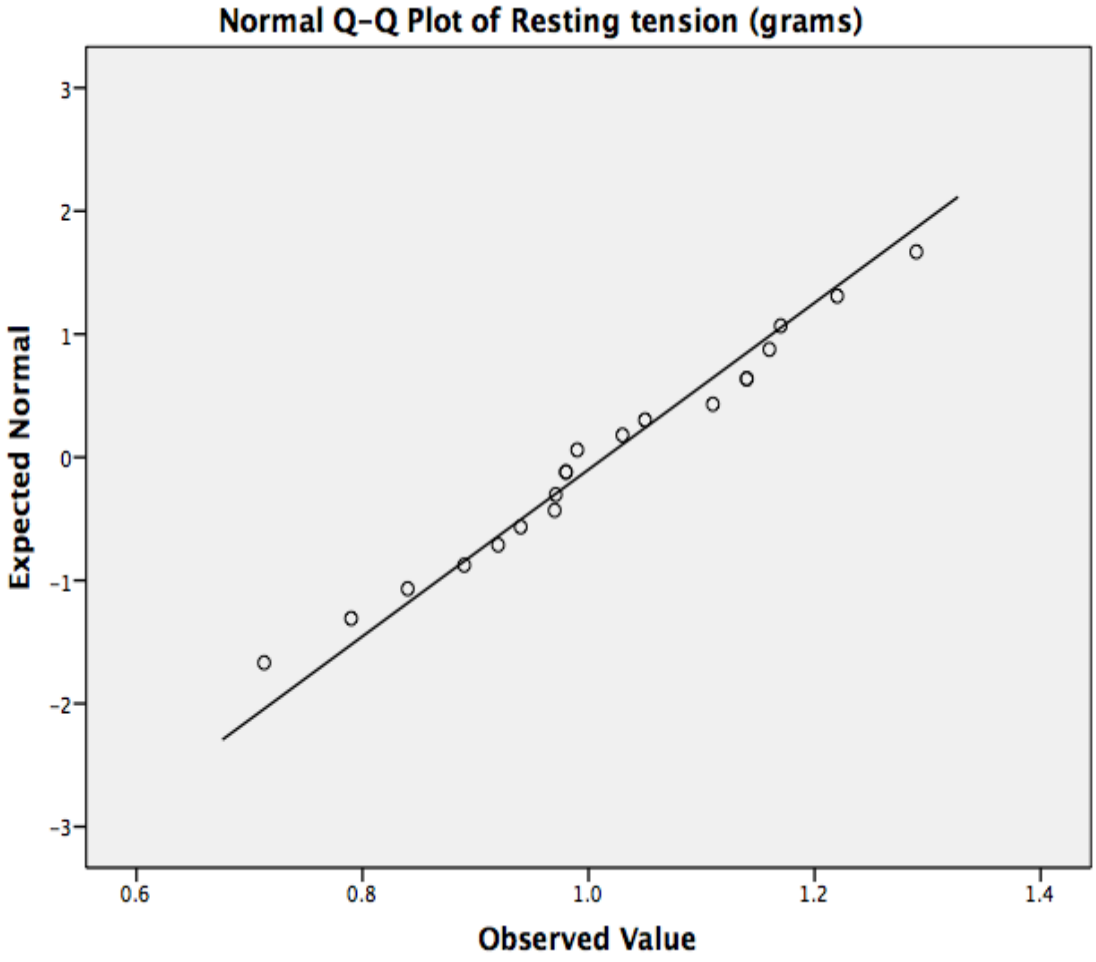
Tests of Normality

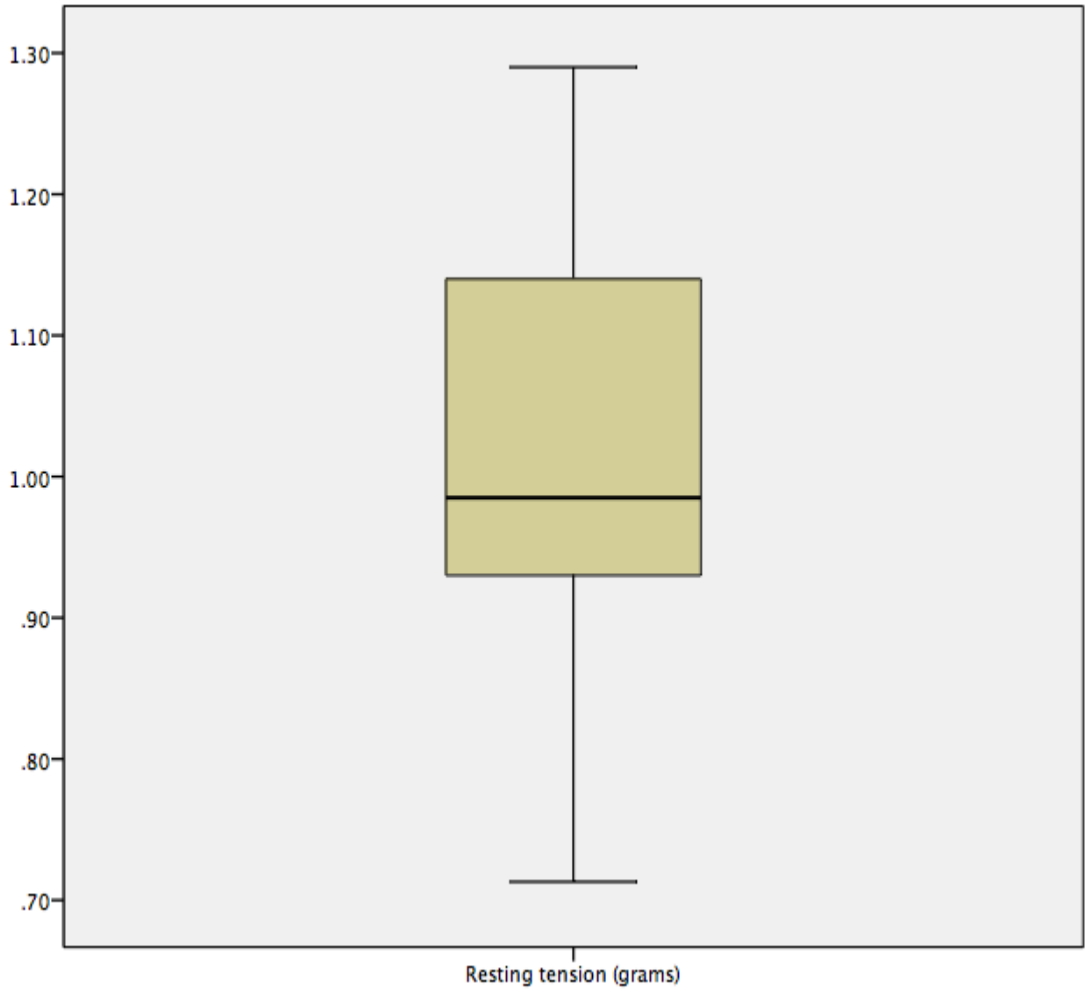
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.116	20	.200*	.983	20	.965

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.01
Std. Dev. = .148
N = 20
Frequency
0
2
4
6
.70
.80
.90
1.00
1.10
1.20
1.30
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
3
2
1
0
-1
-2
-3
0.6
0.8
1.0
1.2
1.4
Observed Value


1.30
1.20
1.10
1.00
.90
.80
.70
Resting tension (grams)

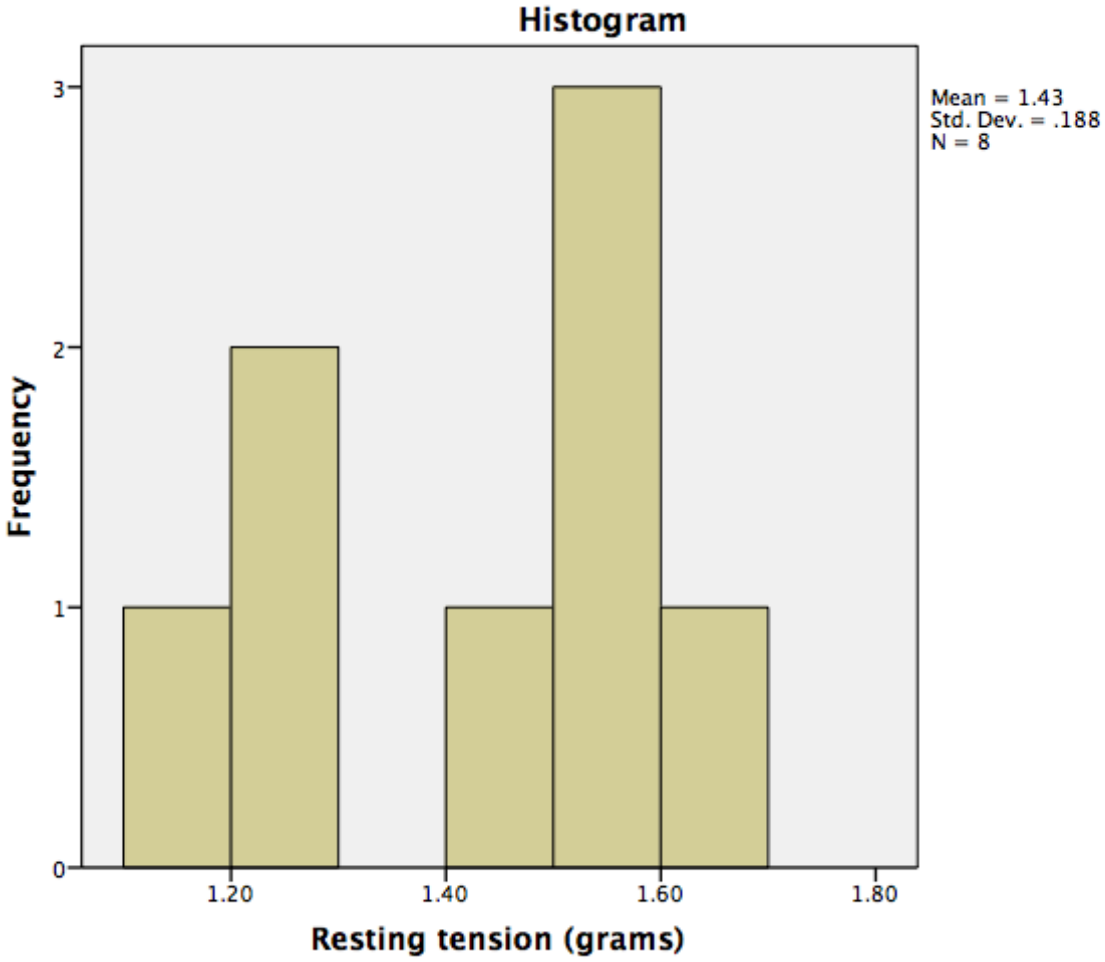
The Differential Effects of Systemic Vasoconstrictors on Human Pulmonary Artery Tension Experiments

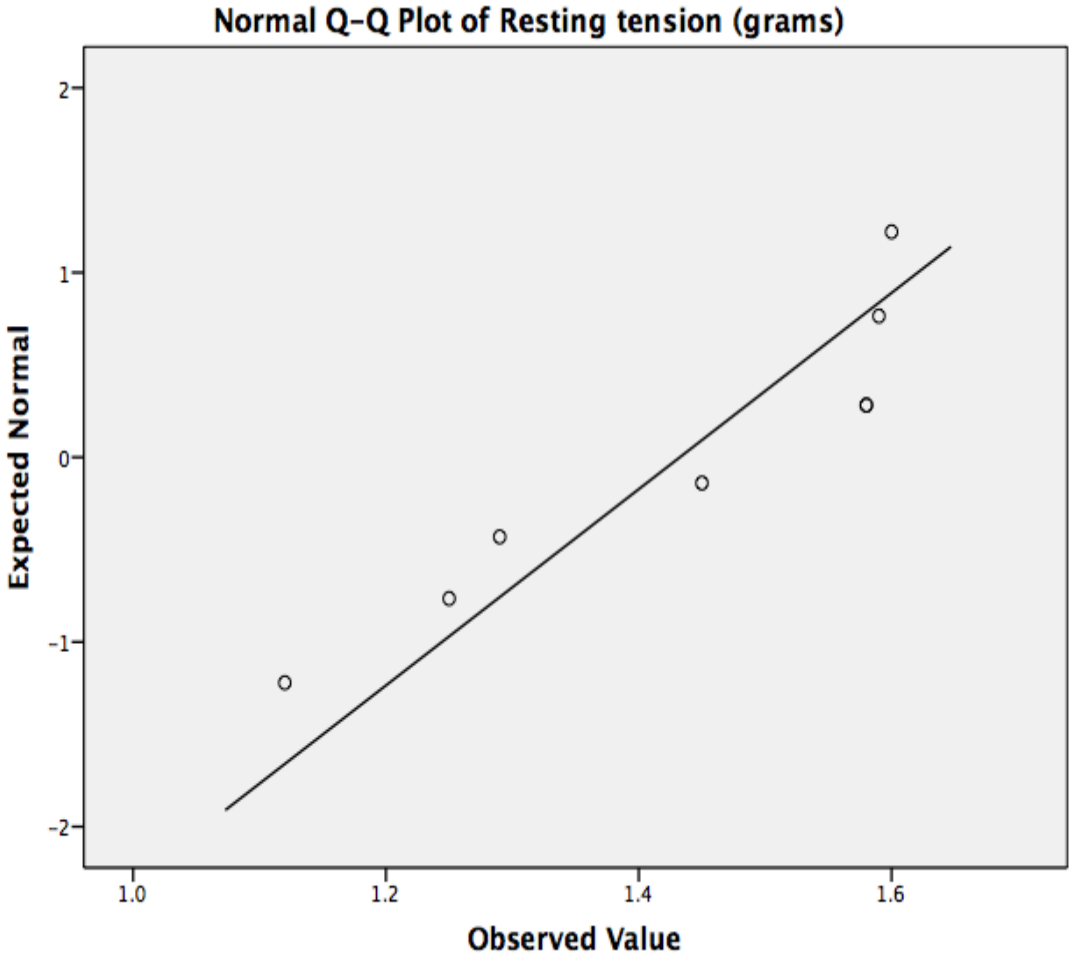
(1) Tensions for Adrenaline experiments:

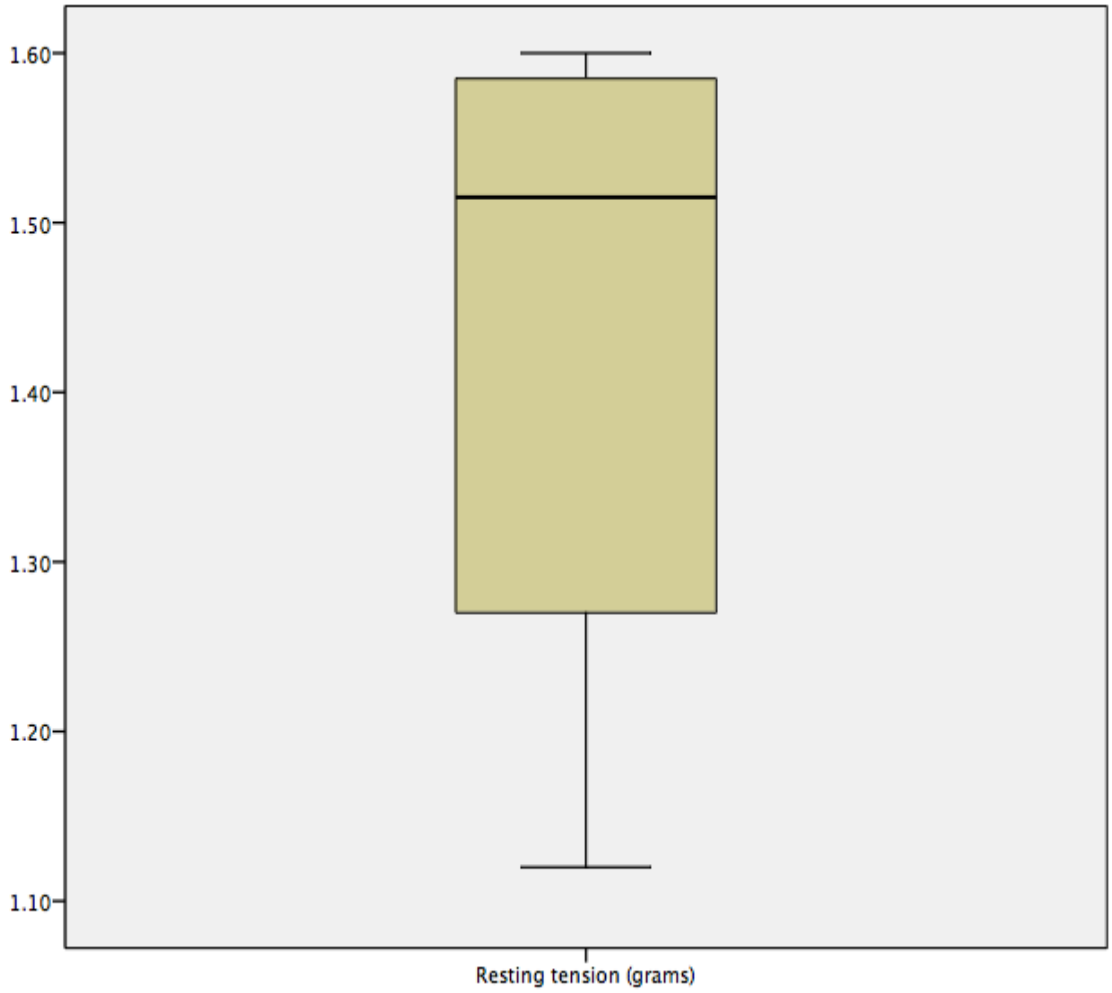
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.283	8	.058	.839	8	.074

a. Lilliefors Significance Correction


Histogram
Mean = 1.43
Std. Dev. = .188
N = 8
Frequency
0
1
2
3
1.20
1.40
1.60
1.80
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.0
1.2
1.4
1.6
Observed Value

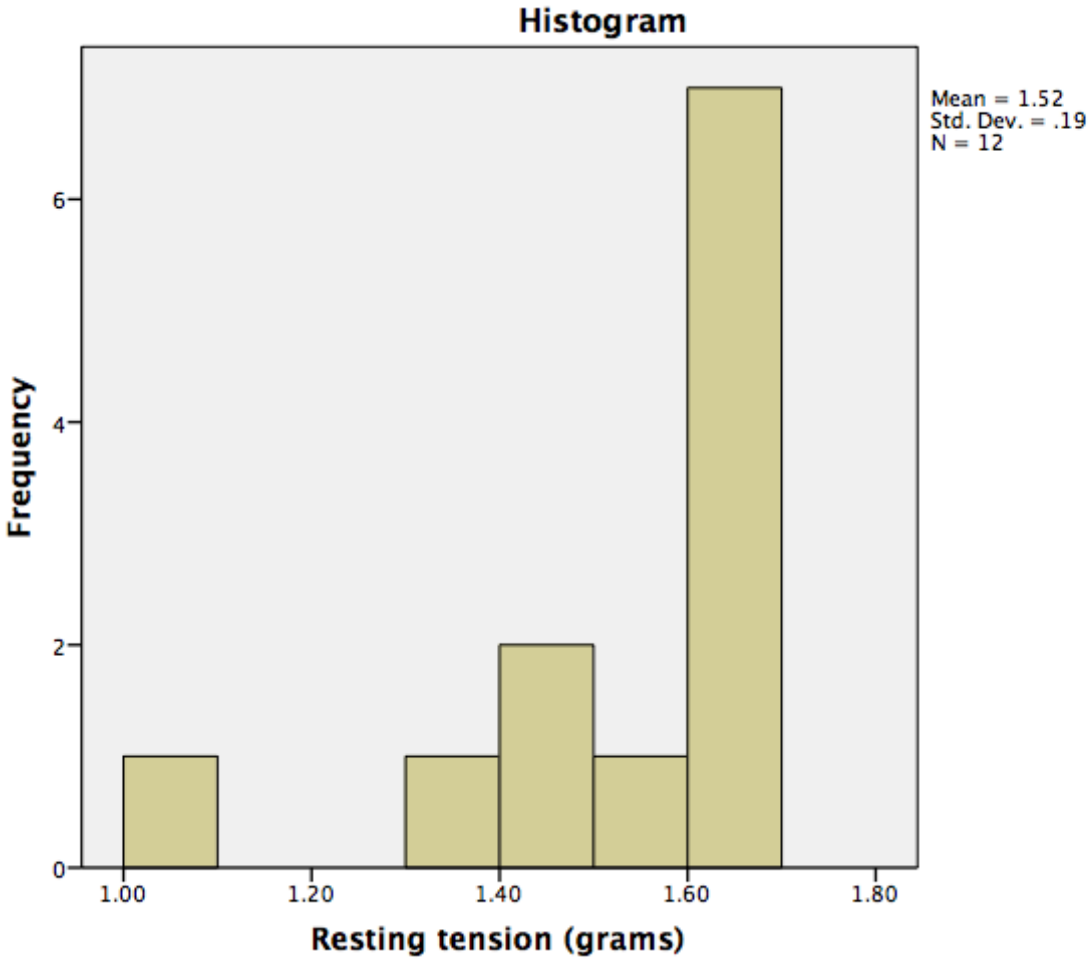

1.60
1.50
1.40
1.30
1.20
1.10
Resting tension (grams)

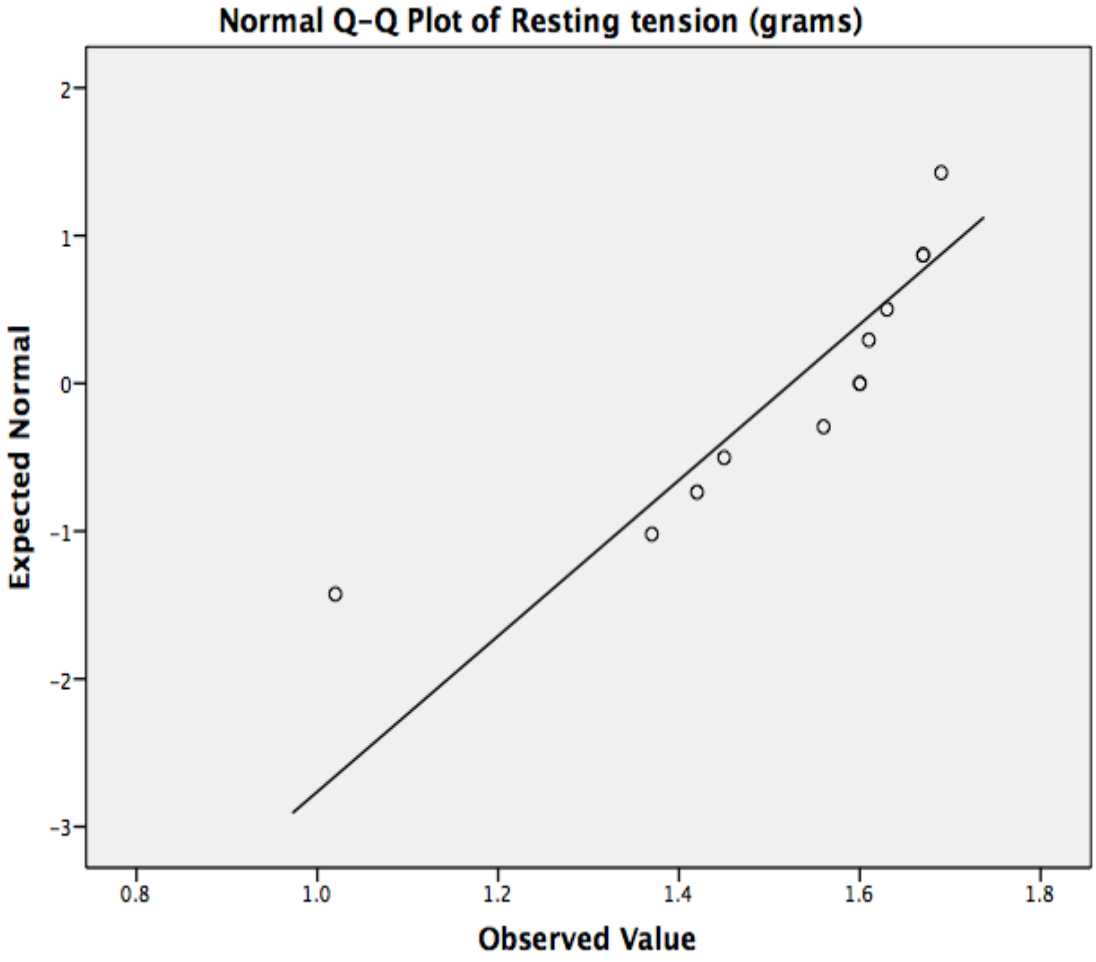
(2) Tensions for Nor-adrenaline experiments:

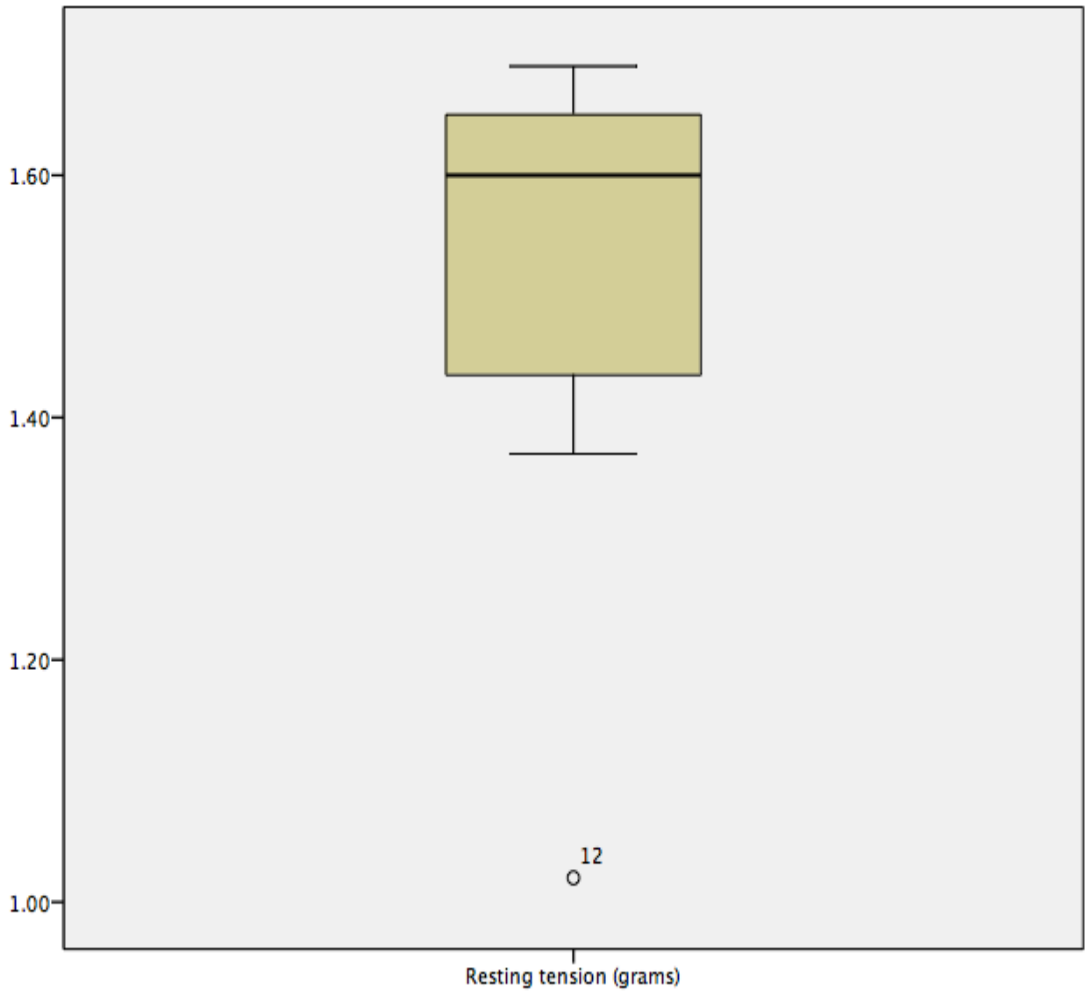
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.242	12	.051	.788	12	.007

a. Lilliefors Significance Correction


Histogram
Mean = 1.52
Std. Dev. = .19
N = 12
Frequency
0
2
4
6
1.00
1.20
1.40
1.60
1.80
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
-3
0.8
1.0
1.2
1.4
1.6
1.8
Observed Value

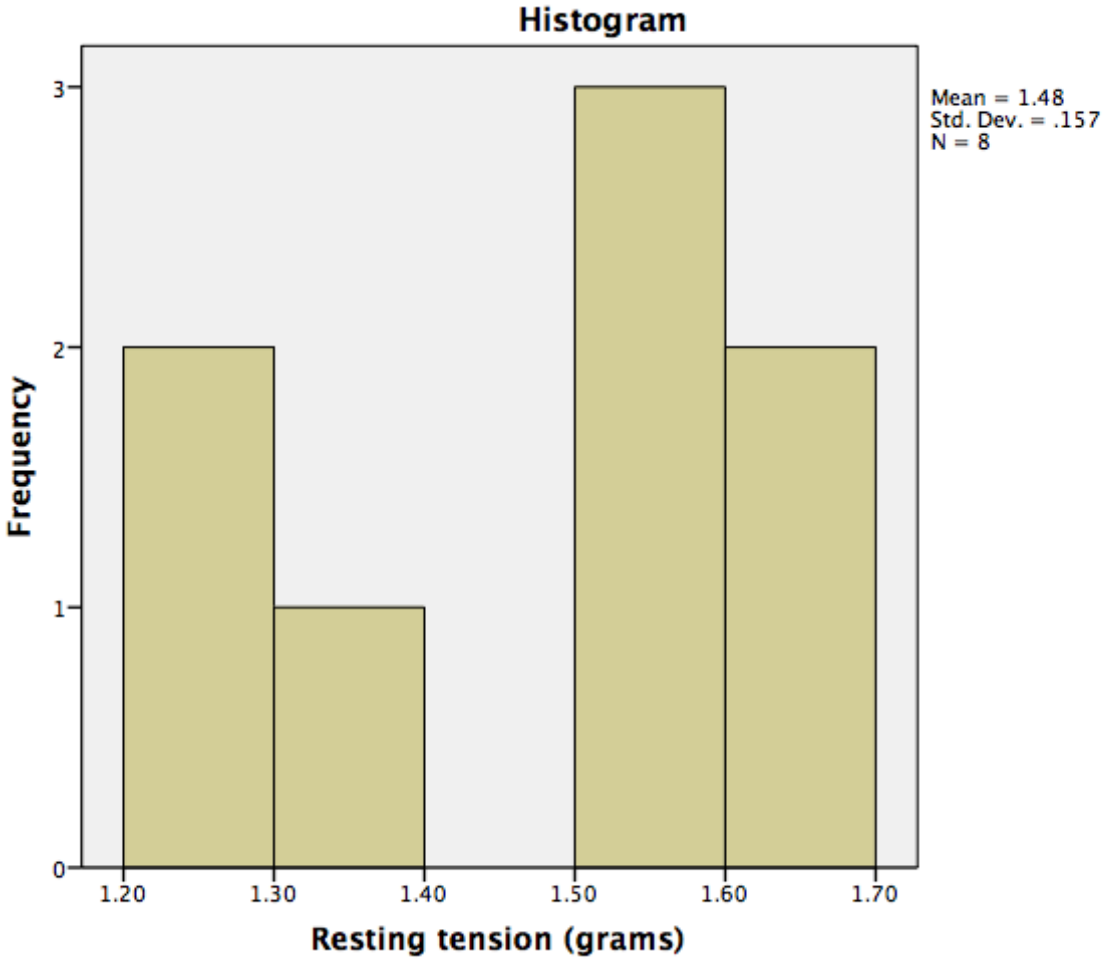

1.60
1.40
1.20
1.00
12
Resting tension (grams)

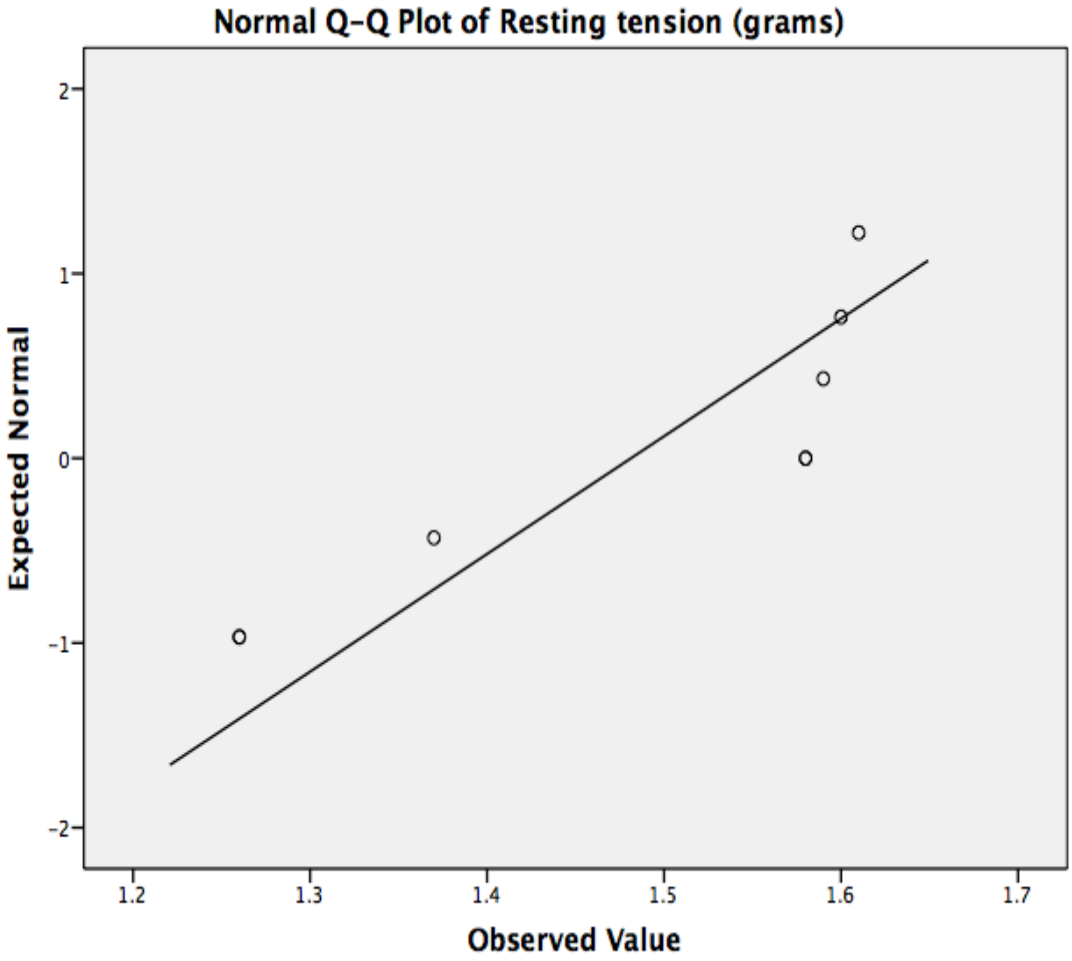
(3) Tensions for Endothelin -1 experiments:

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.360	8	.003	.743	8	.007

a. Lilliefors Significance Correction


Histogram
Mean = 1.48
Std. Dev. = .157
N = 8
Frequency
0
1
2
3
1.20
1.30
1.40
1.50
1.60
1.70
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.2
1.3
1.4
1.5
1.6
1.7
Observed Value

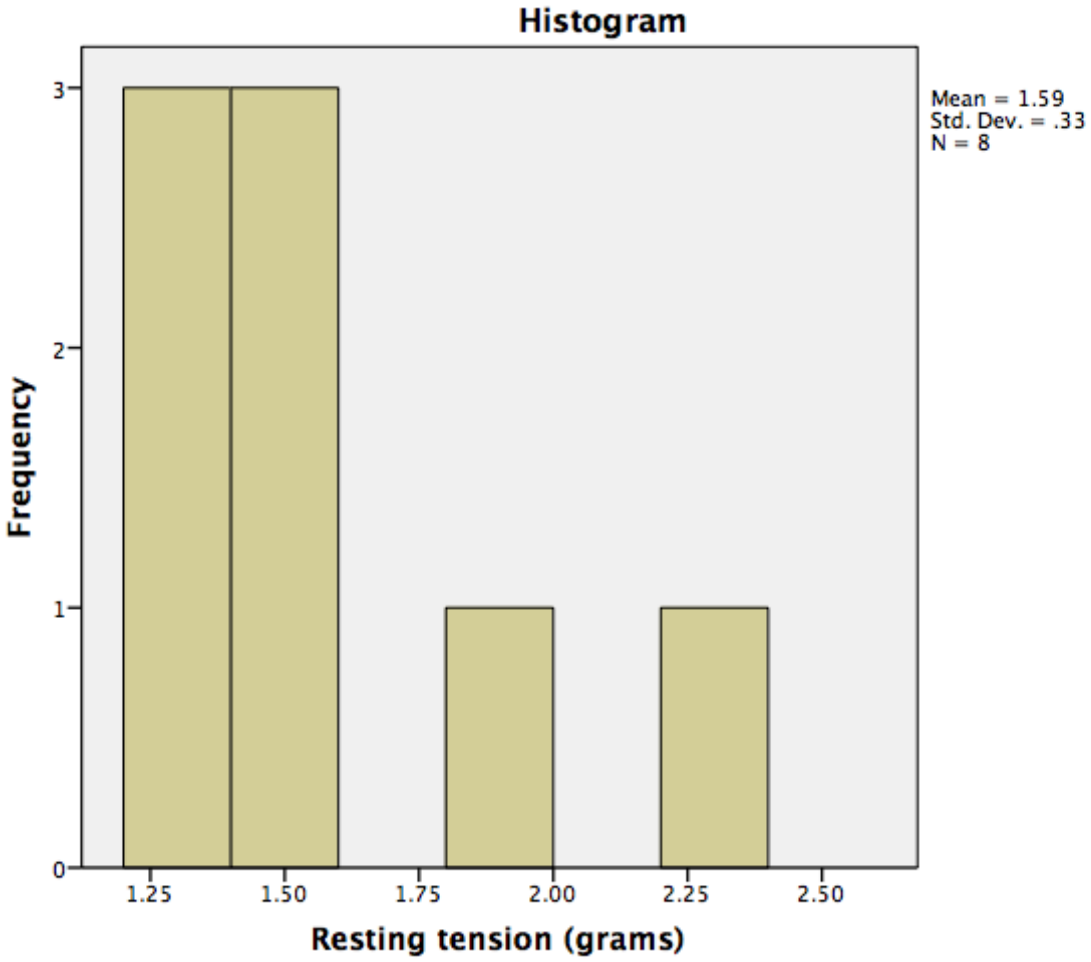

1.70
1.60
1.50
1.40
1.30
1.20
Resting tension (grams)

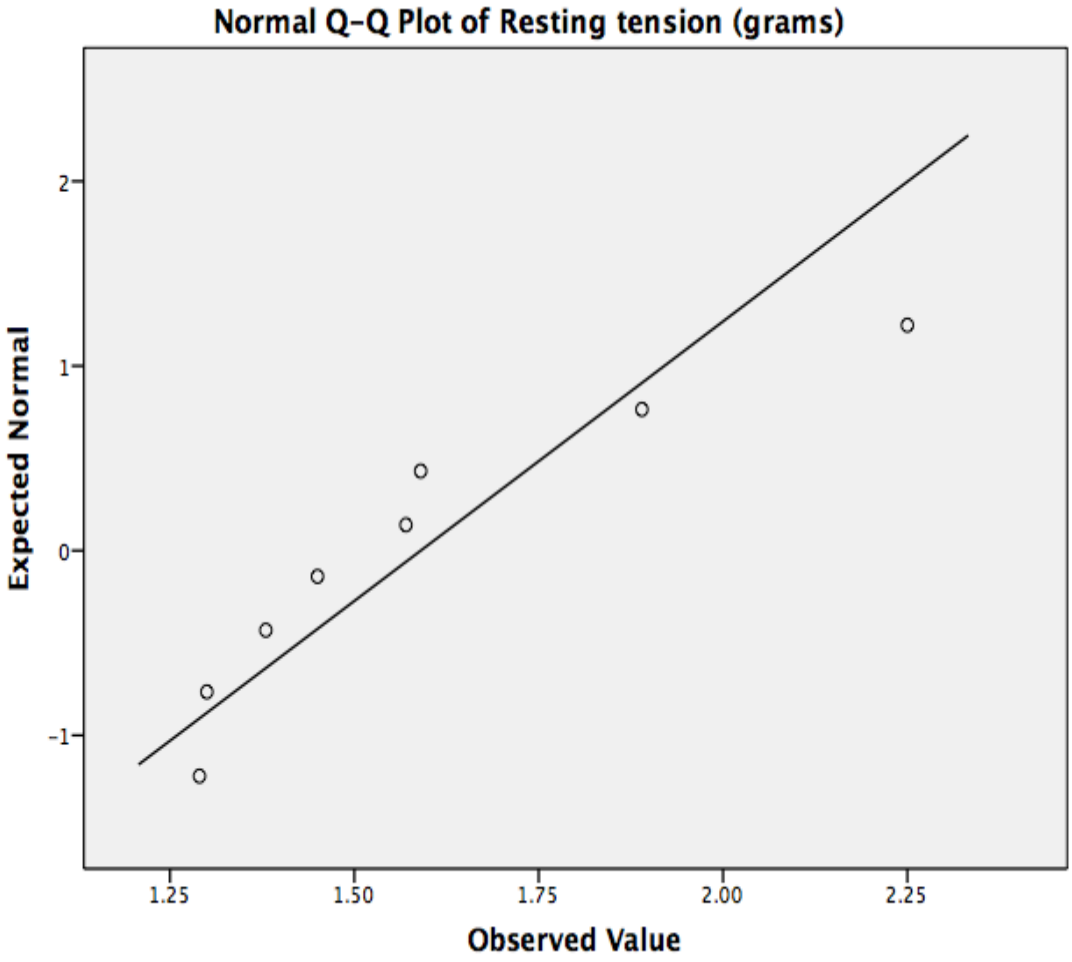
(4) Tensions for Prostaglandin F2α experiments:

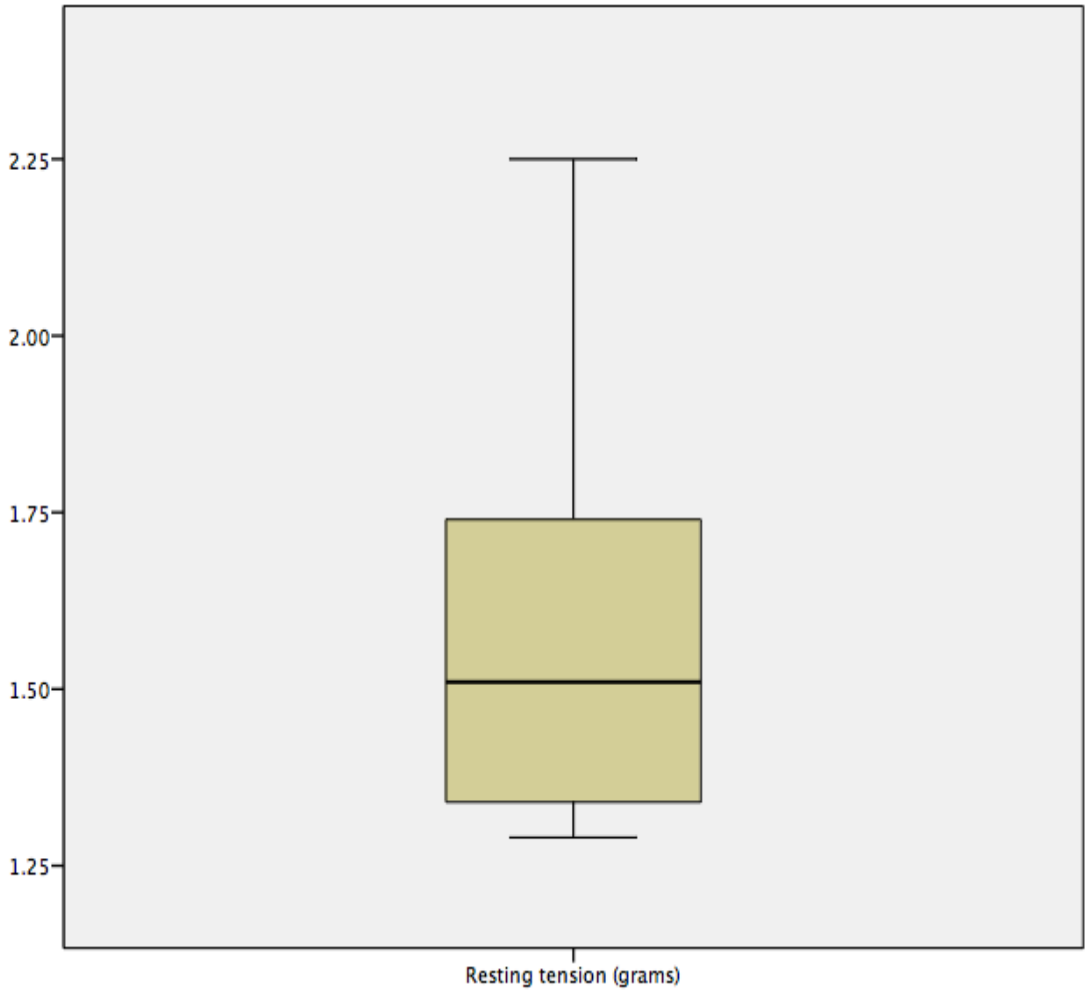
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.250	8	.150	.861	8	.123

a. Lilliefors Significance Correction


Histogram
Mean = 1.59
Std. Dev. = .33
N = 8
Frequency
3
2
1
0
1.25
1.50
1.75
2.00
2.25
2.50
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
1.25
1.50
1.75
2.00
2.25
Observed Value

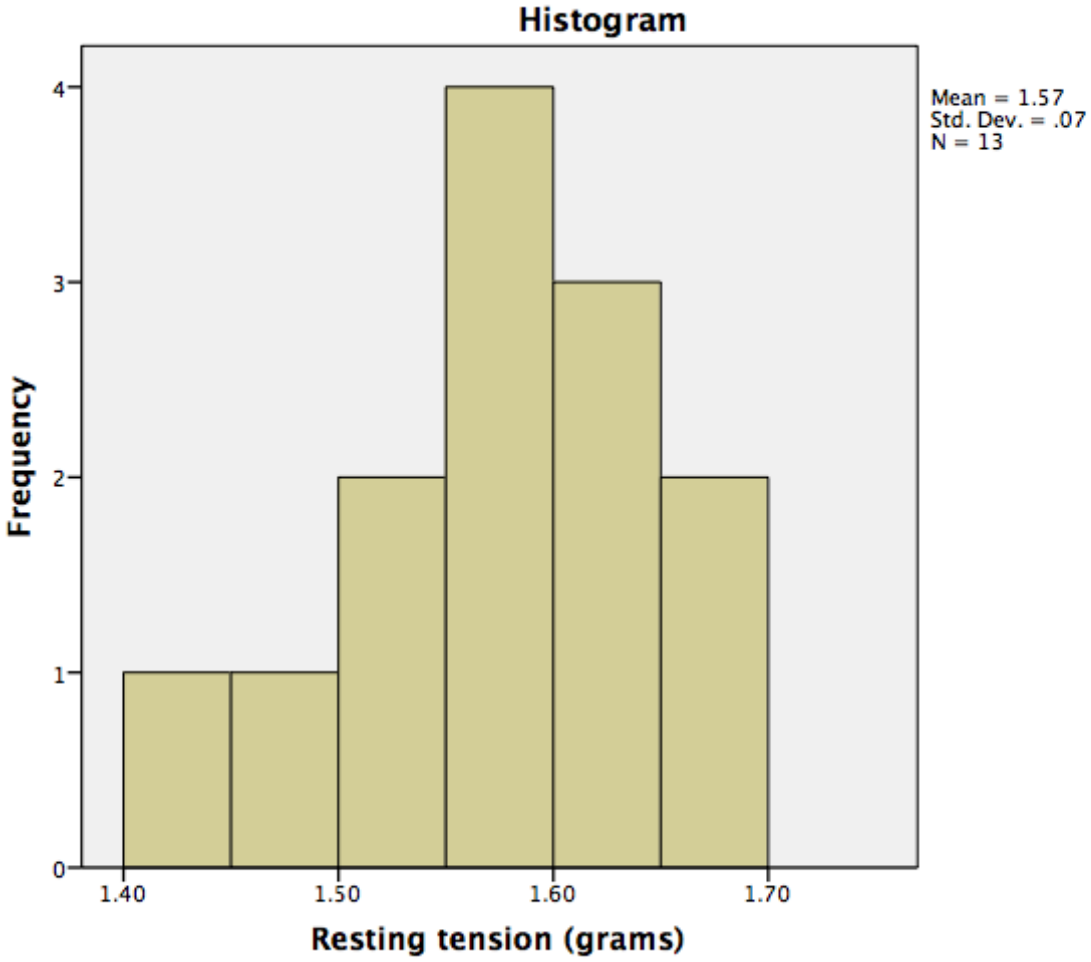

2.25
2.00
1.75
1.50
1.25
Resting tension (grams)

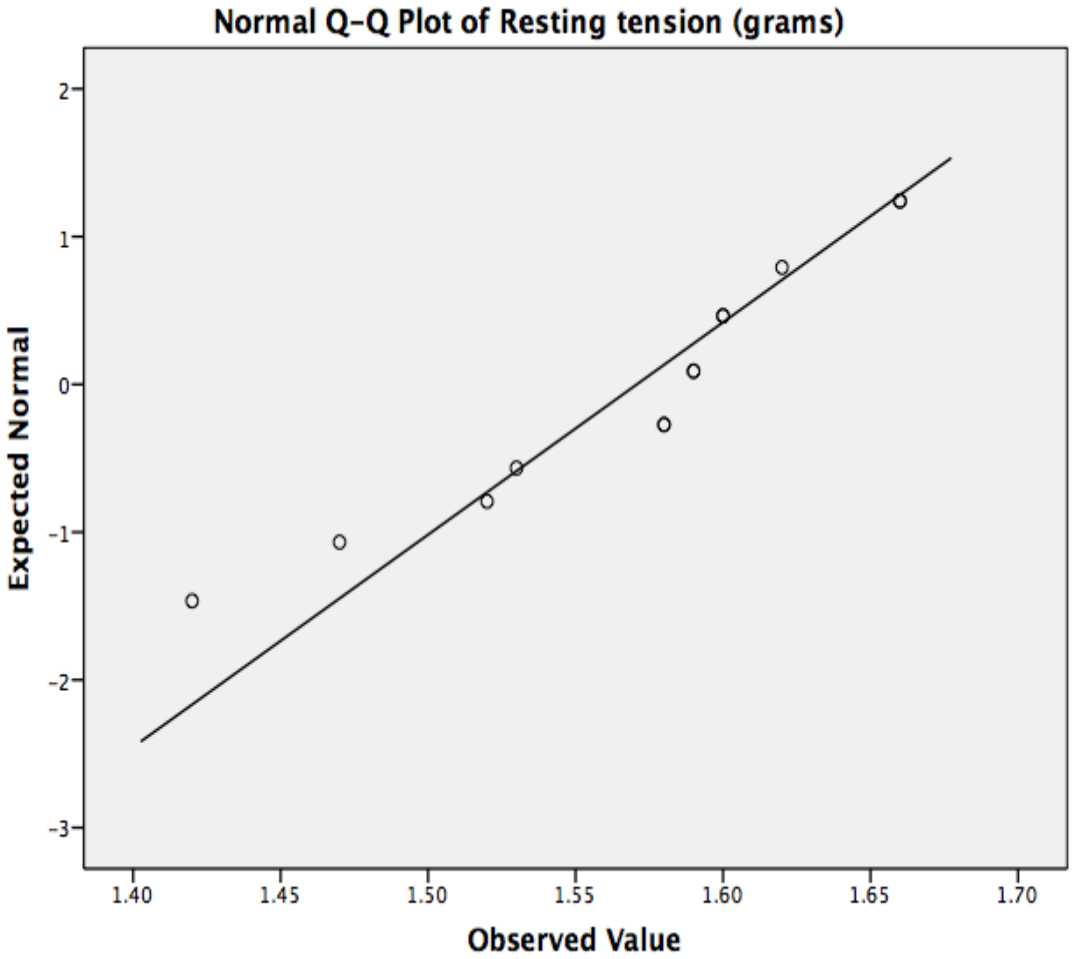
(5) Tensions for Potassium Chloride experiments:

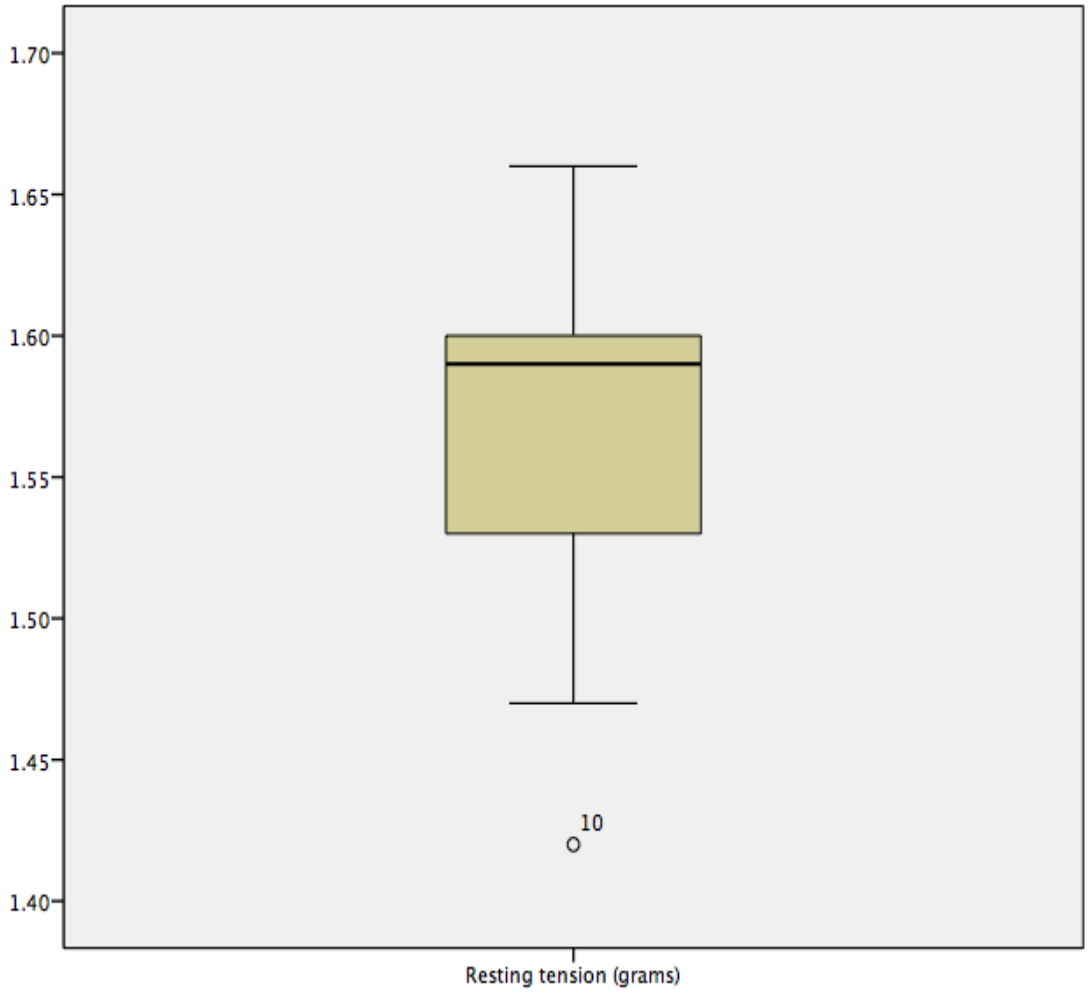
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.245	13	.032	.915	13	.216

a. Lilliefors Significance Correction


Histogram
Mean = 1.57
Std. Dev. = .07
N = 13
Frequency
0
1
2
3
4
1.40
1.50
1.60
1.70
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
-3
1.40
1.45
1.50
1.55
1.60
1.65
1.70
Observed Value


1.70
1.65
1.60
1.55
1.50
1.45
1.40
10
Resting tension (grams)

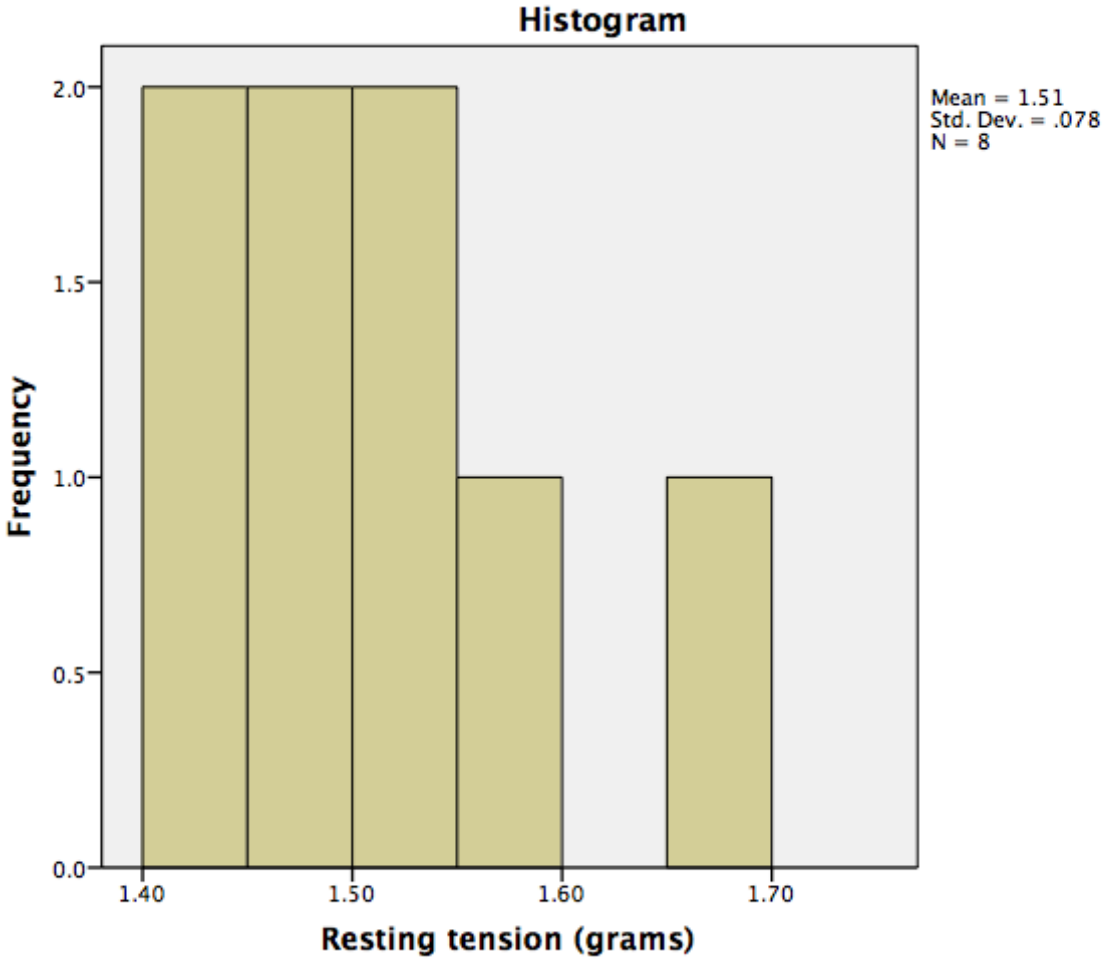
(6) Tensions for Arginine Vasopressin experiments:

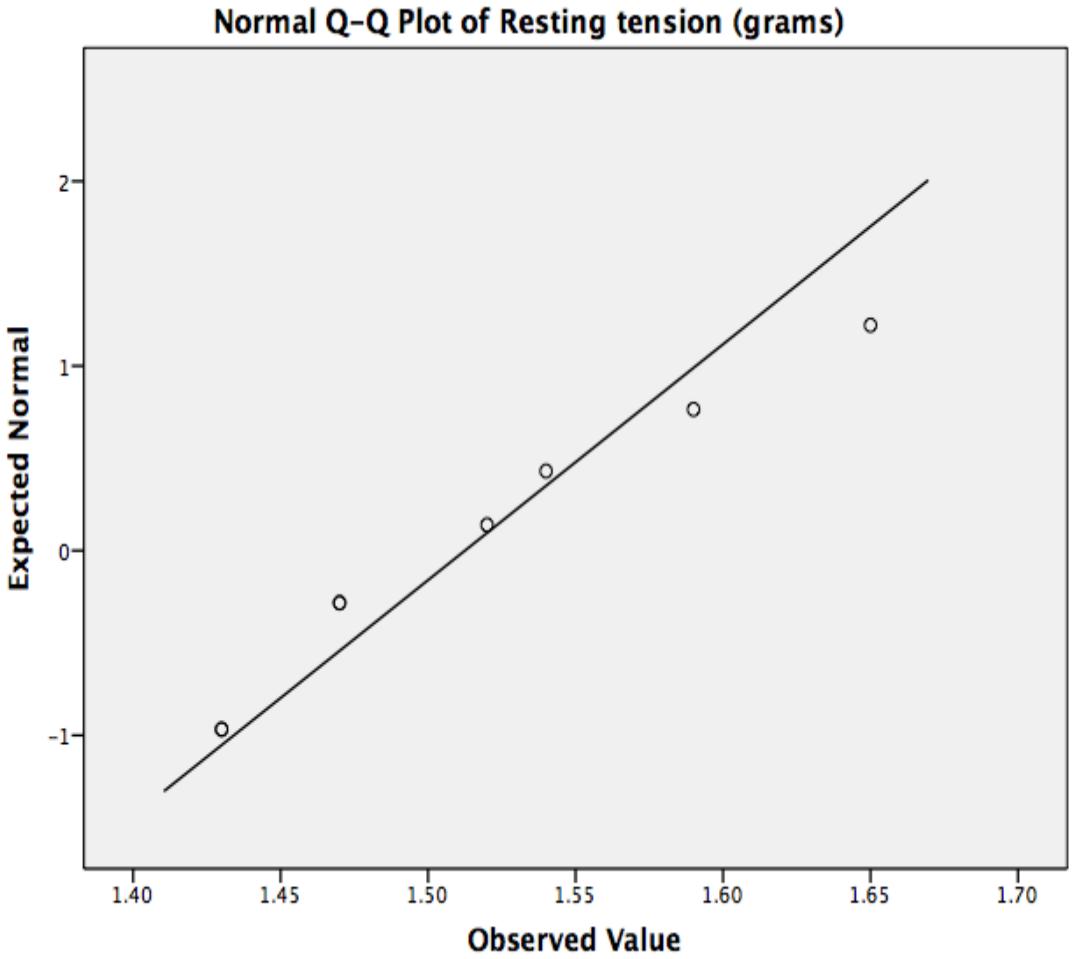
Tests of Normality

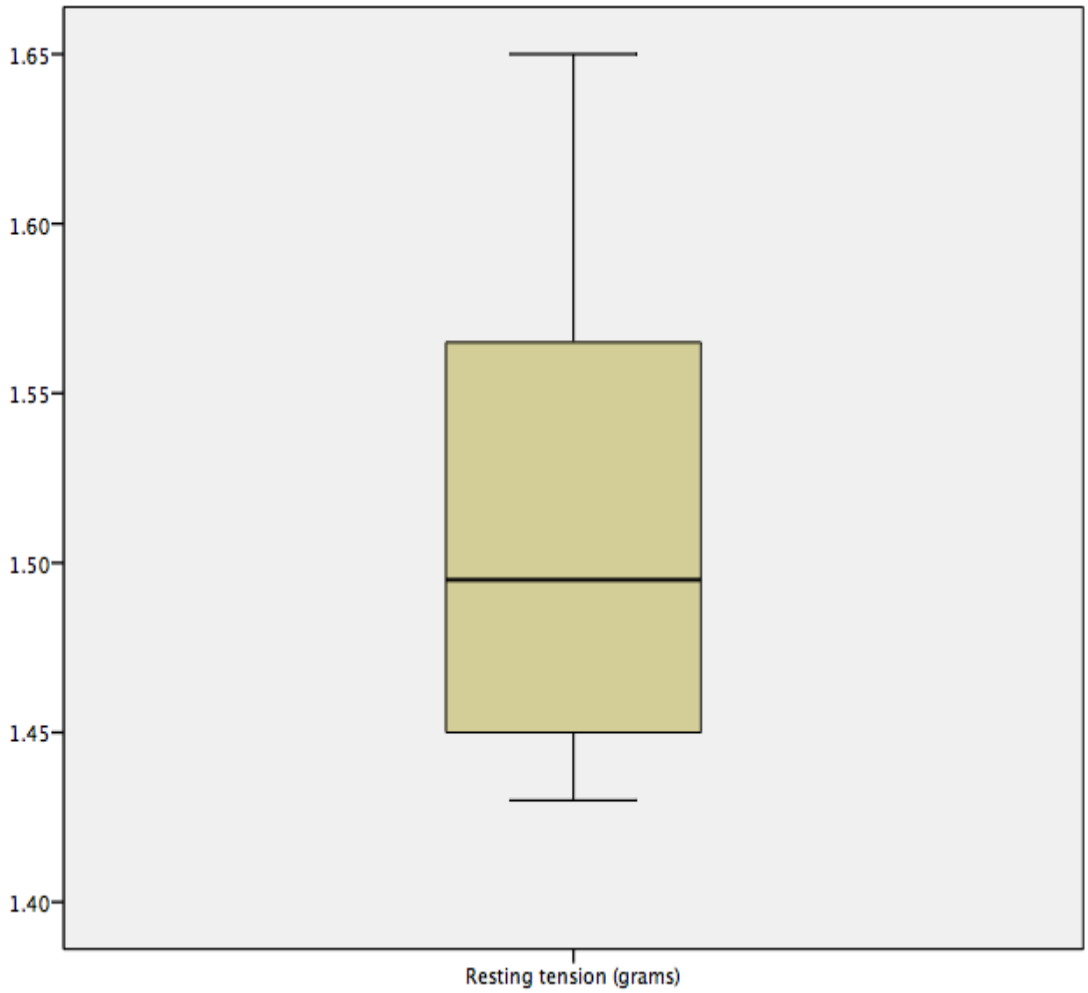
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.206	8	.200*	.920	8	.432

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.51
Std. Dev. = .078
N = 8
Frequency
2.0
1.5
1.0
0.5
0.0
1.40
1.50
1.60
1.70
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
1.40
1.45
1.50
1.55
1.60
1.65
1.70
Observed Value


1.65
1.60
1.55
1.50
1.45
1.40
Resting tension (grams)

In Vitro Characterisation Of Pharmacological Effect Of Prostacyclin Analogues In Comparison To Phosphodiesterase Inhibitors On Small Human Pulmonary Vessels Experiments

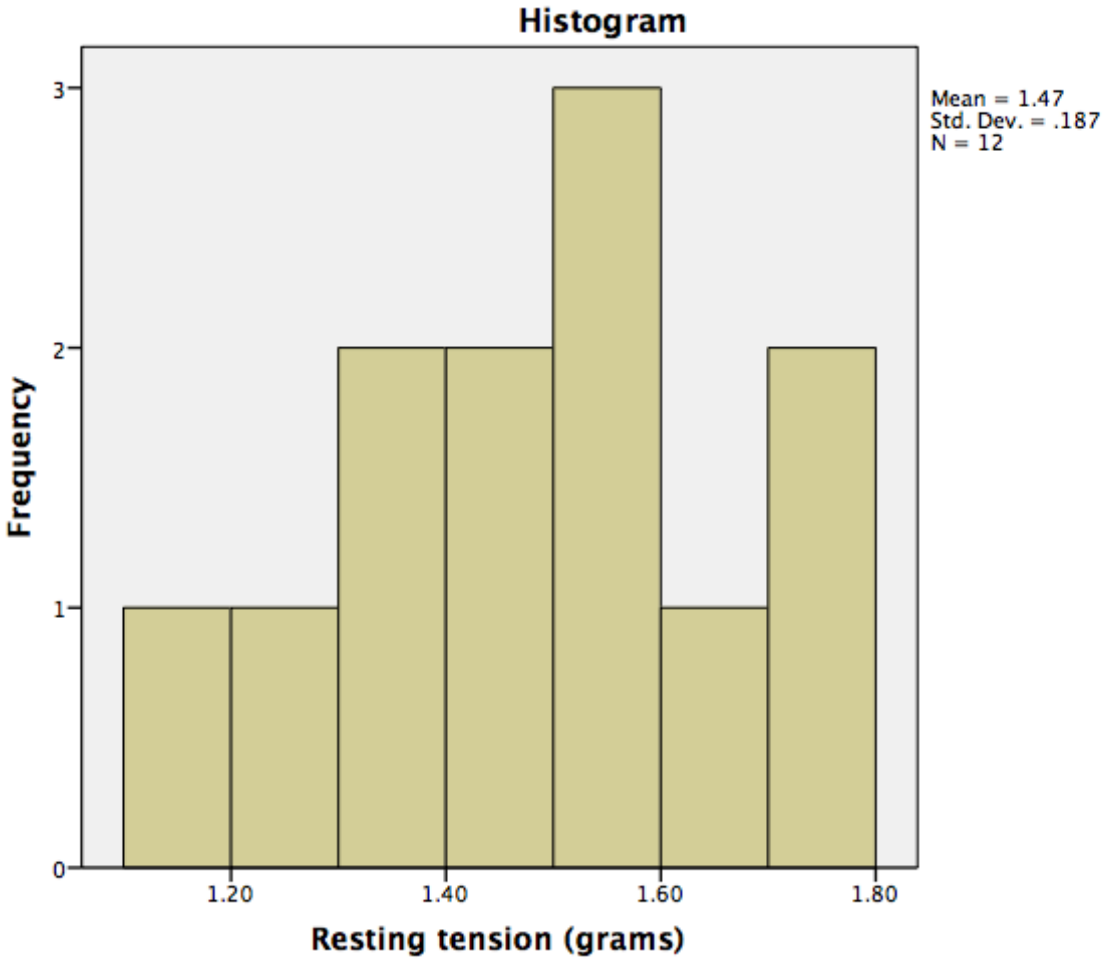
(1) Tensions for Sildenafil experiments:

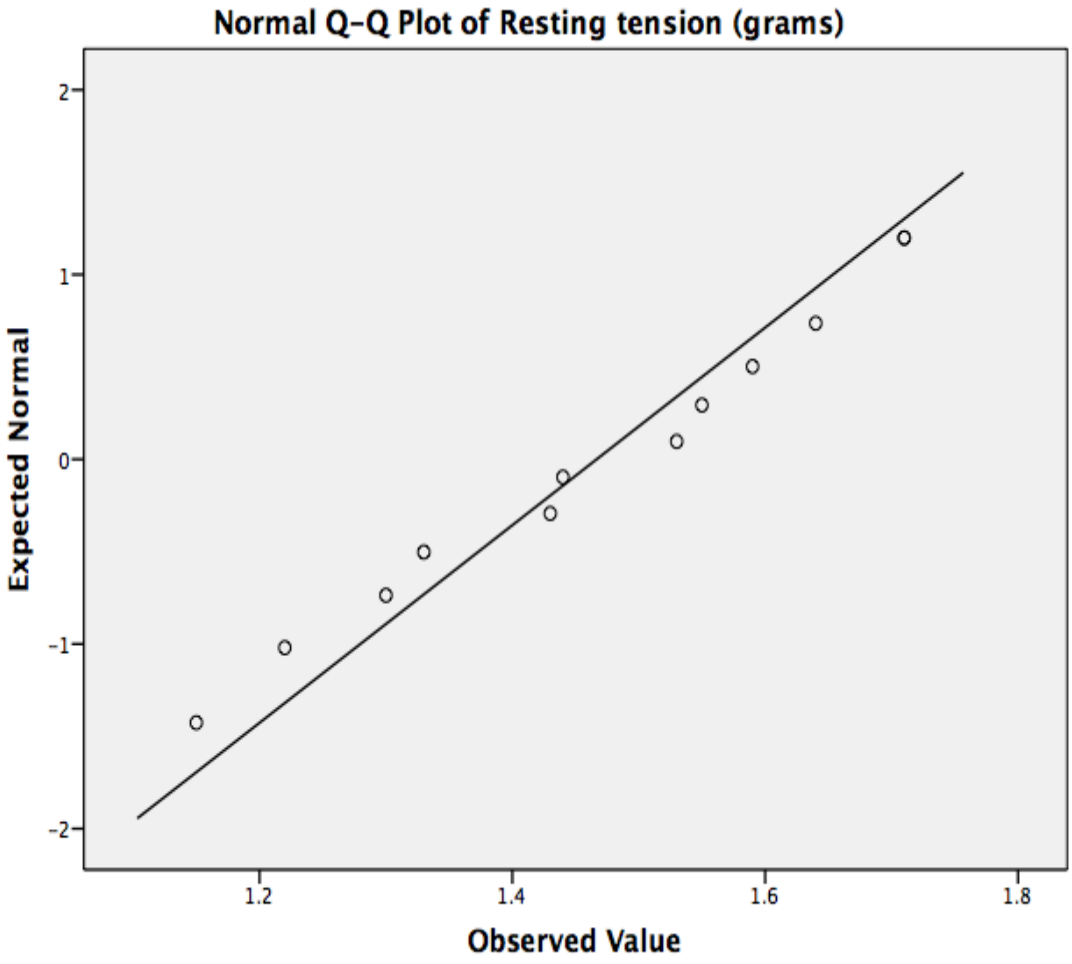
Tests of Normality

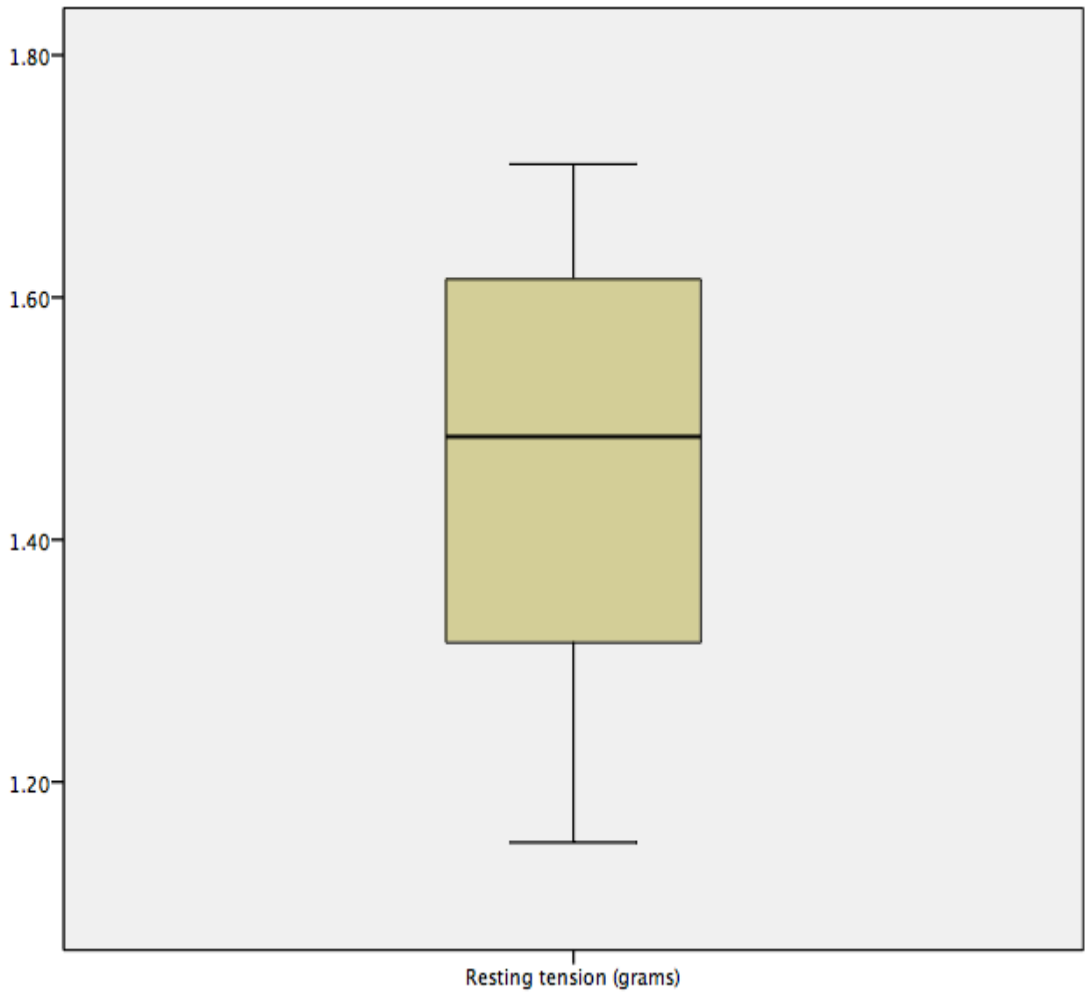
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.133	12	.200*	.951	12	.646

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.47
Std. Dev. = .187
N = 12
Frequency
3
2
1
0
1.20
1.40
1.60
1.80
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.2
1.4
1.6
1.8
Observed Value

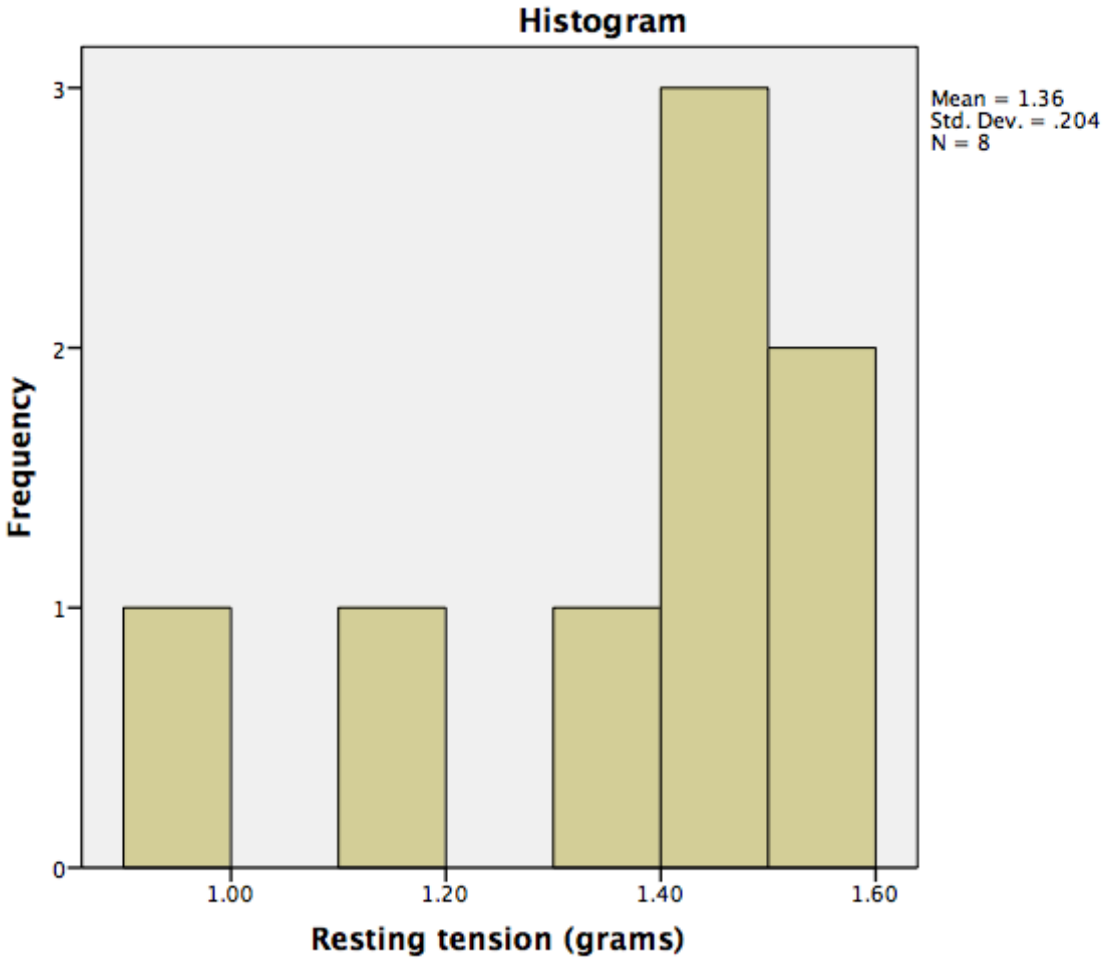

1.80
1.60
1.40
1.20
Resting tension (grams)

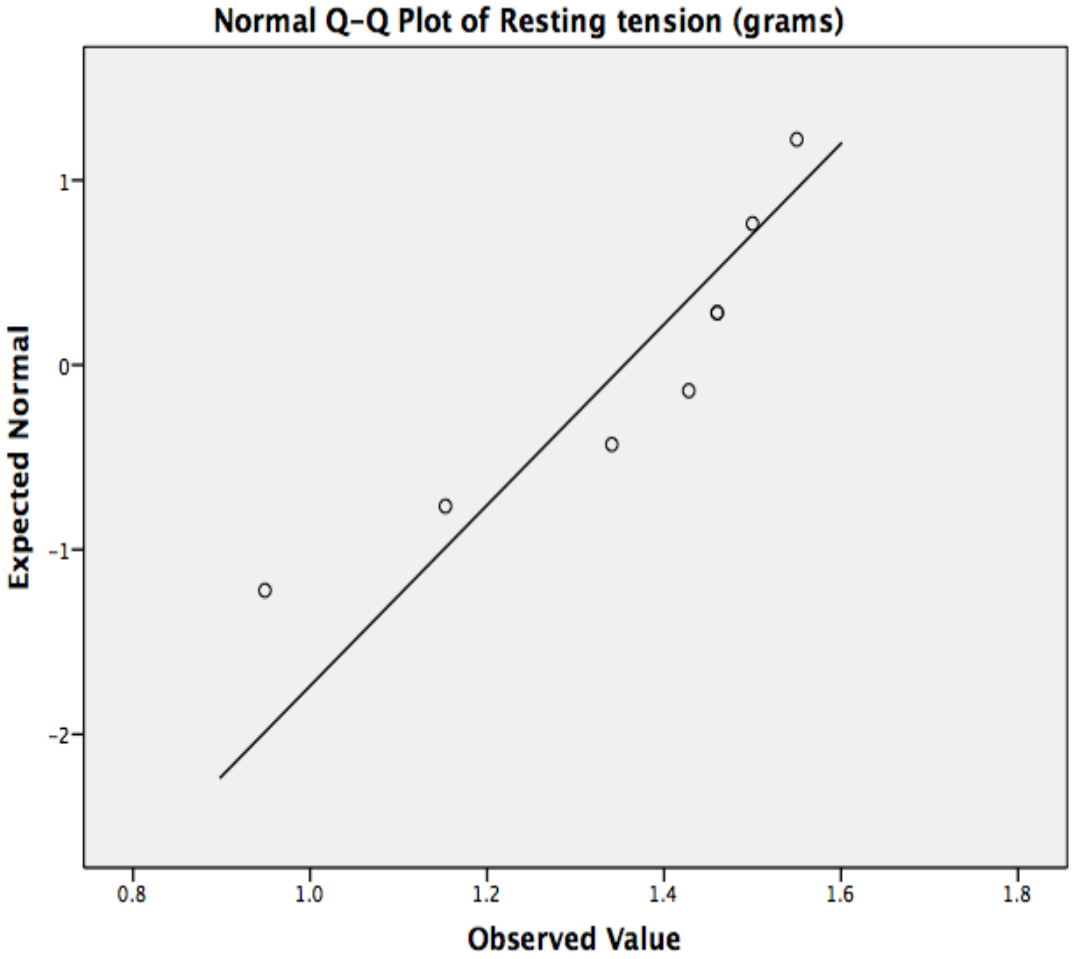
(2) Tensions for Sodium nitroprusside experiments:

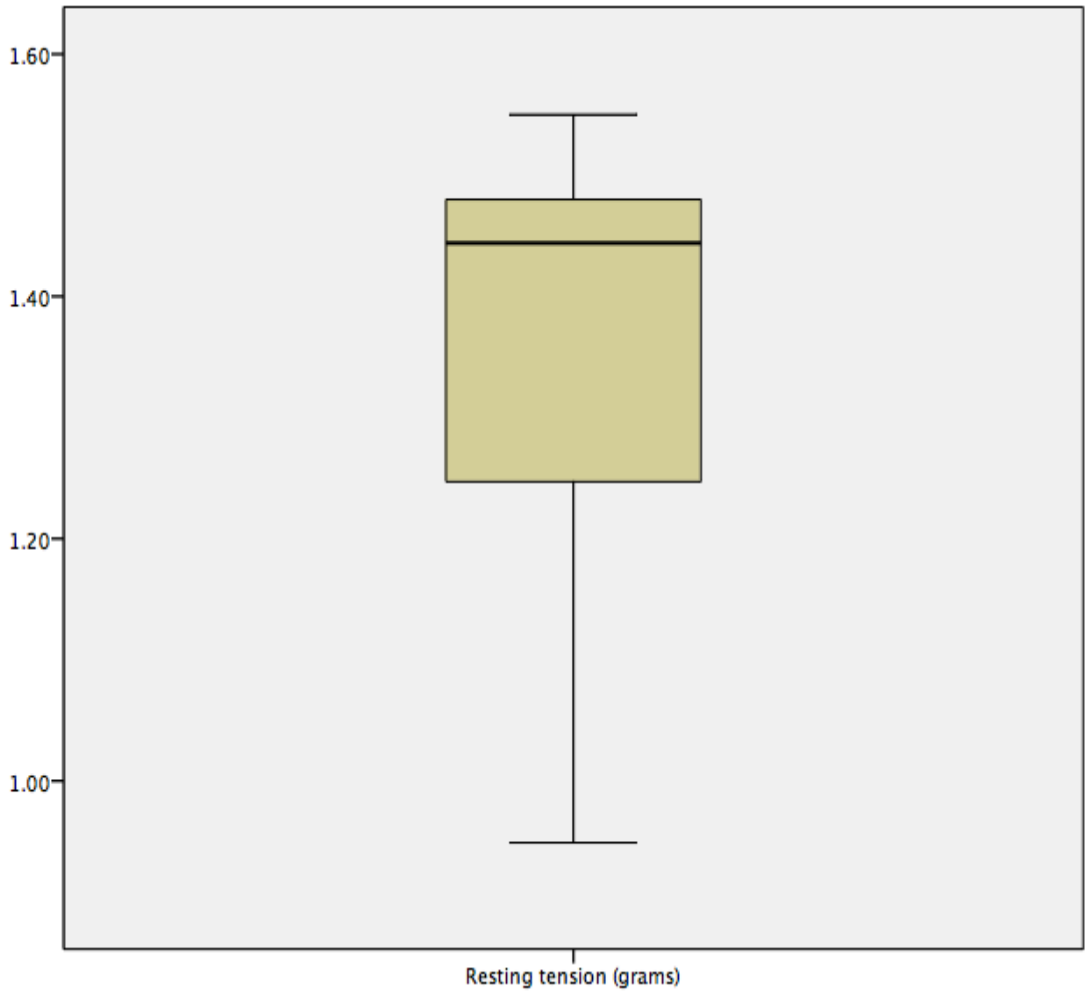
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.264	8	.105	.842	8	.079

a. Lilliefors Significance Correction


Histogram
Mean = 1.36
Std. Dev. = .204
N = 8
Frequency
3
2
1
0
1.00
1.20
1.40
1.60
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
1
0
-1
-2
0.8
1.0
1.2
1.4
1.6
1.8
Observed Value


1.60
1.40
1.20
1.00
Resting tension (grams)

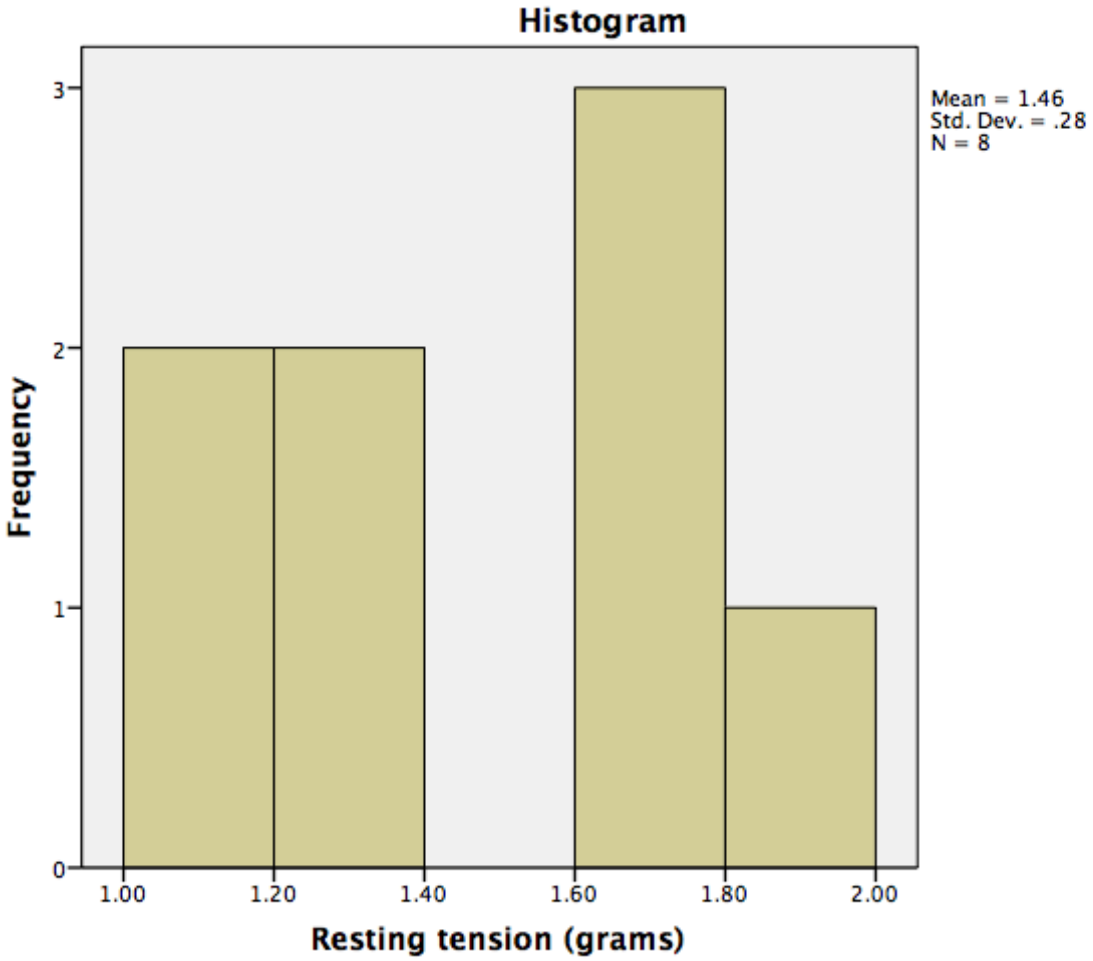
(3) Tensions for Milrinone experiments:

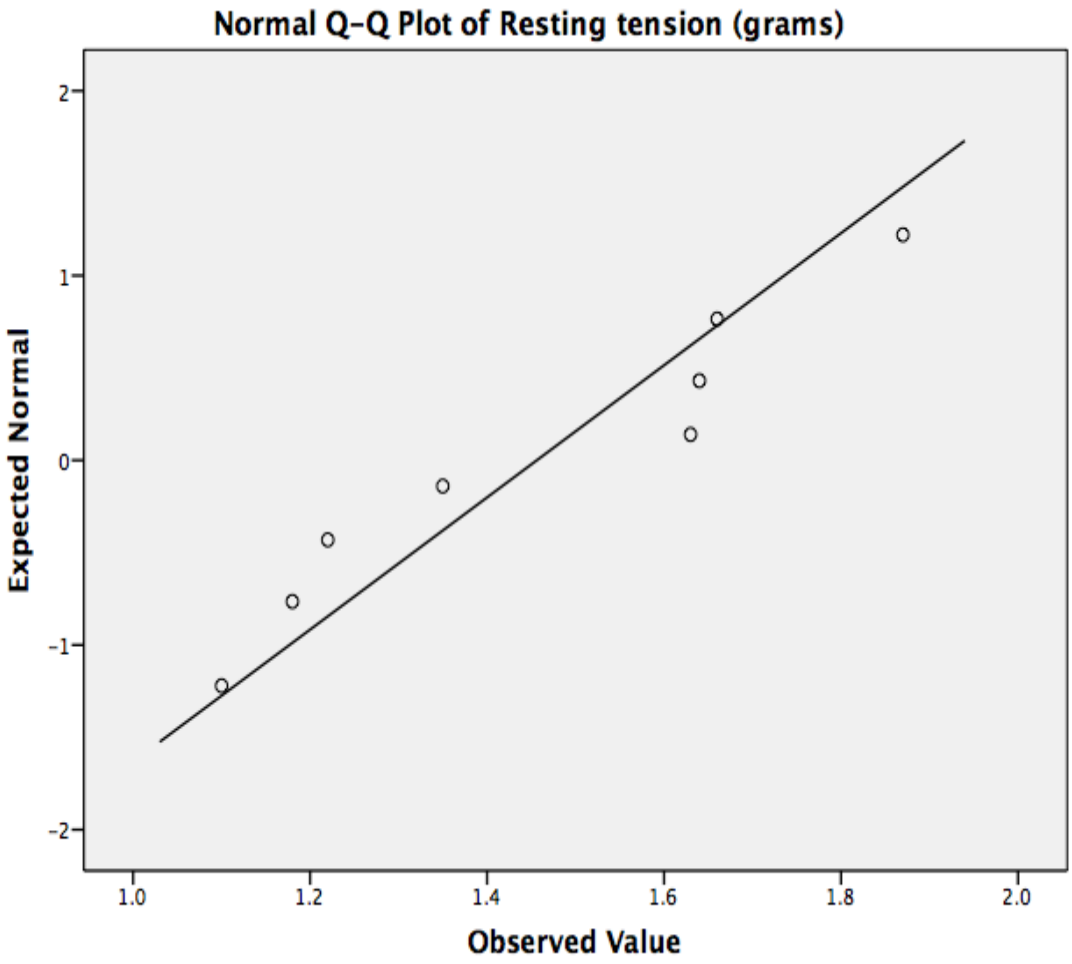
Tests of Normality

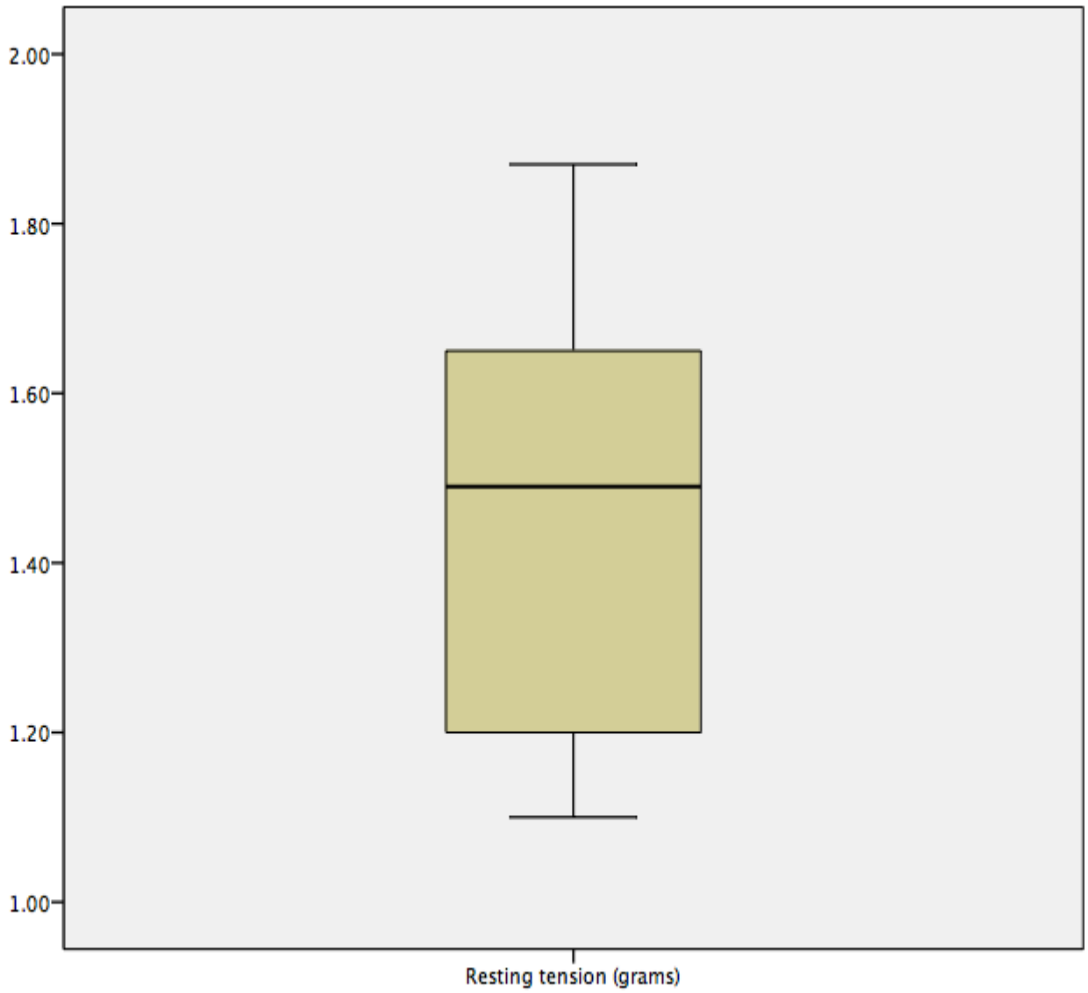
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.233	8	.200*	.914	8	.383

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.46
Std. Dev. = .28
N = 8
Frequency
0
1
2
3
1.00
1.20
1.40
1.60
1.80
2.00
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.0
1.2
1.4
1.6
1.8
2.0
Observed Value


2.00
1.80
1.60
1.40
1.20
1.00
Resting tension (grams)

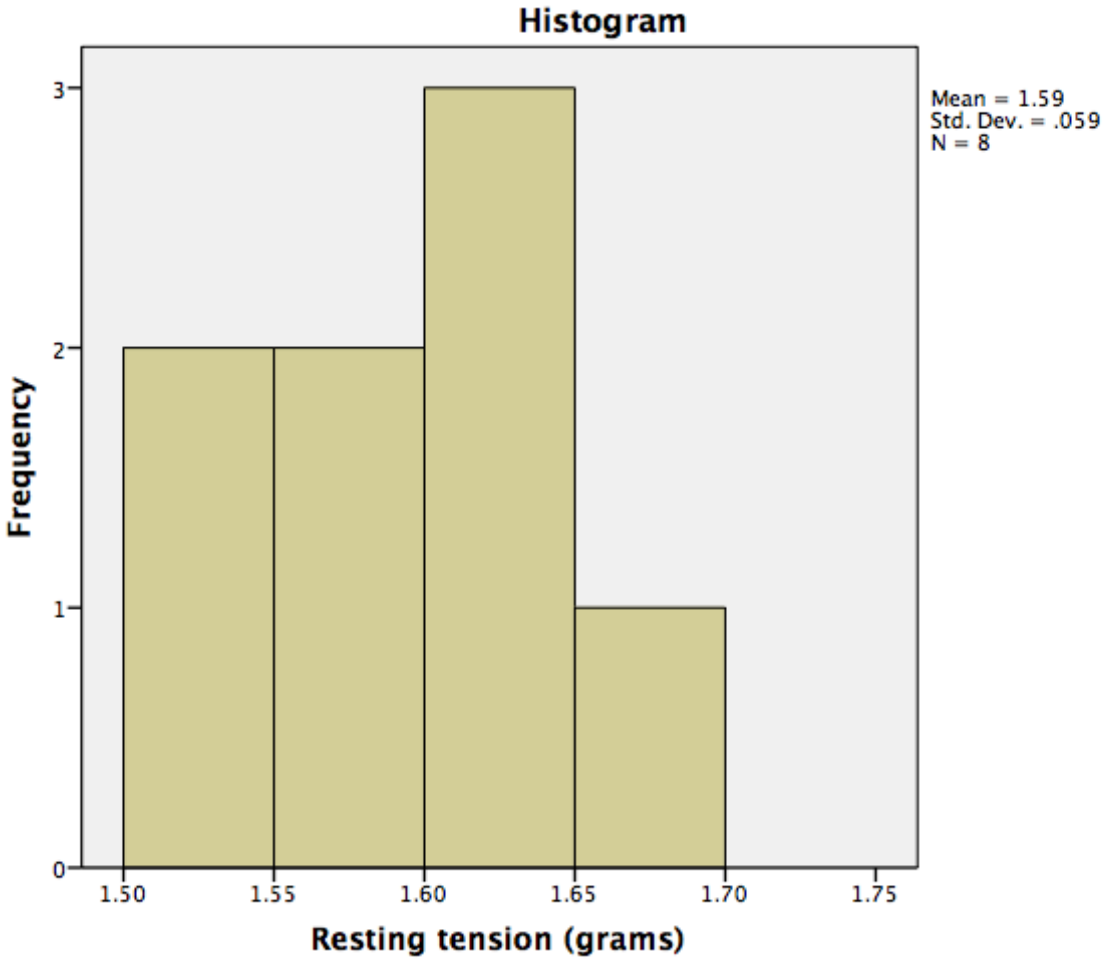
(4) Tensions for Epoprostenol experiments:

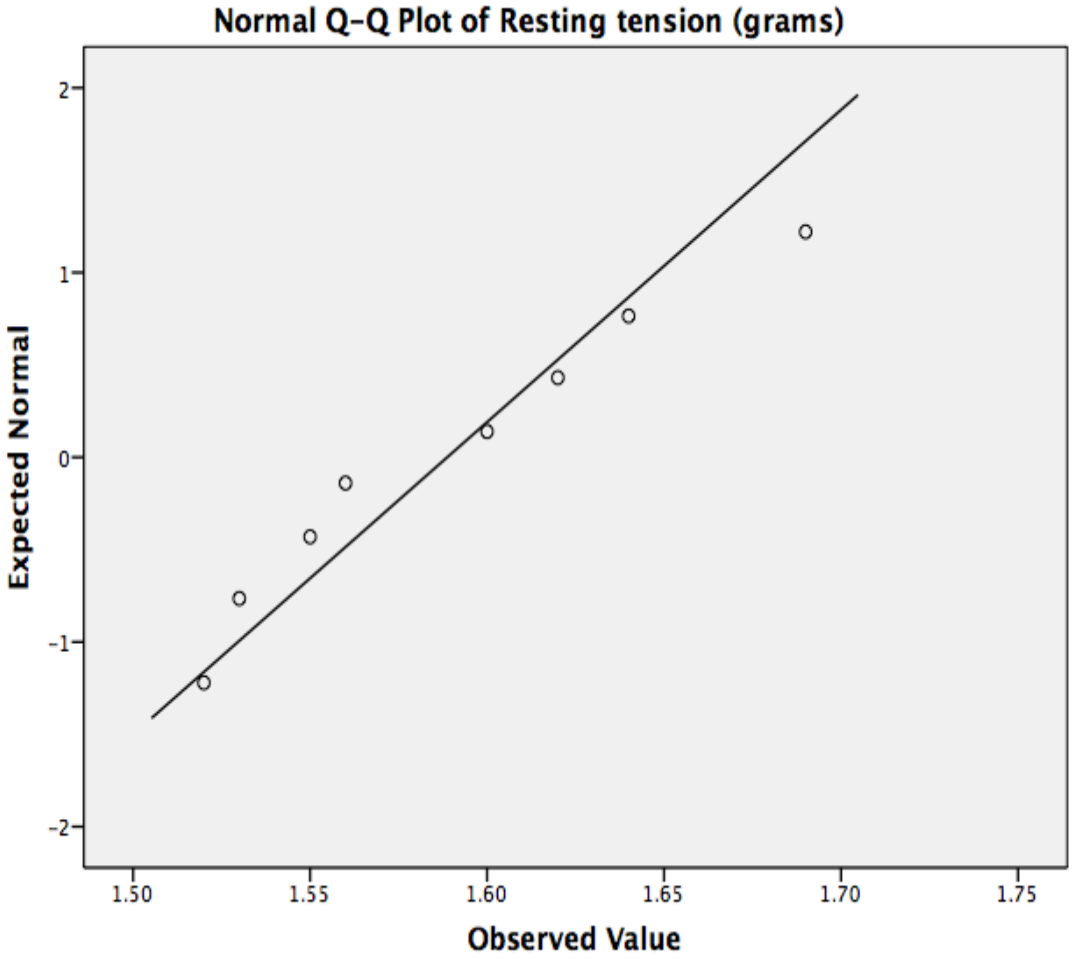
Tests of Normality

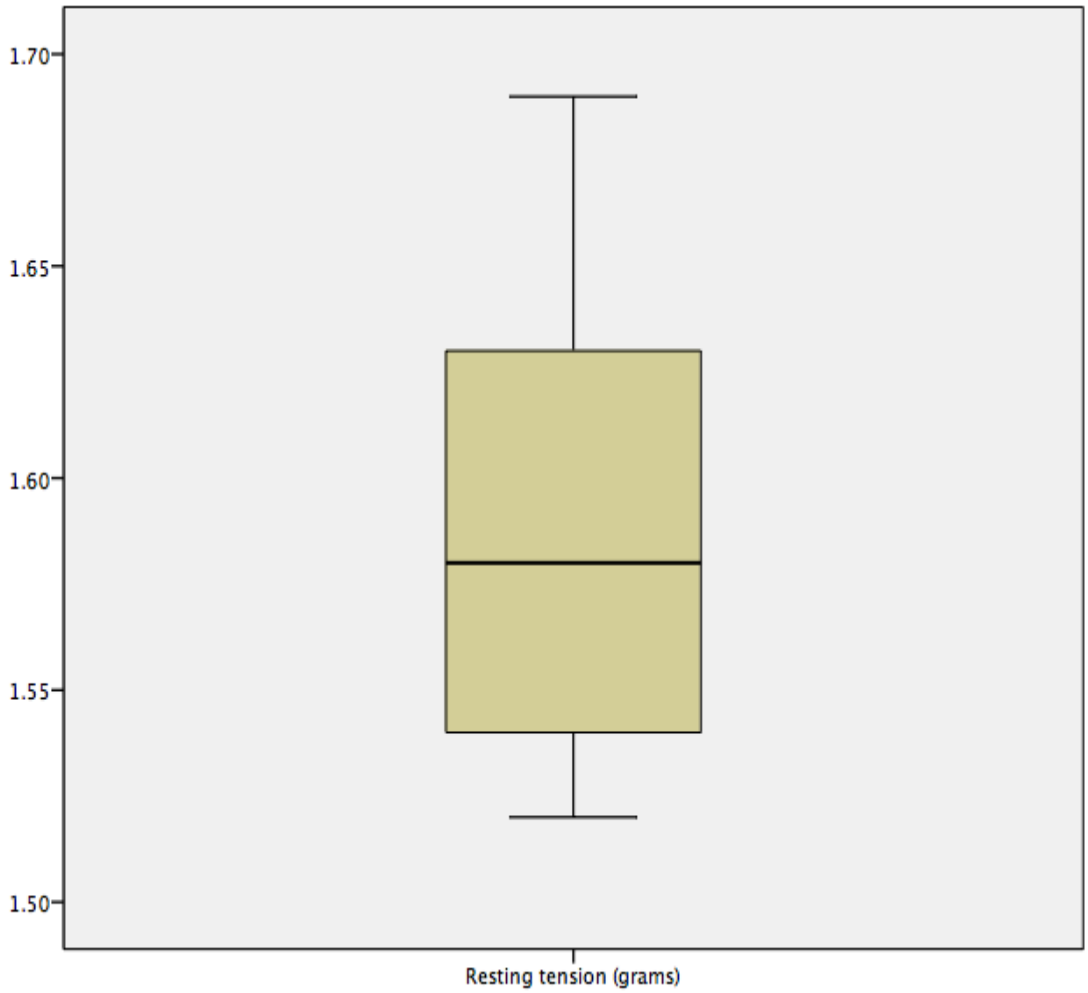
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.187	8	.200*	.946	8	.667

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.59
Std. Dev. = .059
N = 8
Frequency
3
2
1
0
1.50
1.55
1.60
1.65
1.70
1.75
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.50
1.55
1.60
1.65
1.70
1.75
Observed Value


1.70
1.65
1.60
1.55
1.50
Resting tension (grams)

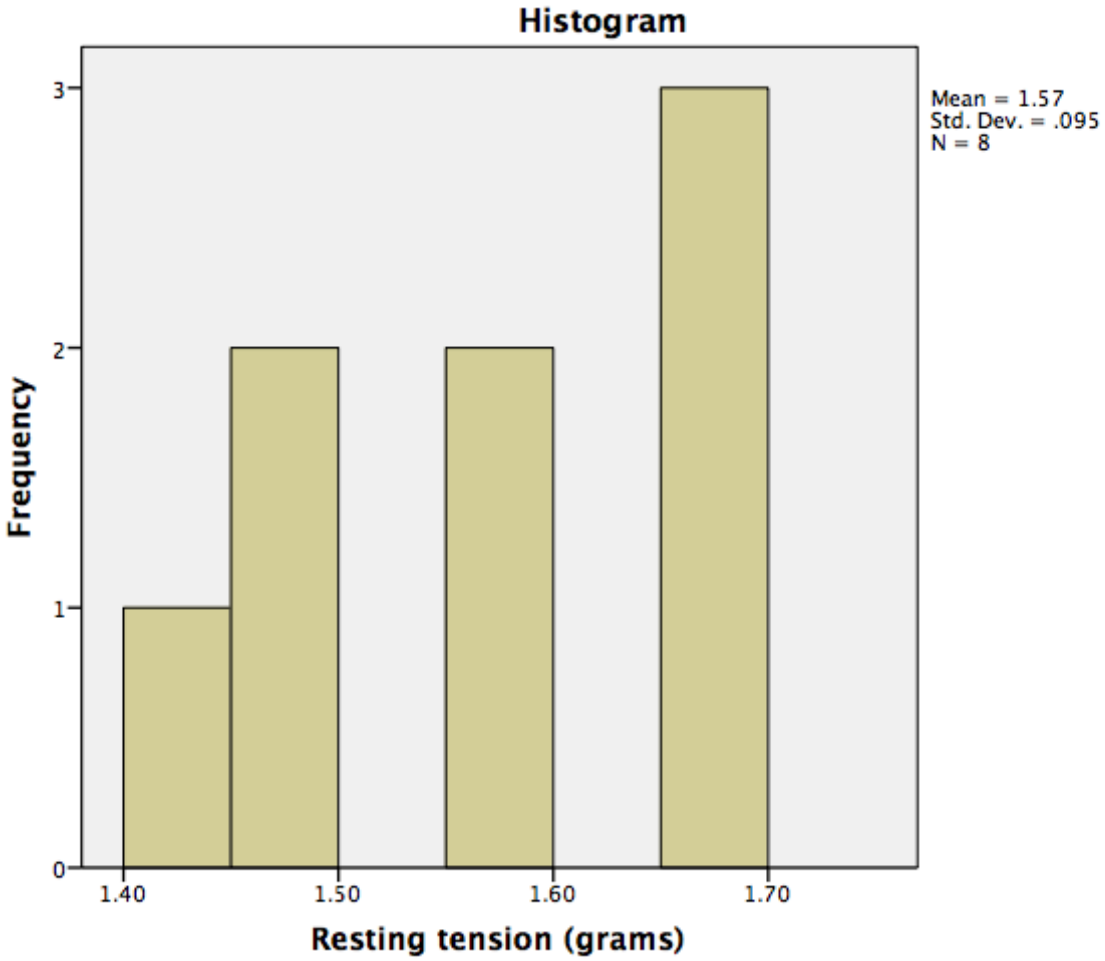
(5) Tensions for Iloprost experiments:

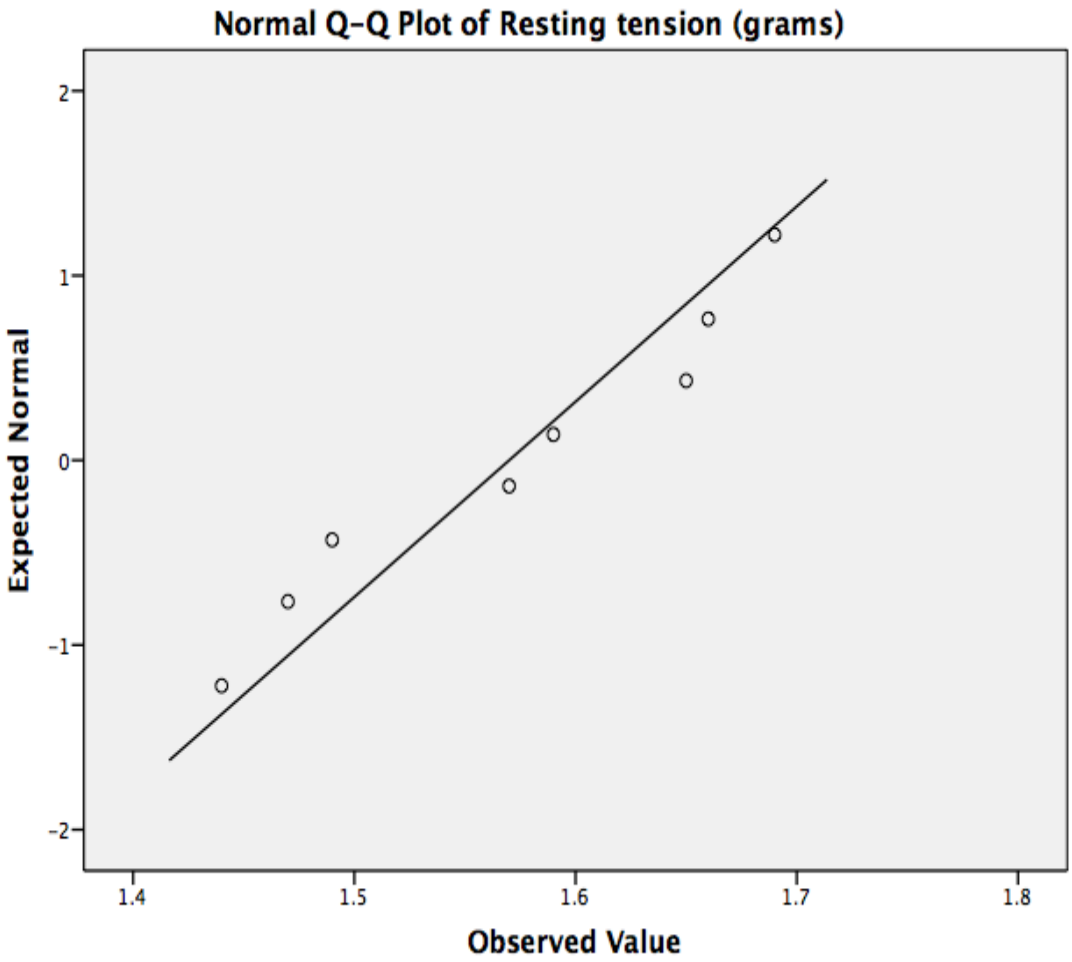
Tests of Normality

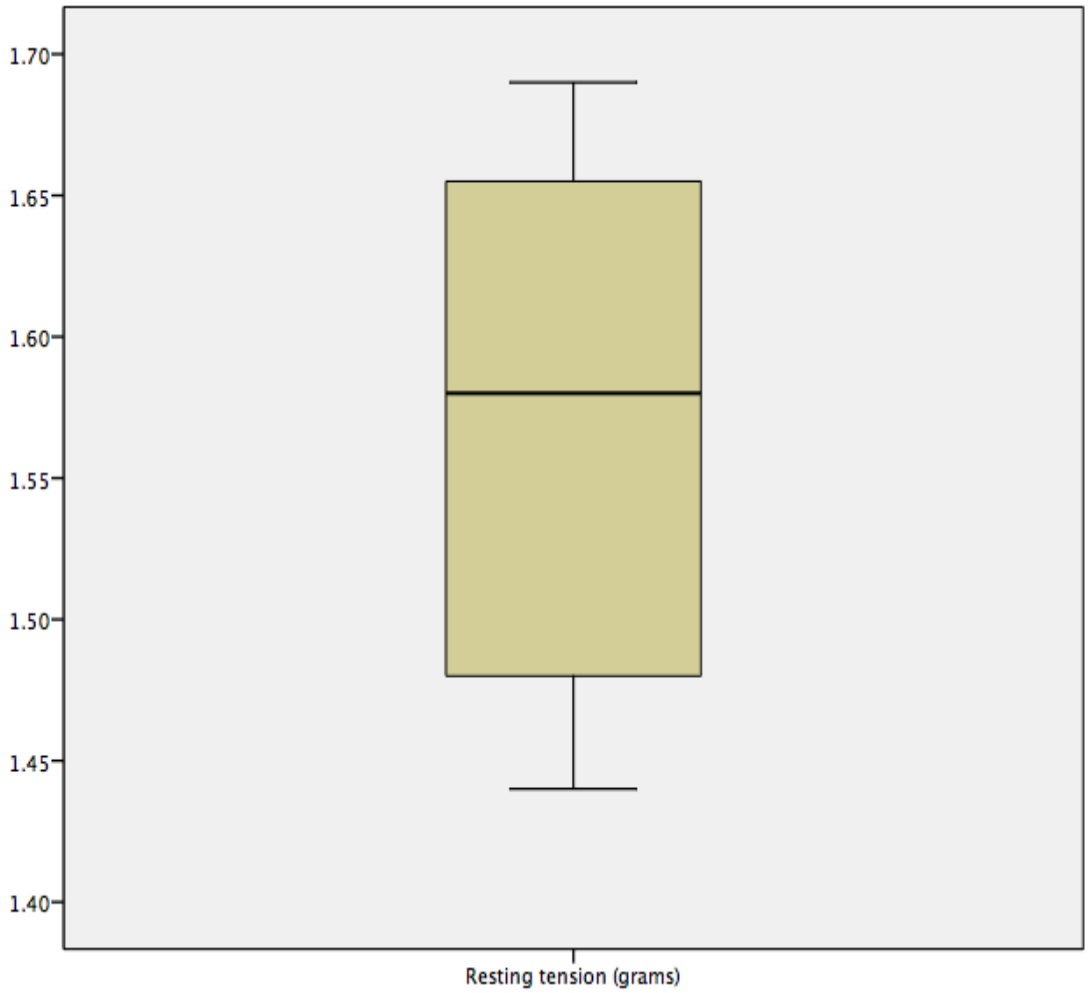
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.176	8	.200*	.924	8	.464

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.57
Std. Dev. = .095
N = 8
Frequency
0
1
2
3
1.40
1.50
1.60
1.70
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.4
1.5
1.6
1.7
1.8
Observed Value


1.70
1.65
1.60
1.55
1.50
1.45
1.40
Resting tension (grams)

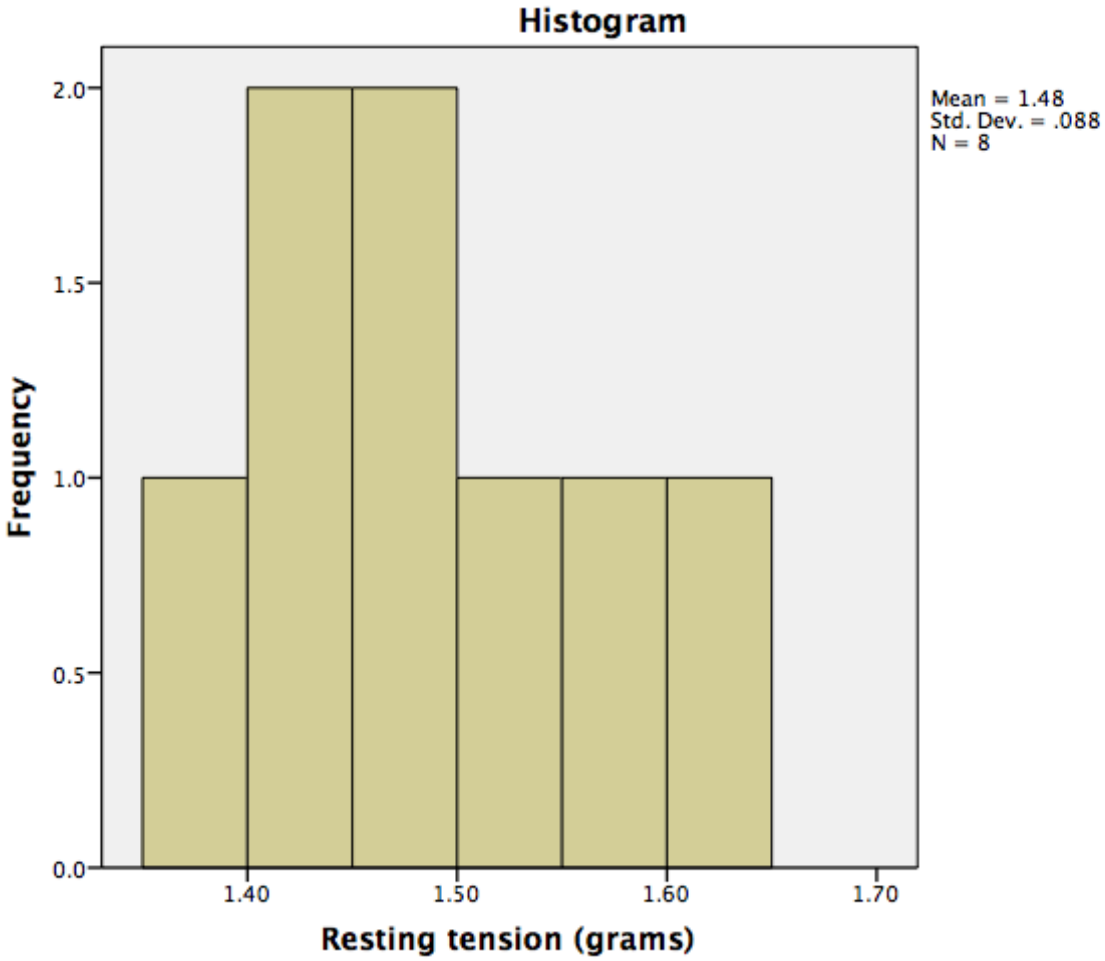
(6) Tensions for Treprostinil experiments:

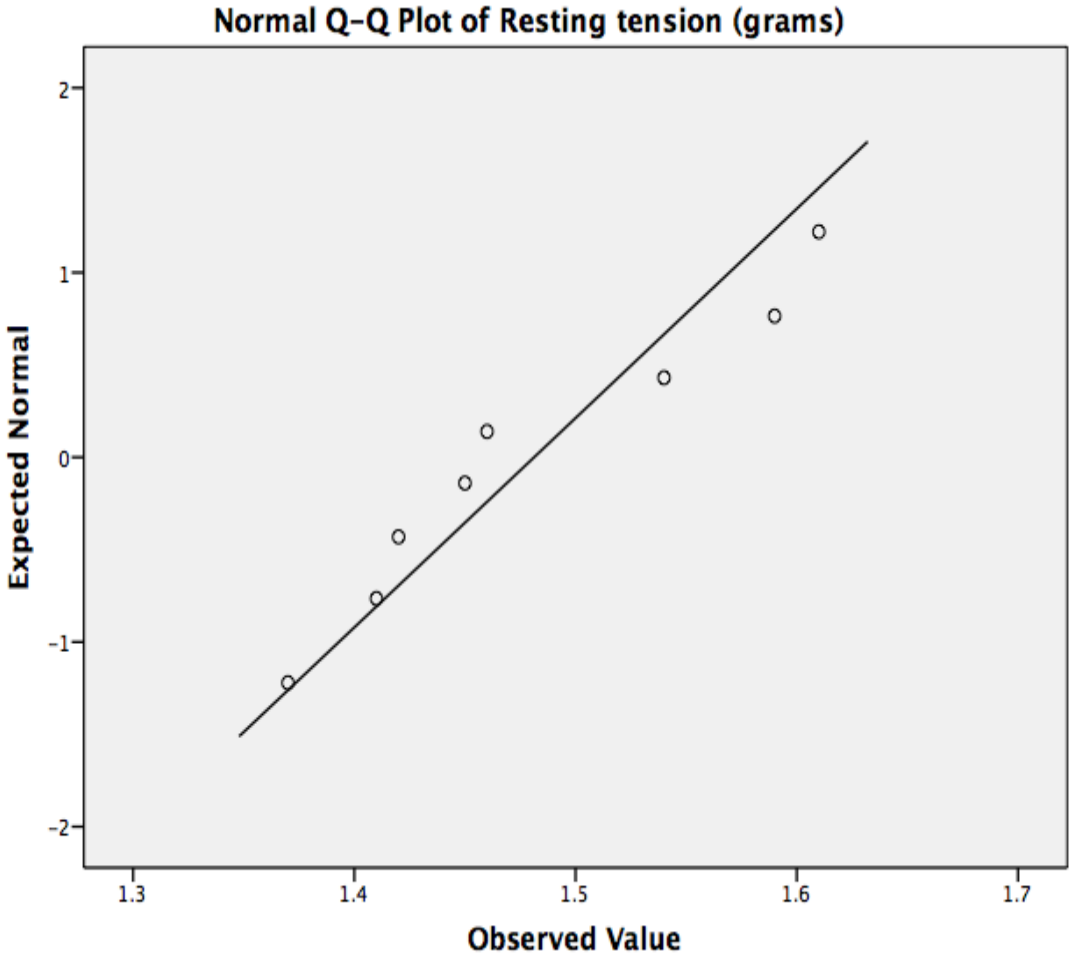
Tests of Normality

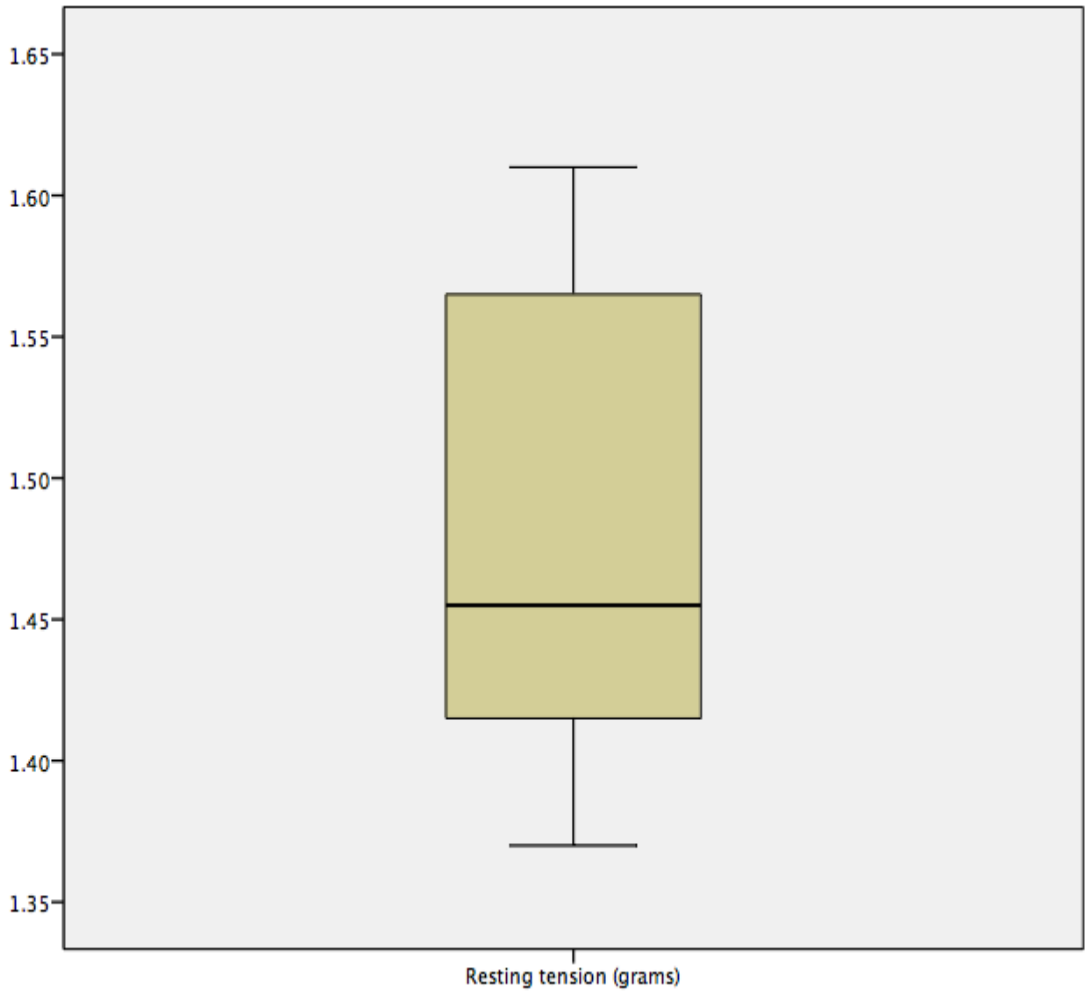
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Resting tension (grams)	.220	8	.200*	.919	8	.419

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction


Histogram
Mean = 1.48
Std. Dev. = .088
N = 8
Frequency
2.0
1.5
1.0
0.5
0.0
1.40
1.50
1.60
1.70
Resting tension (grams)


Normal Q-Q Plot of Resting tension (grams)
Expected Normal
2
1
0
-1
-2
1.3
1.4
1.5
1.6
1.7
Observed Value


1.65
1.60
1.55
1.50
1.45
1.40
1.35
Resting tension (grams)

Appendix VIII: Statistical Test Results For The Differential Effects Of Systemic Vasoconstrictors On Human Pulmonary Artery Tension Experiments

Table 1: Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	441076.637	1	441076.637	54.097	.000
Vasoconstrictor	187180.114	4	46795.028	5.739	.001
Error	358750.242	44	8153.415		

Table 2: Between-Subjects Factors

		N
Vasoconstrictor	Adrenaline	8
	ET-1	8
	Kcl	13
	Noradrenaline	12
	PGF2a	8

Table 3: Vasoconstrictor

Measure: MEASURE_1

Vasoconstrictor	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Adrenaline	34.752	7.046	20.552	48.953
ET-1	8.749	7.046	-5.452	22.950
Kcl	11.219	5.528	.079	22.359
Noradrenaline	26.831	5.753	15.236	38.426
PGF2a	40.130	7.046	25.930	54.331

Table 4: Descriptive Statistics

	Vasoconstrictor	Mean	Std. Deviation	N
Log10	Adrenaline	.0000	.00000	8
	ET-1	10.1000	5.26534	8
	Kcl	.0000	.00000	13
	Noradrenaline	.0000	.00000	12
	PGF2a	.0000	.00000	8
	Total	1.6490	4.27422	49
Log9.5	Adrenaline	.0000	.00000	8
	ET-1	12.2038	6.57395	8
	Kcl	.0000	.00000	13
	Noradrenaline	.0000	.00000	12

	PGF2a	.0000	.00000	8
	Total	1.9924	5.20306	49
Log9	Adrenaline	.0000	.00000	8
	ET-1	14.1662	6.99542	8
	Kcl	.0000	.00000	13
	Noradrenaline	.0000	.00000	12
	PGF2a	.0000	.00000	8
	Total	2.3129	5.92645	49
Log8.5	Adrenaline	-.9236	1.01220	8
	ET-1	11.9075	20.37102	8
	Kcl	.0000	.00000	13
	Noradrenaline	-.6327	1.48392	12
	PGF2a	.0000	.00000	8
	Total	1.6383	9.07223	49
Log8	Adrenaline	-1.2468	1.42486	8
	ET-1	12.8050	18.98367	8
	Kcl	.0000	.00000	13
	Noradrenaline	-.9370	2.28362	12
	PGF2a	.0000	.00000	8
	Total	1.6576	8.89091	49
Log7.5	Adrenaline	.9268	4.48094	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	-.9066	2.21003	12
	PGF2a	.0000	.00000	8
	Total	1.1679	4.18006	49
Log7	Adrenaline	9.3679	8.69707	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	17.9145	26.25342	12
	PGF2a	-4.2754	2.92375	8
	Total	6.4572	15.49318	49
Log6.5	Adrenaline	34.2678	23.43693	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	27.2723	30.69108	12
	PGF2a	-4.3890	3.66904	8
	Total	12.7957	23.02449	49

Log6	Adrenaline	47.9476	24.78193	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	33.7160	34.89744	12
	PGF2a	-5.6431	6.77973	8
	Total	16.4024	28.06847	49
Log5.5	Adrenaline	57.4346	23.84019	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	41.5841	39.11461	12
	PGF2a	6.6489	9.06236	8
	Total	21.8850	30.99157	49
Log5	Adrenaline	56.2048	22.17400	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	40.2096	37.92540	12
	PGF2a	59.9950	64.89964	8
	Total	30.0572	40.47690	49
Log4.5	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	.0000	.00000	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	74.8452	67.14075	8
	Total	32.0378	42.61739	49
Log4	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	.0594	1.39274	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.4640	72.72092	8
	Total	32.9710	44.87829	49
Log3.5	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	-.0503	3.62764	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	32.9290	45.04238	49
Log3	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8

	Kcl	-.1324	6.05649	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	32.9072	45.12388	49
Log2.5	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	2.2206	8.34788	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	33.5315	44.75943	49
Log2	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	18.9869	28.88304	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	37.9797	44.34095	49
Log1.5	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	51.7652	29.00413	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	46.6760	42.94699	49
Log1	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	69.5802	35.01569	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	51.4024	45.30916	49
Log0.5	Adrenaline	53.7557	21.18644	8
	ET-1	7.5863	5.78311	8
	Kcl	63.3372	33.60180	13
	Noradrenaline	40.0297	36.83158	12
	PGF2a	80.3854	73.24875	8
	Total	49.7461	44.44040	49

Table 5: Pairwise Comparisons

Measure: MEASURE_1

(I) Vasoconstrictor	(J) Vasoconstrictor	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
Adrenaline	ET-1	25.640	9.989	.137	-3.883	55.164
	Kcl	24.101	8.978	.102	-2.433	50.634
	Noradrenaline	8.465	9.119	1.000	-18.487	35.416
	PGF2a	-4.128	9.989	1.000	-33.652	25.396
ET-1	Adrenaline	-25.640	9.989	.137	-55.164	3.883
	Kcl	-1.540	8.978	1.000	-28.073	24.994
	Noradrenaline	-17.176	9.119	.663	-44.127	9.776
	PGF2a	-29.768*	9.989	.047	-59.292	-.245
Kcl	Adrenaline	-24.101	8.978	.102	-50.634	2.433
	ET-1	1.540	8.978	1.000	-24.994	28.073
	Noradrenaline	-15.636	7.998	.569	-39.274	8.002
	PGF2a	-28.229*	8.978	.030	-54.762	-1.695
Noradrenaline	Adrenaline	-8.465	9.119	1.000	-35.416	18.487
	ET-1	17.176	9.119	.663	-9.776	44.127
	Kcl	15.636	7.998	.569	-8.002	39.274
	PGF2a	-12.593	9.119	1.000	-39.544	14.359
PGF2a	Adrenaline	4.128	9.989	1.000	-25.396	33.652
	ET-1	29.768*	9.989	.047	.245	59.292
	Kcl	28.229*	8.978	.030	1.695	54.762
	Noradrenaline	12.593	9.119	1.000	-14.359	39.544

Based on estimated marginal means

*. The mean difference is significant at the

b. Adjustment for multiple comparisons: Bonferroni.

Measure: MEASURE_1

Source		Type III Sum of	df	Mean Square	F	Sig.
trial	Sphericity Assumed	292896.400	19	15415.600	41.131	.000
	Greenhouse-Geisser	292896.400	1.721	170213.790	41.131	.000
	Huynh-Feldt	292896.400	1.947	150435.897	41.131	.000
	Lower-bound	292896.400	1.000	292896.400	41.131	.000
trial * Vasoconstrictor	Sphericity Assumed	263284.405	76	3464.268	9.243	.000
	Greenhouse-Geisser	263284.405	6.883	38251.269	9.243	.000
	Huynh-Feldt	263284.405	7.788	33806.685	9.243	.000
	Lower-bound	263284.405	4.000	65821.101	9.243	.000
Error(trial)	Sphericity Assumed	313329.302	836	374.796		
	Greenhouse-Geisser	313329.302	75.713	4138.367		
	Huynh-Feldt	313329.302	85.667	3657.512		
	Lower-bound	313329.302	44.000	7121.121		

Table 6: Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
trial	Pillai's Trace	.847	7.564[b]	19.000	26.000	.000
	Wilks' Lambda	.153	7.564[b]	19.000	26.000	.000
	Hotelling's Trace	5.528	7.564[b]	19.000	26.000	.000
	Roy's Largest Root	5.528	7.564[b]	19.000	26.000	.000
trial * Vasoconstrictor	Pillai's Trace	2.889	3.971	76.000	116.000	.000
	Wilks' Lambda	.002	5.703	76.000	104.806	.000
	Hotelling's Trace	23.300	7.511	76.000	98.000	.000
	Roy's Largest Root	13.525	20.643[c]	19.000	29.000	.000

a. Design: Intercept + Vasoconstrictor

Within Subjects Design: trial

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Table 7: ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Log10	Between Groups	682.842	4	170.711	38.705	.000
	Within Groups	194.067	44	4.411		
	Total	876.909	48			
Log9.5	Between Groups	996.929	4	249.232	36.250	.000
	Within Groups	302.517	44	6.875		
	Total	1299.447	48			
Log9	Between Groups	1343.345	4	335.836	43.138	.000
	Within Groups	342.551	44	7.785		
	Total	1685.896	48			
Log8.5	Between Groups	1014.411	4	253.603	3.800	.010
	Within Groups	2936.244	44	66.733		
	Total	3950.655	48			
Log8	Between Groups	1200.083	4	300.021	5.089	.002
	Within Groups	2594.234	44	58.960		
	Total	3794.317	48			
Log7.5	Between Groups	410.311	4	102.578	10.536	.000
	Within Groups	428.389	44	9.736		
	Total	838.700	48			
Log7	Between Groups	3116.775	4	779.194	4.079	.007
	Within Groups	8405.082	44	191.025		
	Total	11521.857	48			

Log6.5	Between Groups	10911.366	4	2727.841	8.258	.000
	Within Groups	14534.734	44	330.335		
	Total	25446.100	48			
Log6	Between Groups	19565.263	4	4891.316	11.792	.000
	Within Groups	18251.015	44	414.796		
	Total	37816.278	48			
Log5.5	Between Groups	24485.975	4	6121.494	12.460	.000
	Within Groups	21616.955	44	491.294		
	Total	46102.930	48			
Log5	Between Groups	29660.839	4	7415.210	6.661	.000
	Within Groups	48981.355	44	1113.213		
	Total	78642.194	48			
Log4.5	Between Groups	37326.078	4	9331.519	8.236	.000
	Within Groups	49853.544	44	1133.035		
	Total	87179.621	48			
Log4	Between Groups	41334.942	4	10333.736	8.216	.000
	Within Groups	55339.981	44	1257.727		
	Total	96674.923	48			
Log3.5	Between Groups	41369.236	4	10342.309	8.124	.000
	Within Groups	56013.950	44	1273.044		
	Total	97383.186	48			
Log3	Between Groups	41439.712	4	10359.928	8.097	.000
	Within Groups	56296.205	44	1279.459		

	Total	97735.917	48			
Log2.5	Between Groups	39471.227	4	9867.807	7.659	.000
	Within Groups	56692.279	44	1288.461		
	Total	96163.505	48			
Log2	Between Groups	28506.976	4	7126.744	4.761	.003
	Within Groups	65866.794	44	1496.973		
	Total	94373.769	48			
Log1.5	Between Groups	22582.397	4	5645.599	3.767	.010
	Within Groups	65950.910	44	1498.884		
	Total	88533.307	48			
Log1	Between Groups	27970.938	4	6992.734	4.360	.005
	Within Groups	70569.215	44	1603.846		
	Total	98540.153	48			
Log0.5	Between Groups	25392.570	4	6348.143	4.024	.007
	Within Groups	69405.004	44	1577.386		
	Total	94797.574	48			

Appendix IX: Statistical Test Results For In Vitro Characterisation Of Pharmacological Effect Of Prostacyclin Analogues In Comparison To Phosphodiesterase Inhibitors On Small Human Pulmonary Vessels Experiment

Table 1:Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	500291.648	1	500291.648	374.030	.000
Vasodilators	89882.864	5	17976.573	13.440	.000
Error	61528.343	46	1337.573		

Table 2: Between-Subjects Factors

		N
VASODILATORS	Epoprosten	8
	Iloprost	8
	Milrinone	8
	Sildenafil	12
	SNP	8
	Treprosten	8

Table 3: VASODILATORS

Measure: MEASURE_1

Vasodilators	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Epoprostenol	20.438	3.136	14.126	26.751
Iloprost	30.249	3.136	23.937	36.562
Milrinone	15.281	3.136	8.968	21.594
Sildenafil	11.815	2.561	6.660	16.969
SNP	24.857	3.136	18.544	31.169
Treprostenil	41.740	3.136	35.427	48.052

Table 4: Descriptive Statistics

	Vasodilators	Mean	Std. Deviation	N
LOG12	Epoprostenol	.0000000	.00000000	8
	Iloprost	.0000000	.00000000	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	.0000000	.00000000	12
	SNP	.0000000	.00000000	8
	Treprostenil	.1925000	.10620062	8
	Total	.0296154	.08041465	52
LOG11.5	Epoprostenol	.0000000	.00000000	8
	Iloprost	.0000000	.00000000	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	.0000000	.00000000	12
	SNP	.0000000	.00000000	8
	Treprostenil	.9262500	.85034342	8
	Total	.1425000	.46165039	52
LOG11	Epoprostenol	.0000000	.00000000	8
	Iloprost	.2675000	.25206292	8
	Milrinone	.0000000	.00000000	8

	Sildenafil	.0000000	.00000000	12
	SNP	.0000000	.00000000	8
	Treprostenil	2.4350000	2.01874784	8
	Total	.4157692	1.15461112	52
LOG10.5	Epoprostenol	.0000000	.00000000	8
	Iloprost	.6437500	.50111127	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	.0000000	.00000000	12
	SNP	.0000000	.00000000	8
	Treprostenil	11.7325000	11.02709617	8
	Total	1.9040385	5.88941332	52
LOG10	Epoprostenol	.4725000	.41306347	8
	Iloprost	2.0025000	1.31946688	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	.9091667	.85080132	12
	SNP	4.4100000	3.97420612	8
	Treprostenil	20.8662500	18.28380934	8
	Total	4.4792308	10.01103153	52
LOG9.5	Epoprostenol	1.2137500	1.30369735	8
	Iloprost	6.9375000	3.27490349	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	1.5900000	1.78088844	12
	SNP	3.6075000	3.97887188	8
	Treprostenil	24.5687500	20.91576401	8
	Total	5.9557692	11.55610039	52
LOG9	Epoprostenol	1.9400000	1.87714068	8
	Iloprost	21.9250000	15.04725889	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	2.2250000	1.68952118	12
	SNP	3.3437500	3.46779855	8
	Treprostenil	40.6937500	19.80121276	8
	Total	10.9600000	17.43105984	52
LOG8.5	Epoprostenol	3.7325000	4.49251997	8
	Iloprost	35.1937500	19.72397376	8
	Milrinone	.0000000	.00000000	8
	Sildenafil	6.3616667	6.46672298	12
	SNP	8.1075000	5.82223264	8

	Treprostenil	54.2012500	17.93796727	8
	Total	17.0426923	22.24841753	52
LOG8	Epoprostenol	7.9762500	7.46188398	8
	Iloprost	41.6387500	19.80982832	8
	Milrinone	2.4237500	1.49192050	8
	Sildenafil	7.9833333	8.94545322	12
	SNP	17.8850000	13.02945125	8
	Treprostenil	57.8975000	16.50199187	8
	Total	21.5071154	23.28057267	52
LOG7.5	Epoprostenol	21.7637500	13.19431256	8
	Iloprost	49.0262500	23.03347188	8
	Milrinone	3.4550000	1.98068531	8
	Sildenafil	10.7108333	12.65062877	12
	SNP	31.6262500	14.80305657	8
	Treprostenil	59.8850000	14.73094799	8
	Total	27.9726923	24.36092766	52
LOG7	Epoprostenol	35.0112500	16.44587090	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	6.4037500	7.93706844	8
	Sildenafil	12.6341667	14.72132557	12
	SNP	43.4250000	6.09815194	8
	Treprostenil	61.8075000	13.82898483	8
	Total	33.3142308	24.46652850	52
LOG6.5	Epoprostenol	42.1575000	16.75820034	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	11.5962500	14.85048238	8
	Sildenafil	15.0525000	17.25960503	12
	SNP	49.0500000	4.55655258	8
	Treprostenil	62.3950000	13.74327472	8
	Total	36.7263462	24.55354637	52
LOG6	Epoprostenol	46.5887500	13.58438961	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	26.6550000	16.22898289	8
	Sildenafil	16.8850000	17.99800570	12
	SNP	51.5012500	5.54203789	8
	Treprostenil	62.3950000	13.74327472	8
	Total	40.5248077	22.53458766	52

LOG5.5	Epoprostenol	46.6487500	13.03370404	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	41.3925000	14.79332161	8
	Sildenafil	17.7991667	17.98029146	12
	SNP	52.4012500	4.91599558	8
	Treprostenil	62.3950000	13.74327472	8
	Total	43.1507692	21.32898992	52
LOG5	Epoprostenol	46.6487500	13.03370404	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	52.4087500	13.23061648	8
	Sildenafil	25.9358333	16.84478903	12
	SNP	52.4012500	4.91599558	8
	Treprostenil	62.3950000	13.74327472	8
	Total	46.7232692	18.94303646	52
LOG4.5	Epoprostenol	46.6487500	13.03370404	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	56.4975000	10.12504638	8
	Sildenafil	39.4125000	16.29941724	12
	SNP	52.4012500	4.91599558	8
	Treprostenil	62.3950000	13.74327472	8
	Total	50.4623077	15.86021705	52
LOG4	Epoprostenol	46.6487500	13.03370404	8
	Iloprost	50.9437500	22.24687069	8
	Milrinone	58.9450000	8.99845542	8
	Sildenafil	43.3475000	14.50473724	12
	SNP	52.4012500	4.91599558	8
	Treprostenil	62.3950000	13.74327472	8
	Total	51.7469231	14.95788552	52

Table 5: Pairwise Comparisons

Measure: MEASURE_1

(I) Vasodilators	(J) Vasodilators	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Epoprostenol	Iloprost	-9.811	4.435	.479	-23.543	3.921
	Milrinone	5.157	4.435	1.000	-8.575	18.890
	Sildenafil	8.624	4.049	.578	-3.912	21.160
	SNP	-4.418	4.435	1.000	-18.150	9.314
	Treprostenil	-21.301*	4.435	.000	-35.034	-7.569
Iloprost	Epoprostenol	9.811	4.435	.479	-3.921	23.543
	Milrinone	14.968*	4.435	.023	1.236	28.701
	Sildenafil	18.435*	4.049	.001	5.899	30.971
	SNP	5.393	4.435	1.000	-8.339	19.125
	Treprostenil	-11.490	4.435	.192	-25.223	2.242
Milrinone	Epoprostenol	-5.157	4.435	1.000	-18.890	8.575
	Iloprost	-14.968*	4.435	.023	-28.701	-1.236
	Sildenafil	3.467	4.049	1.000	-9.069	16.002
	SNP	-9.576	4.435	.541	-23.308	4.157

	Treprostenil	-26.459*	4.435	.000	-40.191	-12.726
Sildenafil	Epoprostenol	-8.624	4.049	.578	-21.160	3.912
	Iloprost	-18.435*	4.049	.001	-30.971	-5.899
	Milrinone	-3.467	4.049	1.000	-16.002	9.069
	SNP	-13.042*	4.049	.035	-25.578	-.506
	Treprostenil	-29.925*	4.049	.000	-42.461	-17.389
SNP	Epoprostenol	4.418	4.435	1.000	-9.314	18.150
	Iloprost	-5.393	4.435	1.000	-19.125	8.339
	Milrinone	9.576	4.435	.541	-4.157	23.308
	Sildenafil	13.042*	4.049	.035	.506	25.578
	Treprostenil	-16.883*	4.435	.006	-30.615	-3.151
Treprostenil	Epoprostenol	21.301*	4.435	.000	7.569	35.034
	Iloprost	11.490	4.435	.192	-2.242	25.223
	Milrinone	26.459*	4.435	.000	12.726	40.191
	Sildenafil	29.925*	4.049	.000	17.389	42.461
	SNP	16.883*	4.435	.006	3.151	30.615

Based on estimated marginal means. *. The mean difference is significant at the….. b. Adjustment for multiple comparisons: Bonferroni.

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
trial	Sphericity Assumed	323484.453	16	20217.778	323.164	.000
	Greenhouse-Geisser	323484.453	2.220	145681.305	323.164	.000
	Huynh-Feldt	323484.453	2.592	124812.975	323.164	.000
	Lower-bound	323484.453	1.000	323484.453	323.164	.000
trial * Vasodilators	Sphericity Assumed	67876.790	80	848.460	13.562	.000
	Greenhouse-Geisser	67876.790	11.102	6113.666	13.562	.000
	Huynh-Feldt	67876.790	12.959	5237.905	13.562	.000
	Lower-bound	67876.790	5.000	13575.358	13.562	.000
Error(trial)	Sphericity Assumed	46045.646	736	62.562		
	Greenhouse-Geisser	46045.646	102.143	450.797		
	Huynh-Feldt	46045.646	119.221	386.222		
	Lower-bound	46045.646	46.000	1000.992		

Table 6: Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
trial	Pillai's Trace	.970	62.517[b]	16.000	31.000	.000
	Wilks' Lambda	.030	62.517[b]	16.000	31.000	.000
	Hotelling's Trace	32.267	62.517[b]	16.000	31.000	.000
	Roy's Largest Root	32.267	62.517[b]	16.000	31.000	.000
trial * VASODILATORS	Pillai's Trace	3.568	5.452	80.000	175.000	.000
	Wilks' Lambda	.000	7.827	80.000	153.557	.000
	Hotelling's Trace	28.612	10.515	80.000	147.000	.000
	Roy's Largest Root	16.625	36.366[c]	16.000	35.000	.000

a. Design: Intercept + VASODILATORS

Within Subjects Design: trial

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Table 7: ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LOG11	Between Groups	18.792	1	18.792	9.081	.009
	Within Groups	28.972	14	2.069		
	Total	47.764	15			
LOG10.5	Between Groups	491.842	1	491.842	8.073	.013
	Within Groups	852.936	14	60.924		
	Total	1344.777	15			
LOG10	Between Groups	2449.579	4	612.395	9.662	.000
	Within Groups	2471.988	39	63.384		
	Total	4921.567	43			
LOG9.5	Between Groups	3180.389	4	795.097	9.411	.000
	Within Groups	3294.964	39	84.486		
	Total	6475.352	43			
LOG9	Between Groups	9890.439	4	2472.610	21.574	.000
	Within Groups	4469.800	39	114.610		
	Total	14360.239	43			
LOG8.5	Between Groups	16684.280	4	4171.070	27.978	.000
	Within Groups	5814.212	39	149.082		
	Total	22498.492	43			
LOG8	Between Groups	20514.086	5	4102.817	26.480	.000
	Within Groups	7127.153	46	154.938		
	Total	27641.238	51			
LOG7.5	Between Groups	20492.976	5	4098.595	19.291	.000

	Within Groups	9773.218	46	212.461		
	Total	30266.195	51			
LOG7	Between Groups	20747.563	5	4149.513	19.514	.000
	Within Groups	9781.599	46	212.643		
	Total	30529.162	51			
LOG6.5	Between Groups	17117.225	4	4279.306	20.220	.000
	Within Groups	8253.931	39	211.639		
	Total	25371.155	43			
LOG6	Between Groups	7649.598	3	2549.866	11.802	.000
	Within Groups	6913.618	32	216.051		
	Total	14563.216	35			
LOG5.5	Between Groups	7220.060	3	2406.687	11.947	.000
	Within Groups	6446.407	32	201.450		
	Total	13666.468	35			
LOG5	Between Groups	3363.914	1	3363.914	13.931	.002
	Within Groups	4346.561	18	241.476		
	Total	7710.474	19			
LOG4.5	Between Groups	1401.107	1	1401.107	6.929	.017
	Within Groups	3639.997	18	202.222		
	Total	5041.104	19			
LOG4	Between Groups	1396.143	1	1396.143	7.895	.014
	Within Groups	2475.847	14	176.846		
	Total	3871.990	15			