THE UNIVERSITY OF LONDON

THE INVESTIGATION OF CORPUS CAVERNOSUM SMOOTH MUSCLE DYSFUNCTION IN LOW-FLOW PRIAPISM

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Medicine (M.D.) by

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ABSTRACT

Prolonged low-flow (ischaemic) priapism results in a progressive alteration of the microenvironment within the corpus cavernosum with the development of hypoxia, acidosis and glucopenia and a reduction in the responsiveness to α-adrenergic receptor agonists. The aim of this study was to investigate the changes in the corporal microenvironment in patients presenting with refractory low-flow priapism and then develop an *in vitro* model to investigate the effects of hypoxia, acidosis or glucopenia on the tone of the rabbit corpus cavernosum. The recovery of smooth muscle contractility following exposure to these conditions was also investigated. Hypoxia, acidosis or glucopenia alone or in combination showed a sustained and significant reduction in the smooth muscle tone. This was most marked for conditions of hypoxia combined with glucopenia and the combination of hypoxia, acidosis and glucopenia. Reperfusion of tissue strips showed complete recovery of smooth muscle tone for all conditions except when hypoxia and glucopenia were combined or when hypoxia, glucopenia and acidosis were used in combination. Incomplete recovery of tone was not associated with an impairment of nitrergic relaxation responses but was associated with a significant reduction in tissue ATP concentrations and an increase in the number of TUNEL-positive nuclei. This indicates that in the presence of hypoxia, acidosis and glucopenia, failure of conventional α-adrenergic agonists in low-flow priapism is associated with irreversible smooth muscle cell dysfunction which is linked to ATP depletion and smooth muscle cell death.

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CONTENTS

ABBREVIATIONS

A	Acidosis
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ACTH	Adrenocorticotrophic Hormone
Ca^{2+}	Calcium
CICR	Calcium induced calcium release
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monphosphate
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
°C	Degree Celcius
DAG	Diacylglycerol
EFS	Electrical Field Stimulation
EDHF	Endothelium derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
FSH	Follicle stimulating hormone
GABA	γ-aminobutyric acid
g	Gramme
GTP	Guanosine triphosphate
G	Glucopenia
H&E	Haematoxcylin and eosin
Hg	Mercury
Hz	Hertz

HPLC	High pressure liquid chromotography
H	Hypoxia
5-HT	5-Hydroxytryptamine
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
IAA	Iodoacetic acid
K^+	Potassium
LH	Luteinising hormone
L-NAME	N^{ω}-nitro-L-arginine methyl esther
MPOA	Medial Preoptic Area
MHz	Megahertz
MLC	Myosin light chain
MLCK	Myosin light chain kinase
Na^+	Sodium
nNOS	Neuronal nitric oxide synthase
NO	Nitric Oxide
O_2	Oxygen
PCr	Phosphocreatinine
PDE	Phosphodiesterase
PG	Prostaglandin
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5-biphosphate
PVN	Paraventricular Nucleus
P	Probability
ROS	Reactive oxygen species

SEM	Standard error of mean
sGC	Soluble guanylate cyclase
TXA_2	Thromboxane A_2
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
VIP	Vasoactive intestinal polypeptide
V	Volts

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CHAPTER 1

INTRODUCTION

1.1 Historical Background to Priapism

Priapism is a rare condition and is defined as a prolonged penile erection which is maintained in the absence of sexual stimulation and persists despite orgasm. The term is derived from Roman and Greek mythology and the mythological figure Priapus. He was thought to be the son of Aphrodites. Under a spell cast by Hera, Aphrodites gave birth to a son with an enormously and disproportionately enlarged phallus. Later cast out, Priapus became a symbol of the sacredness of birth and life in humans and the vitality of the animal and plant kingdoms. The enlarged phallus came to represent male generative power. Italian fertility cults based in Sicily in the 18th and 19th century attributed magical therapeutic powers to an effigy of genitals displayed to cure or prevent disease affecting the genitals.

The earliest account of the disorder in the medical literature is the citation entitled "Gonorrhoea, Satyriasi et Priapisme" by Petraens (1). However, over the next few centuries, reports were mainly restricted to case studies and management strategies for the condition. It was not until the early 20th century that Hinman through his extensive experience in managing this disorder attempted to classify the disease and suggested a rational approach to its management. He initially classified it into either mechanical or nervous types. The mechanical type was more common and would be equivalent in its presentation to low-flow or ischaemic priapism in modern times. He described this as being related to thrombosis of the veins of the corpora. Multiple clinical conditions were also grouped into this category including pelvic abscesses, penile tumours, perineal and genital injuries and blood dyscrasias. The

second category related to intracerebral disorders affecting the erectile process such as syphilis, brain tumours, epilepsy, spinal cord injuries (2). Following on from his father's work, Frank Hinman Jr hypothesized that the vascular stasis due to a reduction in venous outflow was the primary reason for a failure of detumescence in cases of idiopathic priapism. The identification of dark viscous blood most commonly seen following aspiration or incision of the corpus cavernosum in patients presenting with the condition supported this notion (2).

These observations led to the hypothesis that ischaemia may have a role in the development of priapism. Hinman proposed that deoxygenated blood either due to a reduced oxygen tension or elevated carbon dioxide tension enhanced blood viscosity particularly in patients with deformed erythrocytes such as sickle cell disease (2). The first accounts of the histopathological changes were also proposed namely oedema of the trabeculae and later corporal fibrosis (2).

Although the condition still lacks a complete understanding of the underlying pathophysiology, what is clear is that the overall condition can be classified with each subtype showing a typical course of events. These have developed predominantly from case reports and anecdotal reports of clinical cases over the last 50 years. However, considerable overlap does exist amongst these categories. The main subtypes are:

1. Low-flow (ischaemic) priapism
2. High-flow (non-ischaemic) priapism
3. Recurrent stuttering priapism

In order to understand the underlying dysfunction, which characterizes the condition of priapism, the physiological basis of penile erection is reviewed.

1.2 Physiology of Penile Erection

Penile erection is mediated by a spinal reflex following the central integration of multiple stimuli involving central neurotransmitters as well as autonomic and somatic efferents. The end result is smooth muscle relaxation of the corpus cavernosum which is essential to penile erection. The degree of smooth muscle tone in the penis is dependent on the balance between contractile and relaxant factors (3). An imbalance in the normal regulatory mechanisms of penile smooth muscle tone would result in a spectrum of disorders. At one extreme increased smooth muscle tone as a result of a failure of smooth muscle relaxation can lead to erectile dysfunction. At the other end persistent smooth muscle relaxation or a failure of contraction can present as priapism.

A normal penile erection is a complex haemodynamic event involving local, humoral and neural factors. The initial part of this section deals with normal penile anatomy and is followed by a review of the regulatory pathways culminating in smooth muscle relaxation.

1.2.1 Anatomy of the Human Penis

The human penis is composed of three bodies of erectile tissue; the two corpora cavernosa and the corpus spongiosum. The corpus spongiosum encircles the urethra. Surrounding each cavernosal body is the tunica albuginea which fuses in the midline to form an incomplete septum. The tunica albuginea is a multilayered structure of inner circular and outer longitudinal layers of connective tissue

encompassing the paired corpora cavernosa (Figure 1). The proximal portion of each corpus is known as the crus and is attached to the inferior ramus of the pubic bone. Each crus is surrounded by the ischiocavernosus muscle. Ventral to the corpora is the corpus spongiosum, the distal part of which expands to form the glans penis. The proximal part of the corpus spongiosum is known as the bulb and is enclosed by the bulbospongiosus muscle. Both the ischiocavernosus and the bulbospongiosus muscles are important in producing the rigid phase of an erection and in ejaculation (4;5).

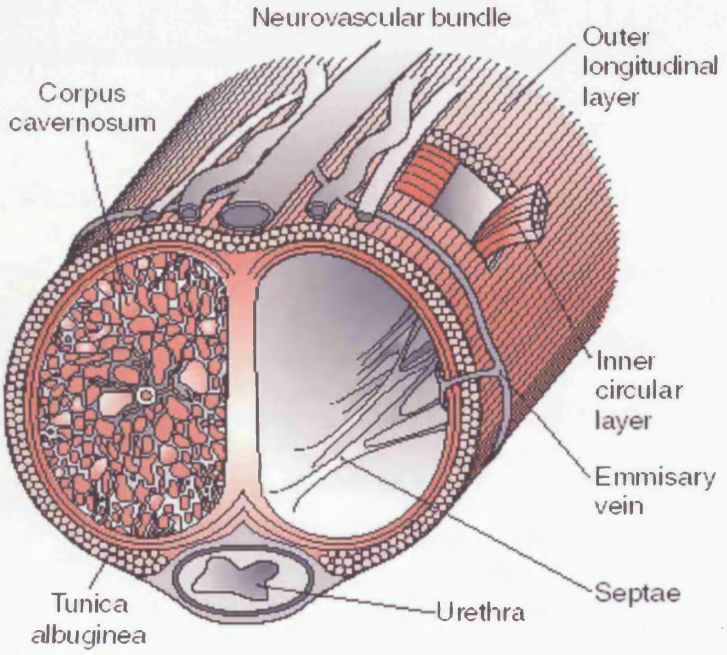


Figure 1: Cross sectional illustration of the human penis illustrating the anatomy of the penis. The inner and outer layers of connective tissue comprising the tunica albuginea are illustrated.

Each cavernosal body is composed primarily of smooth muscle (40-50%) and connective tissue (45%) interspersed with endothelium, fibroblasts and neural tissue. Within each corpus cavernosum there is a network of interconnected sinusoidal spaces (the trabeculae). Each sinusoidal space is surrounded by an endothelial layer,

which in turn is enclosed in bundles of smooth muscle. The smooth muscle is separated by a fine meshwork of collagen and elastin.

Although the corpus spongiosum contains spongy erectile tissue, this does not provide structure to the erection. The smooth muscle is less abundant and the cavernous spaces within the corpus spongiosum are larger in size compared to the corpus cavernosum. It is the ultimate relaxation of the trabecular smooth muscle in the corpus cavernosum and arteriolar smooth muscle which results in an increased blood flow into the cavernous spaces, which in turn causes penile engorgement and erection (4).

The penis receives its arterial blood supply from the internal pudendal artery, which is a branch of the hypogastric artery arising from the internal iliac artery. The internal pudendal artery divides to form the perineal artery and the penile artery, which in turn gives rise to the bulbourethral, dorsal and cavernosal branches. The cavernosal arteries enter the corpora cavernosa at a point where the two crura converge and penetrate the tunica albuginea. The cavernosal arteries then branch to form spiral shaped helicine resistance vessels and it is these arteries, which directly open into the cavernous sinusoidal spaces (Figure 2) (4).

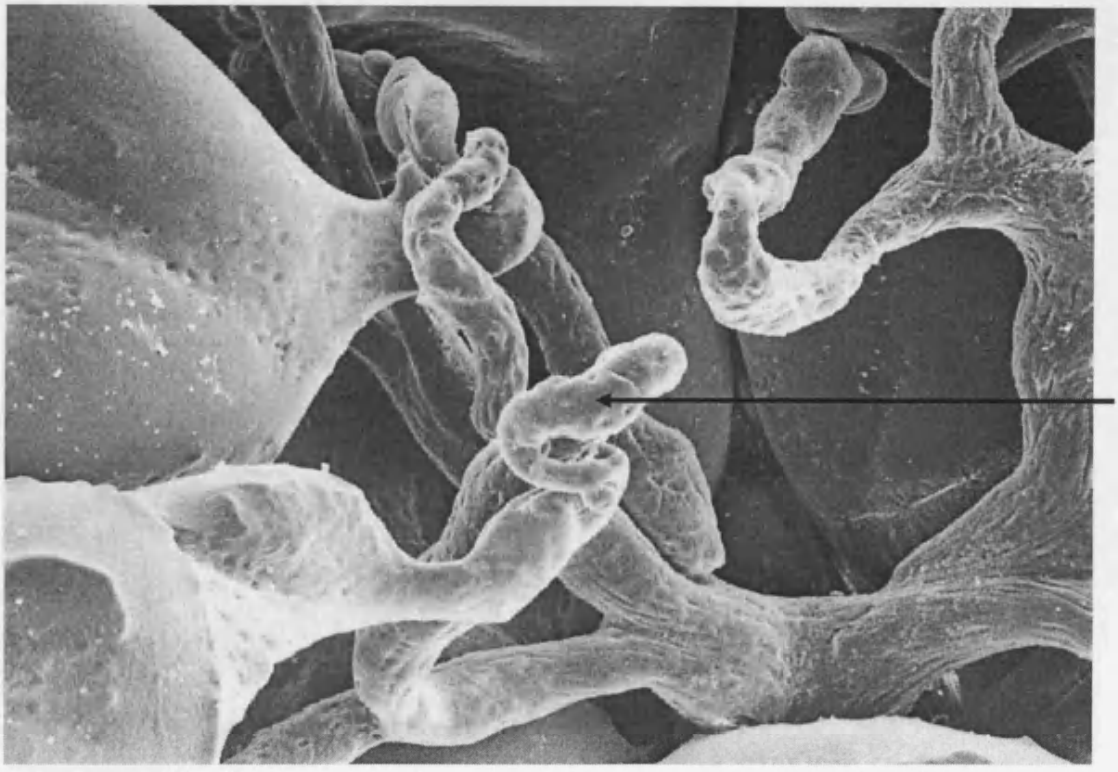


Figure 2: Scanning electron microscopic study of a cast of penile microcirculation. It shows a helicine artery opening directly into a lacunar space. Helicine arteries regulate the amount of blood entering into the lacunar spaces acting as resistance vessels. After the proper stimulus, the muscle surrounding the artery relaxes; increasing the blood flow and pressure delivered to the lacunar spaces. Modified from (6).

The venous drainage system of the penis occurs at three levels: superficial, intermediate and deep. The superficial venous system lies above the tunica albuginea and predominantly drains the penile skin although anastomotic branches to the deep dorsal vein can also occur. The intermediate system consists of the deep dorsal and circumflex veins. The sinusoids of the corpora cavernosa drain into a system of sub tunical venules that coalesce on the outer surface of the cavernous tissue just beneath the tunica albuginea. These venules form a number of veins which traverse the tunica albuginea called the emissary veins which subsequently drain into the circumflex veins on the outer surface of the tunica albuginea and then into the deep dorsal vein of the penis (Figure 3) (5).

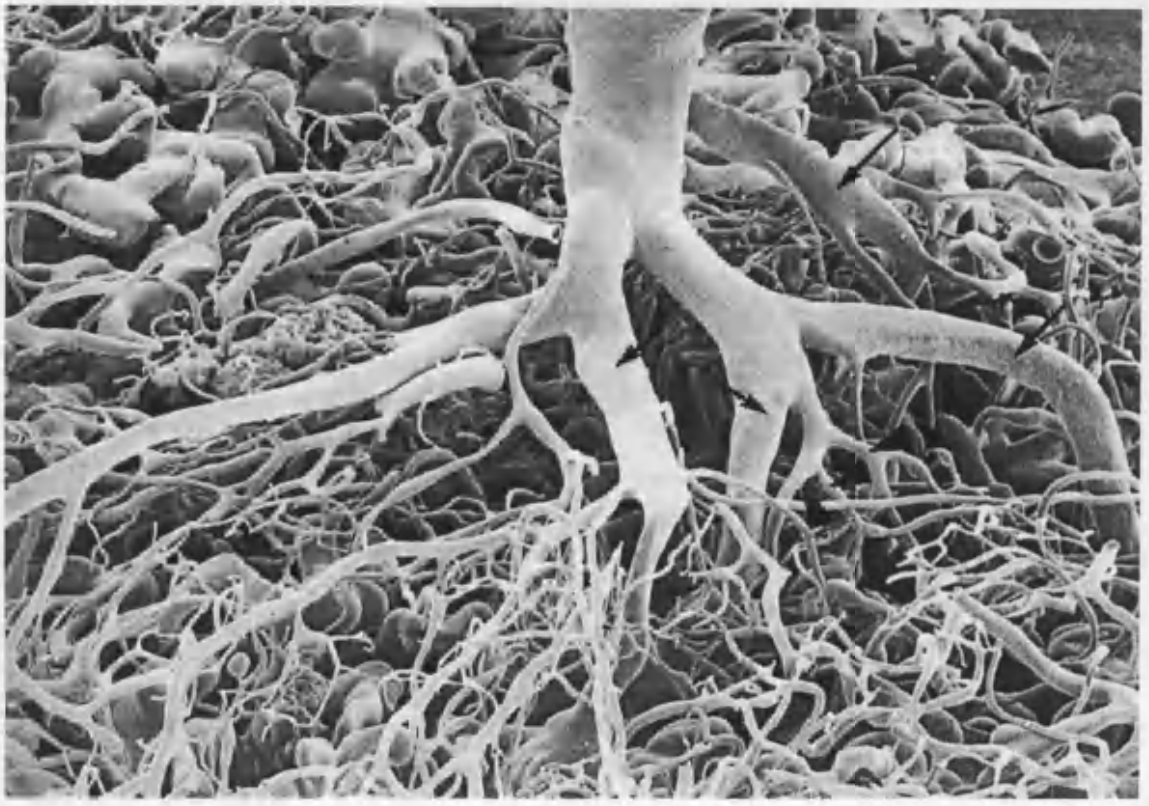


Figure 3: Scanning electron microscopic study of the venous system of the corpus. A dense plexus of venules, which drain the lacunar spaces, can be seen beneath the tunica albuginea. These veins coalesce to form the emissary veins. Arrows indicate the position of the emissary veins. Modified from (6).

1.2.2 Neuroanatomy of the Human Penis

The penis receives an autonomic innervation from both sympathetic and parasympathetic nervous systems. In addition to this, somatic afferent fibres are transmitted from the penis via the dorsal nerve of the penis.

1.2.2a Sympathetic Innervation

Sympathetic noradrenergic fibres emanate from preganglionic cell bodies within the intermediolateral grey matter of the spinal cord between T10 and T12 and pass to the paravertebral sympathetic chain. From here postganglionic fibres pass through the pelvic, cavernous and pudendal nerves to reach the corpora cavernosa. However, some preganglionic nerve fibres pass directly through to the paravertebral ganglia to reach the pelvic plexi, which are situated anterolateral to the rectum.

From the superior hypogastric plexus, the right and left hypogastric nerves connect to the pelvic plexus and it is from here that the main nerve supplies, via the cavernous nerves pass to the penis. In addition, efferent sympathetic fibres travel within the pudendal nerve to supply the ischiocavernosus and bulbospongiosus muscles, the muscles involved in the rigid phase of erection (4). The tonic release of noradrenaline from sympathetic nerves together with the endothelins from the endothelium maintain the penis in a flaccid state (3).

1.2.2b Parasympathetic Innervation

Preganglionic parasympathetic fibres derive from the intermediolateral grey matter of the spinal cord segments S2 to S4. It is from there that preganglionic fibres pass within the 6 paired nervi erigentes to the pelvic plexus. The plexus receives input from both parasympathetic and sympathetic nervous systems and it is here that the efferent and afferent neural pathways to the penis are integrated. The cavernous nerves, which carry the autonomic nerve supply to the penis arise here and pass to the penis in close proximity to the posterolateral surface of the prostate gland. They enter each penile crura along with the terminal branches of the pudendal vessels.

The main role of the parasympathetic nerves is to produce arterial dilatation and penile smooth muscle relaxation. Parasympathetic activity produces penile tumescence and erection by modulating the release of noradrenaline from sympathetic nerve terminals (7) and also by releasing nitric oxide (NO) and several neuropeptides (3).

1.2.2c Somatic Pathways

Afferent information from the penis is transmitted from the penile skin and glans via the dorsal, pudendal and sacral nerves to the spinal cord. The pudendal nerve has its cell bodies located in Onuf's nucleus which lies within the spinal cord segments S2-S4. These fibres convey somatosensory information from the glans penis and penile skin to the spinal cord.

1.2.2d Central Pathways

The hypothalamus contains two key areas involved in penile erection: the medial preoptic area (MPOA) and the paraventricular nucleus (PVN) (8). The MPOA is essential for the recognition of sensory stimuli. Neurones within the PVN are innervated by dopaminergic neurons arising from the caudal hypothalamus which synapse with oxytocinergic neurones (9;10). Multiple inputs, such as audiovisual, cognitive, olfactory and tactile sensation, are integrated within these centres and result in the activation of the autonomic pathways which mediate penile erection. Several neurotransmitters have been implicated in this process. The central pro-erectile neurotransmitters are dopamine, adrenocorticotrophic hormone (ACTH) and oxytocin. Those inhibiting penile erection are 5-Hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), neuropeptide Y and prolactin.

1.2.3 Haemodynamics of Penile Erection

Penile erection is a haemodynamic event, which has been divided into 7 separate phases (5). The overall balance between contractile and relaxant factors determines the overall degree of penile tumescence. The variation in the arterial flow during the phases of penile erection is illustrated in Figure 4.

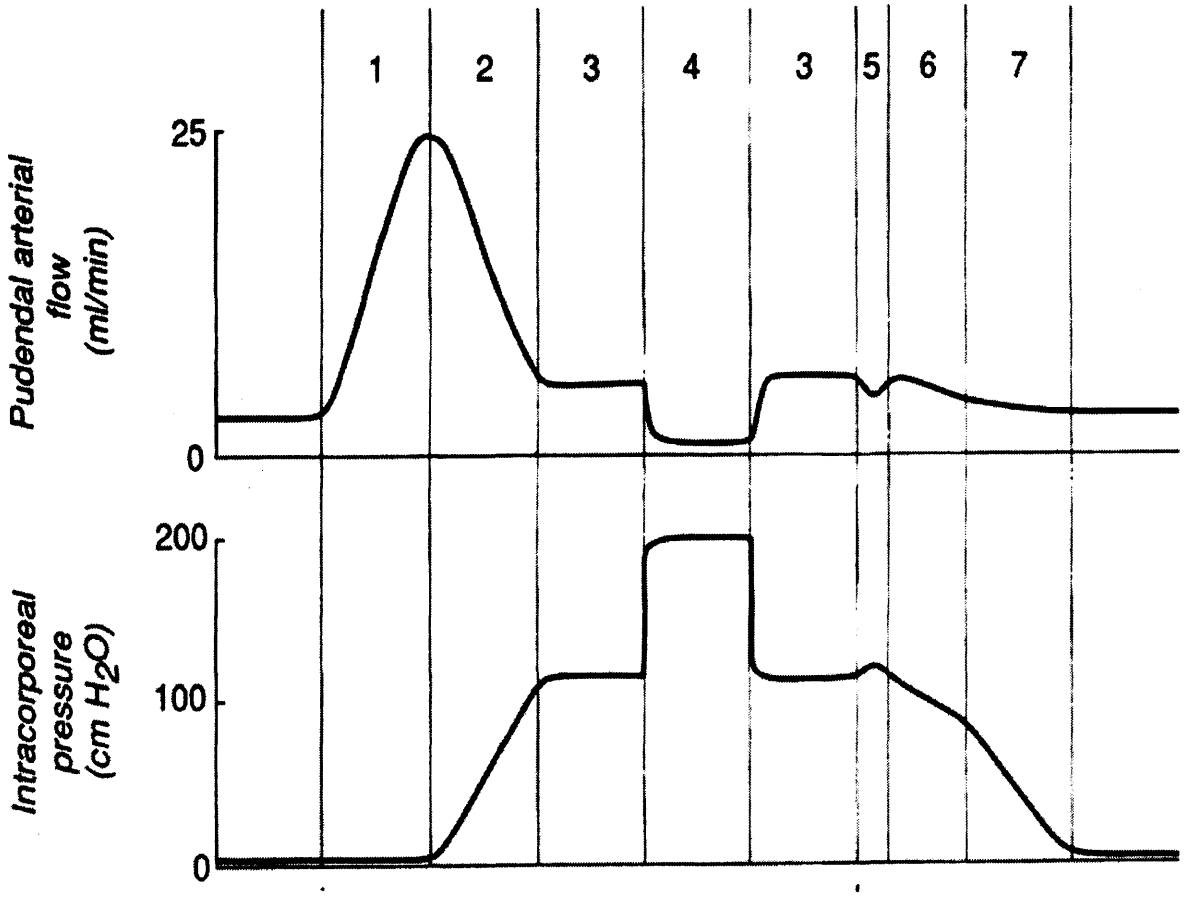


Figure 4. Blood flow and intracavernous pressure changes during the seven phases of penile erection and detumescence: 0, flaccid; 1, latent filling; 2, tumescent; 3, full erection; 4, rigid stage of erection; 5, transient stage; 6, slow detumescence; 7, fast detumescence. Modified from (5).

The physiological changes occurring in each stage are described below:

Phase 0 - Flaccid Stage: During this stage the arteriolar resistance is high and the corporal smooth muscle demonstrates a tonic contraction due to a basal release of noradrenaline from sympathetic nerves and endothelins from the endothelium. The penis therefore remains flaccid under resting conditions indicating that at this point the sympathetic system predominates.

Phase 1 - Latent Filling Stage: A combination of centrally located stimuli and sexual stimulation results in corporal smooth muscle relaxation and an increase in corporal blood flow. This is followed by a rapid increase in intracavernous pressure. In addition, there is increased flow through the cavernous and pudendal arteries.

Phase 2 - Tumescent Stage: The corporal smooth muscle relaxation is followed by filling of the sinusoidal spaces with blood resulting in penile tumescence. The penis is now both expanded and elongated.

Phase 3 - Erection Stage: The engorgement of the cavernous spaces leads to compression of the sub-tunical venules. The subsequent reduction in venous outflow results in a further increase of intra-cavernous pressure. A full erection is now achieved.

Phase 4 - Rigid Stage of Erection: Contraction of the ischiocavernosus and bulbospongiosus muscles leads to the rigid phase of erection. Intracavernous pressure is now well above systolic arterial pressure.

Phase 5 - Transient Stage: The reestablishment of sympathetic vasomotor tone leads to corporal smooth muscle contraction and contraction of the helicine vessels. The penis returns to the flaccid state.

Phase 6 - Detumescent Stage: Re-opening of the venous outflow channels leads to a reduction in intracavernous pressure.

Phase 7 - Fast Detumescent Stage: The veno-occlusive mechanism is no longer in operation and arterial inflow decreases leading to flaccidity of the penis.

1.2.4 Physiology of Ejaculation

Ejaculation involves the emission of semen into the prostatic urethra with the simultaneous closure of the bladder neck and propulsion of semen out of the urethra. Central control of ejaculation is likely to be at the hypothalamus and is triggered once sensory stimuli reach enough intensity to initiate the effector mechanism through the thoracic sympathetic outlets and chains (4). Contraction of the smooth muscle cells in the epididymes, vasa, seminal vesicles and bladder neck is via α-adrenergic receptors (4;5). Ejaculation can be divided into three phases (4):

Phase I: Under sympathetic control contraction of the epididymes and vasa result in the emission of seminal fluid into the urethra. This is followed by contraction of the seminal vesicles and the prostate.

Phase II: Closure of the bladder neck and contraction of the posterior urethra to prevent retrograde flow into the bladder.

Phase III: Closure of the external sphincter followed by rhythmic contraction of the bulbospongiosus along the urethra.

1.2.5 Pharmacology of Penile Erection

1.2.5a Adrenoceptors in Penile Erection

Although both α and β- adrenergic receptors have been identified within the penis, α- adrenoceptors outnumber β adrenoceptors in a ratio of 10:1 (11). The α adrenoceptors are further subdivided into α_1 and α_2 subtypes (12-14). The α_1 adrenoceptors subtypes α_{1A}, α_{1B} and α_{1D} have been demonstrated in human corporal tissue (15) with α_{1A} and α_{1D} receptors being the predominant ones (14;16).

α_2 adrenoceptors are subdivided into α_{2A}, α_{2B}, α_{2C} subtypes (13) and are more abundant on the arterioles supplying the cavernosal spaces (17). Functional studies have suggested the occurrence of postjunctional α_2 receptors in the corpus cavernosum (3) and selective α_2 agonists have induced concentration-dependent contraction of isolated strips of corpus cavernosum (13). Whether these are of importance in the regulation of corporal smooth muscle tone is still unclear.

In addition to this, prejunctional α_2 receptors may be involved in the modulation of NO release from autonomic nerves. Stimulation of prejunctional α_2 receptors inhibits NO release in horse penile resistance arteries (18).

The effects of α-receptor stimulation have been investigated using both phenylephrine and noradrenaline which have been shown to be potent contractors of human corpus cavernosum (19). These contractions were sensitive to the α_1 adrenoceptor antagonist prazosin and the non-selective α–adrenoceptor antagonist phentolamine. *In vitro* contractions were sensitive to inhibition by both Ca^{2+} channel antagonists nifedipine and rauwolscine (19;20). These studies suggest that α_1-mediated contraction results in the release of intracellular Ca^{2+} stores followed by the extracellular entry of Ca^{2+} for the maintenance of tone. Therefore α-adrenoceptor stimulation in the penis results in smooth muscle contraction and subsequent

detumescence of the penis. This is the basis for the use of α-adrenoceptor agonists in priapism in order to promote smooth muscle contraction and subsequent penile detumescence.

β-adrenergic stimulation appears to cause relaxation of both corporal and penile arteriolar vascular smooth muscle (19). Both β_1 and β_2 adrenoceptors are expressed in the cavernosal smooth muscle (11). Both procaterol, a selective β_2 adrenoceptor agonist and the β_1 and β_2 adrenoceptor agonist, isoprenaline relaxed noradrenaline contracted strips of human corpus cavernosum, which were effectively inhibited by the β antagonist propranolol (19). These results suggest that both β_1 and β_2 adrenoceptor activation causes relaxation of corporal smooth muscle. However, β receptors are markedly outnumbered by α receptors on cavernosal smooth muscle and their functional role in the maintenance of smooth muscle tone is still largely unknown.

1.2.5b Modulation of Calcium Sensitivity

The contraction of vascular smooth muscle is regulated by the intracellular Ca^{2+} concentration (21). However, the degree of elevation in intracellular Ca^{2+} concentration does not always correlate with the extent of contraction (22). *In vitro* experiments utilising FURA-PE3 loaded rabbit corpus cavernosum strips with simultaneous measurement of intracellular Ca^{2+} concentration and tension showed that the α_1 adrenoceptor-mediated contraction results from an increase in the intracellular Ca^{2+} as well as an increase in the Ca^{2+} sensitivity of the contractile apparatus (21). Ca^{2+} sensitising pathways involve the guanosine triphosphatase RhoA/Rho kinase pathway. The GTP bound active form of RhoA stimulates a downstream kinase, Rho kinase, which phosphorylates the myosin binding subunit of

myosin light chain phosphatase to inhibit myosin light chain phosphatase activity (23). An alternative pathway involves protein kinase C potentiated protein phosphatase-1 inhibitory protein (CPI-17) (21). This protein inhibits myosin light chain phosphatase when phosphorylated by protein kinase C. As the extent of myosin light chain phosphorylation depends on the balance of activities of myosin light chain kinase and myosin light chain phosphatase, myosin light chain phosphatase inhibition by these pathways results in increased myosin light chain phosphorylation, which accounts for the increased contractile response occurring at a fixed Ca^{2+} concentration (21).

1.2.5c Cholinergic Mechanisms in Penile Erection

The rich cholinergic innervation of penile tissue has been demonstrated by histochemistry (acetylcholinesterase staining) or immunohistochemistry (24-26). These nerves have been shown to express neuronal NO synthase (nNOS) (27). Muscarinic receptors have been found on both the corporal smooth muscle cell and the endothelium lining of the cavernous spaces in human penile tissue (28). *In vitro* studies using cultured membranes of human corpus cavernosum and endothelial cells suggest that human corpus cavernosum expresses M_1, M_2, M_3 and M_4 subtypes of muscarinic receptor (28). The M_3 subtype appears to be the predominant receptor on the endothelium (29), whilst the M_2 and M_4 subtypes are expressed mainly on the smooth muscle cell. *In vitro* experiments demonstrate that acetylcholine causes relaxation of smooth muscle by releasing NO from the endothelium (19;25;30) and other vasodilator substances which comprise the endothelium-derived hyperpolarizing factor (EDHF) via stimulation of M_3 receptors (31). Cultured human corpus cavernosum smooth muscle cells express M_2 and M_4 subtypes (29). It is not known as

to how cholinergic fibres could influence endothelial function *in situ* despite their wide anatomical separation (32-34). Alternatively, stimulation of preganglionic muscarinic receptors on adjacent adrenergic terminals may inhibit the release of noradrenaline (35).

1.2.5d Nitric Oxide and Penile Erection:

Nitric oxide (NO) is produced from the conversion of the amino acid L-arginine to L-citrulline by the enzyme NO synthase (NOS). There are three isoforms of NOS, namely endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS is expressed in the endothelium and nNOS in the postganglionic parasympathetic nerve fibres innervating penile smooth muscle (25;27;36). nNOS has also been demonstrated in preganglionic parasympathetic nerves as well as preganglionic sympathetic nerves (37-39).

The term 'nitrergic' applies to nerves whose transmitter function depends on the release of NO or to transmission mechanisms that are brought about by NO (40). The nitrergic pathway is now accepted as the predominant pathway mediating cavernosal smooth muscle relaxation (41).

With regards to the role of NO in the physiology of penile erection, the primary attention is initially given to the neuronal source of NO. The liberation of NO into the erectile tissue from nNOS requires neuronal depolarisation resulting from electrical impulses transmitted neurally in response to psychogenic and reflexive erogenous stimuli that converge upon nNOS containing efferent nerve fibres coursing within the corporal bodies (42). Acetylcholine as well as other neurotransmitters such as substance P and bradykinin stimulates NO release from endothelial sources in the penis (43;44). These neurotransmitters exert their effects by inducing a transient

increase of calcium entry into endothelial cells which stimulates NO production by eNOS. Acute increases in 'shear stress' which is the term used to describe pressure forces exerted on endothelial cells by the flow of blood over them, act to drive rapid but limited amounts of NO release by similar biochemical mechanisms (45;46). The release of NO from the nitrergic nerves and endothelium ultimately results in smooth muscle relaxation. NO activates the intracellular enzyme soluble guanylate cyclase (sGC) which increases intracellular cGMP concentrations. The resulting smooth muscle relaxation is thought to involve several intracellular mechanisms. These include the following:

1. cGMP may inhibit the L-type Ca^{2+} channel and reduce the influx of Ca^{2+} into the cell (47)
2. the activation of a cGMP-dependent protein kinase may subsequently mediate several events in the cascade including

- inhibition of voltage-gated Ca^{2+} channels (48)
- activation of Ca^{2+}-sensitive K^{+} channels resulting in hyperpolarisation of the cell membrane (49)
- activation of the Na^{+}-Ca^{2+} exchanger (50)
- activation of membrane bound Ca^{2+}ATPase (51)
- inhibition of the IP_3 receptor on the sarcoplasmic reticulum (52)
- inhibition of the IP_3 receptor on the sarcoplasmic reticulum membrane by phosphorylating IP_3 receptor related regulatory proteins or IP_3 receptor associated cGMP kinase substrate (53)
- binding to PIP_2 phosphatidyl inositol 4, 5 bisphosphate and inhibiting production of IP_3 (54)
- sensitisation of myofilaments to intracellular Ca^{2+} by Rho-kinase is involved in maintaining smooth muscle contraction. This is modulated by Rho A/cGMP interaction (55)

1.2.5e Endogenous Modulators of Penile Erection

Various other neurotransmitters have been identified in the corpus cavernosum but the effects are predominantly modulatory as opposed to having significant relaxant or contractile effects. These are mentioned briefly:

Vasoactive intestinal polypeptide (VIP): VIP-immunoreactivity has been demonstrated in human penile erectile tissue (56). Nerves containing VIP also contain immunoreactivity to NOS and this colocalization of NOS and VIP has been demonstrated in both humans and animals (57;58). Although intracavernosal administration of VIP has been shown to cause tumescence in some individuals (59), it has been difficult to convincingly demonstrate that VIP released from nerves is responsible for the relaxation of penile smooth muscle *in vitro* or *in vivo* (41). Studies utilising rabbit corpus cavernosum showed that a VIP antagonist inhibited electrically induced contractions suggesting that VIP was released from nerves during stimulation and concluded that the mechanism of relaxation was dependent on prostanoids and involved the generation of NO (60).

Histamine: The role of histamine in penile erection has received very little attention and is thus poorly understood. Three types of histamine receptor have been described, H_1, H_2 and H_3. The principal receptor in corpus cavernosum smooth muscle is the H_1 subtype (61). There have been very few studies which have investigated the action of histamine on isolated corpus cavernosum. These studies have also been contradictory. One study supports the contractile role of H_1 receptor activation using isolated rabbit corpus cavernosum (61). However, a study utilising an *in vitro* preparation of human corpus cavernosum found that histamine (concentration 3-100μg) produced a dose

dependent relaxation (62). Furthermore, an intracavernosal injection of histamine was shown to produce a penile erection in humans which is shorter in duration compared to papaverine use (62).

5-Hydroxytryptamine (5-HT, serotonin): The use of selective 5-HT agonists on isolated strips of rabbit corpus cavernosum failed to induce smooth muscle relaxation in one study (63). A further study utilising human corpus cavernosum concluded that peripherally 5-HT may have a physiological role in the control of penile detumescence and maintenance of flaccidity. Further studies utilising rabbit corpus cavernosum demonstrated a dual response of contraction mediated by type 1 and 2 receptor subtypes and relaxation mediated by type 4 receptor subtypes (64). Therefore the role of peripheral 5-HT is still uncertain.

Purinergic mechanisms: The role of purines such as ATP and adenosine in penile erection is not fully established, although both appear to lead to relaxation of strips of rabbit corpus cavernosum and canine penile artery pre-contracted with noradrenaline (65). Further work is required to clarify their role in the control of corporal smooth muscle tone.

EDHF: The relaxation of vascular tissue evoked by acetylcholine and other neurohumeral substances are mediated by endothelium-dependent mechanisms which involve NO as well as other endothelium-derived relaxing factors (66). Studies on vascular tissue with blockade of the L-arginine-NO pathway by inhibiting NO synthase and the prostaglandin pathway by cyclooxygenase inhibition have demonstrated that endothelium-dependent relaxations still occur and are associated

with hyperpolarisation (67). The alternative EDHFs involved in relaxation pathways may be related to products of arachidonic acid metabolism or metabolites of P-450 (67). EDHF is reported to be an important contributor to vasodilation in coronary arteries (68). EDHF contributes to smooth muscle relaxation during penile erection. It has been proposed that EDHF has an important role in the endothelium-dependent relaxation of human penile resistance arteries as opposed to the trabecular smooth muscle in the corpus cavernosum (31).

Endothelins: Endothelins are 21 amino-acid polypeptides, which are produced by all endothelial cells. Endothelin-1 is the only subtype produced by vascular endothelial cells and causes potent contraction of all types of vascular smooth muscle, including corporal smooth muscle (69). Endothelin-2 and endothelin-3 are less potent peptides.

Endothelin-1 is not stored in discrete granules but synthesized directly from mRNA in response to stimuli such as hypoxia, ischaemia, catecholamines, angiotensin II, thrombin or shear stress.

Receptors for endothelins namely ET_A and ET_B have been demonstrated on the endothelium and smooth muscle of blood vessels, including those of human erectile tissue (70). ET_B receptors are mainly expressed on the endothelial cells, whilst the ET_A receptors have been localised to the endothelium lining the cavernosal lacunar spaces in the penis (71). ET_A receptors mediate the contractile response of the endothelium (72) while ET_B mediates the endothelium dependent vasodilator response possibly through the action of ET-3 and local NO release (73).

ET-1 has been shown to potently induce long lasting contractions in penile corpus cavernosum (69;70;74). Therefore the main role of endothelins in penile

smooth muscle physiology is to sustain smooth muscle tone and maintain penile detumescence (75).

Prostaglandins: Prostaglandins form a heterogeneous group of substances with diverse physiological effects. It has been known for some time that exogenous prostaglandin (PGE_1) application results in cavernosal smooth muscle relaxation and indeed it has been the mainstay of treatment for men with erectile dysfunction for some time (76). More recently researchers have tried to focus on the possible physiological and pathophysiological role for prostaglandins in the penile vasculature. Shear stress or changes in oxygen tension can result in stimulation of the endothelium and activation of phospholipase A_2 and/or phospholipase C, which in turn liberate arachidonic acid from membrane-bound phospholipids in vascular endothelial cells. Arachidonic acid is converted to PGH_2 by PGH synthase or cyclo-oxygenase. There are two subtypes of this enzyme, namely the constitutive (COX 1) and inducible forms (COX 2). In turn, PGH_2 is acted upon by a specific endoperoxidase leading to the formation of the corresponding prostanoid of which $PGF_{2\alpha}$, thromboxane A_2 (TXA_2), PGI_2 (prostacyclin), PGE_2 and PGD_2 are the primary active metabolites. In addition, arachidonic acid may be converted into leukotrienes via the action of lipo-oxygenases. The action and role of leukotrienes in the control of corporal muscle tone is unknown.

Pharmacological studies in other vascular beds have enabled the classification of prostanoid receptors on functional characteristics. Hence PGD_2 mainly acts on a specific G-protein coupled receptor termed the DP receptor, whilst PGE_2, $PGF_{2\alpha}$ and TXA_2 act via specific receptors, namely EP, FP and TP. Recently, the EP receptor has been further typed into 6 different subtypes, PGE 1-6. Receptor mRNA corresponding

to EP2, EP3I, EP3II and TP subtypes have been isolated from both human corpus cavernosal tissue and cultured human cavernosal smooth muscle cells (77). The effects of a specific PG appear to not only depend upon the specific tissue studied but also the distribution of the various receptor subtypes within that tissue. Furthermore, there is also a considerable degree of receptor cross specificity in all vascular smooth muscles, including the penis.

The overall contribution of prostanoids in the regulation of penile erection is complex and not completely understood. However, endogenous and exogenous prostanoids regulate penile smooth muscle contraction via specific receptors. The TP receptors mediate smooth muscle contraction and the EP and IP receptors mediate smooth muscle relaxation (78).

1.2.5 Mechanisms Involved in Penile Smooth Muscle Contraction

Contraction of human penile arteries and trabecular smooth muscle is largely mediated by the stimulation of α_1 adrenergic receptors by circulating noradrenaline (14;35) or noradrenaline release from autonomic nerves (35) as well as endothelins from endothelium (79).

Stimulation of α_1 adrenoceptors activates G protein-coupled phospholipase C. Once activated phospholipase C converts inositol 4,5 bisphosphate (IP_2) into inositol 1,4,5 triphosphate (IP_3) and diacylglycerol. IP_3 binds to its receptor on the sarcoplasmic reticulum releasing Ca^{2+} into the cytosol. Although vascular smooth muscle can utilise extracellular Ca^{2+} which enters via voltage gated L-type channels, IP_3 -dependent Ca^{2+} release from internal stores is predominantly responsible for smooth muscle contraction in the corpus cavernosum. The contractile process involves the binding of calcium to calmodulin which then activates myosin light chain

kinase (MLCK). The activated MLCK catalyses the phosphorylation of the regulatory light chain subunits of myosin (MLC_{20}). Phosphorylated MLC_{20} activates myosin ATPase triggering myosin heads to form cross bridges with actin filaments resulting in smooth muscle contraction (22;80).

1.2.6 Role of Oxygen and pH in Regulation of Penile Smooth Muscle Tone

The penile arterial inflow determines oxygen tension and the erectile tissue in the penis demonstrates a situation whereby the oxygen tension changes with function. During the flaccid state the cavernous tissue oxygen tension is similar to that of venous blood which is approximately 20-40mmHg (81;82). During erection the increased arterial blood in the sinusoidal spaces increases the pO_2 to approximately 100mmHg (82). The synthesis of NO by NOS requires both L-arginine and oxygen as substrates. The variation in pO_2 during a penile erection has been proposed to regulate the synthesis of NO (81-83). Therefore at the relatively low pO_2 levels which occur during the flaccid state, the basal levels of NO are low (82). As cavernous levels of O_2 increase following vasodilatation in response to sexual stimulation, increased levels of NO are synthesised with estimates of approximately 50-60mmHg O_2 required in order for NO synthase to reach full activity (81). In addition to reduced NO synthesis at low oxygen tension, the formation of PGE_2 is also impaired and contributes to the maintenance of penile flaccidity and detumescence (84).

1.3 Priapism and its Classification

Priapism can be subdivided into three main subheadings; low-flow (ischaemic), high-flow (non-ischaemic) and stuttering (recurrent) (85). Each group has several underlying aetiological factors which are described below.

1.3.1 Low-flow (Ischaemic) Priapism

This is the most common subtype of priapism (86). Prolonged penile erection results in the stasis of corporal blood in a closed compartment. This results in a progressive alteration of the tissue microenvironment consistent with stagnant ischaemia (87;88). This is regarded as an acute medical emergency but in practice medical attention is often delayed (86;89;90).

During this condition, there is impairment in the outflow of blood from the corpus cavernosum. Eventually this results in a failure of replenishment of the cavernous spaces by arterial blood. Approximately 50-70 millilitres of blood are trapped within the cavernosal space. The venoocclusive mechanism remains compressed and there is very little replenishment by arterial blood. As the duration of this blood stasis increases, the extracellular milieu becomes increasingly acidotic and hypoxic (91). The degree of hypoxia and acidosis in corporal blood aspirates is one method utilised to differentiate between low-flow and high-flow priapism (87;92).

1.3.1a Aetiology of Low-flow Priapism

Idiopathic: Approximately 30% of cases are idiopathic (86;93)

Hypercellular haematological disorders: Haematological conditions which result in blood hyperviscosity may induce priapism. These include leukaemia (commonest association with chronic granulocytic leukaemia) (94), thalasaemia (95),

polycythaemia, fat emboli and Fabry's disease. Total parental nutrition which contains 20% fat emulsion may result in blood hypercoaguability (96). By far the commonest group of patients suffering from low-flow priapism are those with sickle cell anaemia. The most commonly affected are those with the homozygous form. Between 38-42% of men with sickle cell disease have at least one episode of priapism in their lifetime. Sickle cell crises are associated with red cell adhesion followed by venoocclusion in the penis. The subsequent failure of blood to drain from the penis is manifested as prolonged priapism (97).

Iatrogenic: The most common association is with antipsychotics (chlorpramazine, clozapine), antihypertensives (hydralazine, prazosin, guanethidine) and oral coumarins. The mechanism is considered secondary to α-adrenergic blockade (98) Intracavernosal agents utilised in erectile dysfunction and directly instilled into the corpora (e.g. papaverine, prostaglandin E_1, phentolamine) are commonly associated with low-flow priapism and account for 0.5-6% of cases (89). Drugs which have the following pharmacological effects may also be associated with priapism due to either a peripheral or centrally mediated action:

1. α adrenergic blockers
2. serotoninergic agonist
3. dopamine agonist
4. parasympathetic agonist

Malignancy: Various metastasic lesions are associated with priapism. Again metastatic deposits result in venoocclusion and the subsequent development of

priapism. Those reported include prostate carcinoma (99), bladder cancer (100;101), urethral metastases (102).

Neurological: Although several neurological conditions are associated with the onset of priapism the underlying mechanism is not entirely clear. Examples of neurological disorders include cauda equina syndrome, spinal cord lesions and spinal stenosis. One suggestion is that neurological conditions may result in the potentiation of erectogenic neurotransmitters from the parasympathetic system or reduce the tonic discharge from the sympathetic neurones (103).

Others: Other rare causes which have been reported include the systemic diseases amyloidosis and rabies (104;105).

1.3.1b Clinical Presentation of Low-flow Priapism

Patients presenting with low-flow priapism commonly present as a delayed medical emergency. Clinically the patients describe a prolonged painful erection in excess of several hours. There is a failure of detumescence despite initial conservative techniques employed by the patients which include ejaculation, cold baths and the application of ice directly onto the erect penis. Failure of these measures and the increasing distress attributed to the pain, leads one to seek appropriate medical intervention (89;104).

Although a large number of these cases are idiopathic in nature, a number of predisposing conditions have been reported (see section 1.3.1a). Classically the penis is erect and may show signs of oedema. Patients are distressed and anxious (89;97).

1.3.1c Medical Management of Low-flow Priapism

The initial management involves the administration of analgesia. As the duration of the erection increases, the tissue becomes increasingly more hypoxic and acidotic which results in painful stimuli via nociceptors in the penis and opioid analgesia is often required (86).

Once adequate pain relief has been administered, aspiration of stagnant corporal blood is performed. Using a large gauge needle and local anaesthesia, corporal blood is aspirated by inserting the needle directly into the corpus cavernosum. The blood appears deoxygenated and viscous. In some instances this may be adequate in itself to resolve the problem, venoocclusion is successfully reversed and reperfusion allows penile detumescence (85;87). The aspirated blood is sent for biochemical analysis in order to determine the pO_2 and pH of the aspirated blood and this helps to verify that this is indeed a low-flow priapism as opposed to a high-flow priapism (85;87;92). However this simple procedure may not completely resolve the problem and additional measures are required.

The next step involves the instillation of α-adrenergic agonists to induce contraction of the corpus cavernosum and the helicine arteries in order to reduce the volume of stagnated blood and relieve the pressure on the subtunical venules and ultimately lead to detumescence. Despite the uncertainty in the initiating events leading to low-flow priapism, the cavernosal smooth muscle may still fail to contract due to the metabolic alteration in the immediate microenvironment of the corporal smooth muscle.

The most common agent utilised is the α-adrenergic agonist phenylephrine (92). Careful monitoring of the patient's blood pressure is required as the adrenergic effects of the drug on the systemic circulation can lead to systemic hypertension (92).

One method used to minimise this is the use of a tourniquet around the base of the penis in order to reduce the systemic absorption. Alternative α adrenergic agents which have successfully been utilised include epinephrine and norepinephrine (92). Oral terbutaline has also been reported to be successful (106;107) although it does also have β_2 agonist effects and minor β_1 activity (85).

1.3.1d Alternative Pharmacotherapies for Low-flow Priapism

A combination of cavernosal blood aspiration and instillation of phenylephrine alone is successful in a large number of cases, particularly those presenting after a short period of priapism. However, alternative medical treatments have been utilised in those patients who fail to respond to these measures:

Methylene Blue: Methylene blue is a compound with several biological effects. It is a well known inhibitor of soluble guanylate cyclase (sGC) (108). By inhibiting intracellular sGC, and therefore reducing cGMP levels, cavernosal smooth muscle tone is increased. The use of methylene blue has been limited to anecdotal reports and small series (109-111).

Etilefrine: This compound is an α-adrenergic agonist which has the advantage of oral administration as well as intracavernous use. It is commonly utilised in sickle cell patients. Although not widely used at present, there have been successful reports in the literature when used sickle cell patients (112-114).

Intracorporal thrombolytic agents: Failure of conventional aspiration and drainage techniques has led to some investigators utilizing thrombolytic agents such as

streptokinase in order to allow reperfusion of the erectile tissue (115). Again this technique has only been described in a single case report and does not have widespread acceptance as a therapeutic option (85).

1.3.1e Surgical Management of Low-flow Priapism

Surgical intervention has not changed significantly over time. The underlying physiological basis of surgical interventions is to divert ischaemic corporal blood into the venous system by developing a new channel of blood flow. The main surgical procedures which have been utilised are listed in Table 1 and then described below.

Year	Technique
1964	**Quackels procedure**
1964	**Grayhack procedure**
1975	**Ebbehoj procedure**
1976	**Winter Shunt**
1976	**Barry procedure**
1981	**Al Ghorab procedure**

Table 1. Chronological development of surgical procedures utilised in low-flow priapism.

Quackels procedure: This procedure was first described in 1964 (116). The procedure involves the formation of a shunt between the cavernosal and spongiosal systems and can be performed by using the proximal part of the corpus spongiosum where 1cm windows of tissue are removed. Once the flow of oxygenated blood is established the openings in each body are anastomosed using absorbable 5-0 PDS

sutures. If the intracavernosal pressure falls below 40mmHg after 10 – 15 minutes then the procedure can be deemed successful. However, the procedure can be performed bilaterally if there is failure of detumescence following a unilateral procedure.

Saphenous vein – corpus cavernosum shunt: This procedure was described by Grayhack in 1964 (117). In this procedure the saphenous vein is isolated and exposed for approximately 10cm. The proximal aspect is detached so that it is long enough to reach the penis. An ellipse of tunica is excised and an end to side anastomosis is created after irrigating the corpus cavernosum until oxygenated blood is obtained.

Winter Shunt and Ebbehoj Procedure: This is the simplest shunt formed between the corpus spongiosum and the corpus cavernosum initially described by Ebbehoj in 1975 (118) and later modified by Winter in 1976 (119). A narrow blade scalpel is inserted into the glans dorsal to the urethral meatus into the corpus cavernosum. Multiple incisions are made through the same entry site by rotating the scalpel blade 90°. The overlying glans is then closed using sutures. In contrast Winters procedure uses a Trucut biopsy needle to remove cores of tunica albuginea to create fistulous communications. Several passes may be required before successful detumescence occurs.

Cavernoso-dorsal vein shunt (Barry procedure): This procedure was described in 1976 and involves a 4cm skin incision at the penile base. The superficial or deep dorsal vein is identified and mobilized. Ligation of the distal aspect is followed by an

anastomosis of the proximal vein to the corpus cavernosum after spatulation. The size of the vein has meant that the procedure has not been as successful as expected.

Al Ghorab procedure: This procedure was first described in 1981 (120). The procedure allows the formation of a larger shunt. A 2cm dorsal semi circular incision of the glans at the coronal level exposes the distal corpora. A further circular core of tissue which is 5mm in diameter is excised from each corpora to create a cavernous-glandular shunt. The glans is then resutured to the penis after the penis has been squeezed until oxygenated blood is seen to flow.

Despite the use of these surgical procedures, approximately 50% of patients develop erectile dysfunction following successful detumescence (86). If the insertion of a penile prosthesis is required in the future, placement is very difficult due to the presence of significant corporal fibrosis (121).

1.3.2 High-Flow Priapism

1.3.2a Aetiology of High-Flow Priapism

Although this is less common compared to low-flow priapism, it is usually a consequence of perineal trauma (122;123). It commonly occurs following traumatic injuries such as straddle-type injuries where the cavernosal artery is compressed against the pubic bone. The injury is not realized until several hours or days following the trauma have elapsed and unrestricted arterial flow enters the cavernous space. A variable degree of tumescence occurs depending on the size and location of the arterial defect and the degree of venous drainage (122). Intracavernous injections can also result in the formation of a cavernosal artery-corporal fistula and subsequent

high-flow priapism (87;123). Rarer causes have also been reported such as sickle cell disease (124), cavernosal artery pseudoaneurysms (125), Fabry's disease (126).

1.3.2b Clinical Presentation of High-Flow Priapism

This condition has a lower incidence than low-flow priapism. One of the differentiating features between high-flow and low-flow types is the increased arterial blood flow which occurs in high-flow priapism as shown by Doppler studies. The blood is well oxygenated and the risk of ischaemic damage with eventual corporal fibrosis and impotence is low (85;127). Potency is reported to be preserved in 77%-86% of patients with long term follow up (122;127). However, diagnosing the condition is of paramount importance, particularly differentiating the condition from the more serious low-flow priapism. Although corporal blood aspiration here will generally reveal the presence of oxygenated corporal blood, on occasions this may be misleading. By inserting a needle into the corpus cavernosum of a low-flow priapism, an iatrogenic shunt can be created and erroneously convert a previous low-flow priapism which has a poorly oxygenated and acidotic microenvironment into a high-flow priapism as diagnosed on Doppler studies. Although the blood parameters may improve slightly the corpus cavernosum smooth muscle is still exposed to a hypoxic and acidotic microenvironment. Therefore a thorough history of each condition should be ascertained. The investigation of choice is a Doppler ultrasound. This would help differentiate high velocity corporal blood flow as would occur in high-flow priapism from stagnant blood flow which occurs in low-flow priapism.

1.3.2c Management of High-Flow Priapism

Medical management generally does not have a role in high-flow priapism although some conservative methods employed have been reported to lead to successful detumescence. These involve the use of ice packs and observation (127). The use of methylene blue by intracavernosal injection has been reported to achieve partial detumescence (128). By using contrast injected into the systemic arterial tree, images of the internal iliac vessels and the corporal vessels can be evaluated as illustrated in Figure 5. Superselective embolisation can then be utilised using material such as gelfoam which is absorbable or metallic coils (129-132). Although spontaneous resolution can occur after observation, immediate embolisation is both successful and avoids the risk of long term venous leakage (129). In the presence of fistulae at the base of the corpora, duplex ultrasound guided compression can also be successful in reversing the high-flow priapism. Open arterial ligation using intraoperative ultrasound techniques has also been described (133).

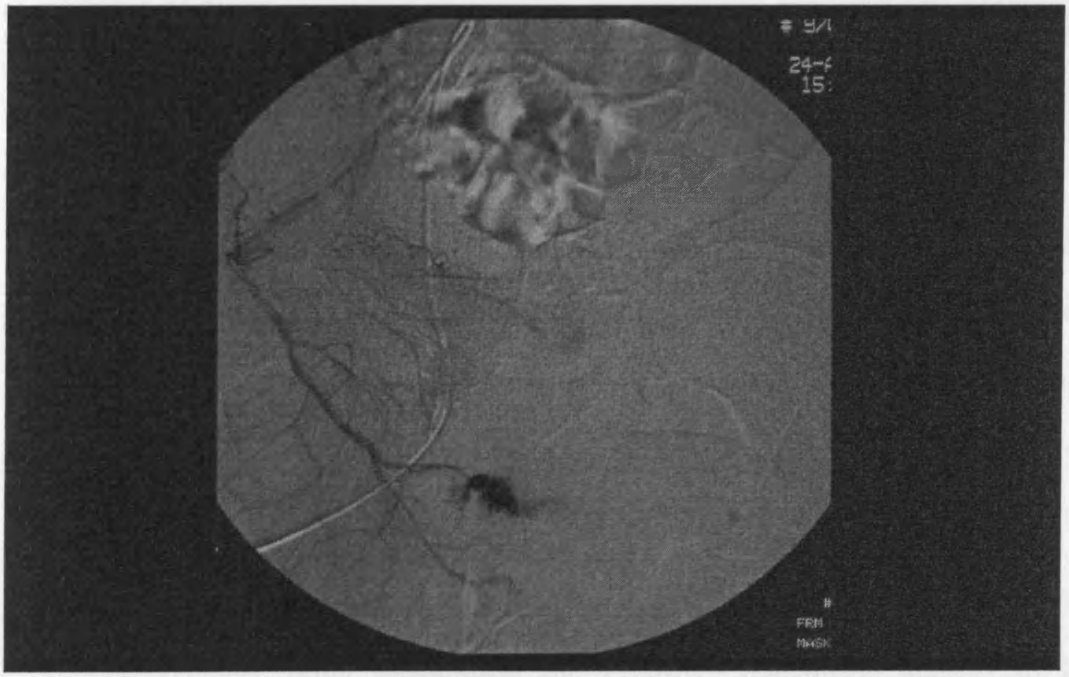


Figure 5. Arteriogram illustrating traumatic fistula in a patient with high-flow priapism (Illustration courtesy of Mr D.J. Ralph and reproduced with permission)

1.3.3 Stuttering Priapism

1.3.3a Aetiology of Stuttering Priapism

This condition is most prevalent in patients with sickle cell anaemia although idiopathic stuttering priapism is now also increasingly recognised (98). There is no proven aetiology and the condition is characterised by having features of low-flow priapism with a recurrent nature (98). As with low-flow priapism there is an underlying alteration in the balance of relaxation and contraction within the corporal smooth muscle which is weighted heavily in favour of relaxation resulting in prolonged penile erections which are generally self limiting (98).

1.3.3b Clinical Presentation of Stuttering Priapism:

This condition presents as recurrent episodes of prolonged painful erections which are venoocclusive in nature. Despite the frequency and duration, they spontaneously resolve although medical intervention is required if there is a failure of spontaneous resolution (98). The majority of patients report recurrent painful nocturnal erections which can last for several hours in duration. Symptoms of pain indicate that the corporal blood is becoming increasingly hypoxia and acidotic with the subsequent stimulation of nociceptors in the cavernous tissue. Although previously interpreted as probably being physiological nocturnal erections in poor sleepers, the presence of the problem in individuals without warning and lasting several days with spontaneous resolution shows that this condition is distinct (98).

1.3.3c Medical Management of Stuttering Priapism

Management using rehydration and exchange transfusion appears to be sufficient in a vast majority of cases where this is a manifestation sickle cell disease.

Hormone analogues and subcapsular orchidectomy attempt to abolish erectile function altogether by reducing testosterone circulation which then reduces the levels of NO synthase in nitrergic neurones (134). As the understanding of this condition is limited the therapeutic options tried thus far include:

Digoxin This is a cardiac glycoside and chronic digoxin administration is associated with erectile dysfunction (135). Digoxin inhibits the smooth muscle membrane Na^+-K^+-ATPase (136) which prevents the efflux of Ca^{2+} from the smooth muscle cell and increases the intracellular Ca^{2+} level. Smooth muscle contraction occurs which then results in penile detumescence. Whether there is a significant role in priapism is still unclear. However, an *in vitro* study showed that digoxin impairs NO-mediated smooth muscle relaxation (135).

Procyclidine: This is a muscarinic antagonist most commonly used in the treatment of drug-induced extra pyramidal disorders and Parkinson's disease. It has also been used as a first-line treatment in stuttering priapism at a dose of 2.5mg with very limited success. The precise mechanism of action is unknown with only anecdotal reports of success existing.

Cyproterone acetate: Cyproterone acetate has a strong gonadotropin-inhibiting effect and has a well established clinical use as an anti-androgen for the treatment of hyperandrogenic disorders such as hirsutism, acne and seborrhea in women. Cyproterone acetate is also used in the treatment of prostate cancer and hypersexuality disorders. The administration of cyproterone acetate to young males results in a 50% reduction in the plasma levels of FSH and LH together with a 75% reduction in the

plasma levels of testosterone. The effects are mediated by the inhibition of testosterone production and direct anti-androgen action. Gonadotropin releasing hormone analogues have been used with success in treating recurrent priapism (134). In this series the frequency of priapism episodes was reduced and libido and sexual function was preserved (134).

Stilboestrol: Although most commonly utilized in sickle cell patients who suffer from repeated episodes of priapism, those patients with idiopathic stuttering priapism who can not tolerate the alternative pharmacotherapies can be successfully controlled on stilboestrol. A double blind placebo controlled crossover study found that 5mg daily was better than placebo in preventing attacks in patients with sickle cell disease (137).

Pseudoephedrine: More commonly used as an oral decongestant, pseudoephedrine is often used as a first line treatment in stuttering priapism. Pseudoephedrine acts on β_2 receptors in bronchial smooth muscle to cause relaxation and via the α-adrenergic receptors/cAMP pathway in the respiratory mucosa to produce vasoconstriction. The effects on corporal smooth muscle have not been investigated although it is likely that smooth muscle contraction in the corpus cavernosum is mediated by the α adrenergic receptors.

Phosphodiesterase type 5 (PDE5) inhibitors: This is a novel treatment option in cases of idiopathic stuttering priapism (138). Studies utilizing rat myocytes incubated with the PDE5 inhibitor, sildenafil, have found an up-regulation of the enzyme PDE5 (138) suggesting that in stuttering priapism a reduced level of PDE5 in the corpus

cavernosum may potentiate the intracellular action of NO and prolong the penile erection. However it is debatable whether after the initial NO-mediated corpus cavernosum smooth muscle relaxation, impaired smooth muscle contraction is due to NO or due to the changes in the corpus cavernosum smooth muscle microenvironment.

1.3.3d Surgical Management of Stuttering Priapism

Drug Delivery Systems: Drug delivery systems were first described in 1987 (139). This technique involves the use of a drug delivery system positioned into a dependent part of the scrotum. A cannula is inserted into the lateral aspect of the corpus cavernosum and sutured to the tunica with a non-absorbable suture. Either phenylephrine (140) or etilefrine (141) is instilled into the corpus cavernosum by squeezing a pump and promoting detumescence.

Penile Prosthesis: Individuals who fail to control their symptoms by using conventional pharmacotherapies and are still troubled by the condition have the option of undergoing surgery to insert a penile prosthesis. A variety of implants are now available and are classified into malleable or inflatable implants. These can be inserted electively. However, in a small proportion of patients a prolonged priapic episode fails to respond to conventional treatment and in this scenario the option of an immediate penile prosthesis insertion is acceptable (90).

Embolisation: A Doppler study during the course of an acute episode of priapism in an individual who is initially thought to be suffering from idiopathic stuttering priapism may reveal a high-flow priapism (142). In this situation, intermittent high-

flow episodes manifest as short lived episodes which leads to an initial diagnosis of stuttering priapism. Therefore if the history isn't typical of classical predominantly nocturnal episodes with pain then a Doppler study during an acute episode can delineate between the two. If the underlying diagnosis is of a high-flow priapism then arteriography followed by embolisation of an identified fistula would be the preferred management.

Subcapsular orchidectomy: With the development and improved availability of penile prosthesis, the use of subcapsular orchidectomy is now almost obsolete in this condition.

1.4 Pathophysiology of Low-Flow Priapism

Of the common subtypes described previously, low-flow priapism is the most prevalent form and one which is characterised by the development of ischaemia with potentially devastating consequences for erectile function. The complete pathophysiology of low-flow priapism and the consequences of delayed treatment are still not fully understood. As it is a condition which can afflict male individuals of any age and potentially result in the development of impotence, by understanding the underlying alteration in the corpus cavernosum function we can develop an improved algorithm in the management of the disease in order to reduce the comorbidity.

1.4.1 Histological Changes in the Corpus Cavernosum following Priapism

Histologically the components of the corpus cavernosum undergo progressive changes as the duration of priapism increases. Electron microscopy has been used to retrospectively examine tissue samples from patients with low-flow priapism (143).

Although the study only consisted of 22 patients it found that interstitial oedema occurs in the early stages of the disease process. In cases where priapism is of a short duration (less than 12 hours) the tissue consisted of minor endothelial defects with occasional lymphocytic infiltration with no alteration in the smooth muscle cells. It is only after 12 to 14 hours of low-flow priapism that trabecular smooth muscle cells show the beginning of focal cytoplasmic transformation which manifests as an increase in size of the perinuclear cytoplasm, endoplasmic reticulum, ribosomes and Golgi apparatus. At between 24 to 48 hours duration widespread endothelial destruction and exposure of the basement membrane occurs with subsequent thrombocyte adherence. In addition to this the smooth muscle cells undergo a transformation as described above as well as necrosis. Persistent blood stasis for longer than two days is associated with infiltration of the trabecular tissue with inflammatory cells and smooth muscle cells undergoing necrosis or phenotypic change into fibroblast like cells.

From this description the first cells to show histological alteration are the smooth muscle cells followed by the endothelial cells.

1.4.2 Dysregulatory Mechanisms in Penile Smooth Muscle Contraction during Priapism:

As the duration of the penile erection becomes pathologically prolonged as in the case of low-flow priapism, the pO_2 progressively falls as the closed compartment prevents replenishment of stagnant blood with freshly oxygenated arterial blood (144). Broderick and Harkaway (91) analysed the change in the cavernous blood gas parameters in patients presenting with prolonged penile erection following

pharmacologically induced erections. There were time-dependent alterations in the pO_2, pH and pCO_2 during the erection. After 240 minutes the cavernous tissue is no longer perfused by highly oxygenated blood and the pH is reduced to less than 7.3, pO_2<6kPa and pCO_2 >8kPa. After 360 minutes the parameters altered further and it was found that pH<7.27, pO_2<5.3kPa and pCO_2 >8.7kPa. By 720 minutes the pH had deteriorated further to 6.89 and pO_2<2kPa and pCO_2>16kPa. This confirmed time-dependent changes in pH , pO_2 and pCO_2 in the corpus cavernosum during prolonged erections. The progressive changes in these parameters are illustrated in Figures 6 and 7. This is the only study which has directly measured changes in the pH and pO_2 levels in prolonged erections. However, as yet there have been no studies which have investigated the alteration in the glucose concentration of the blood in the corpus cavernosum.

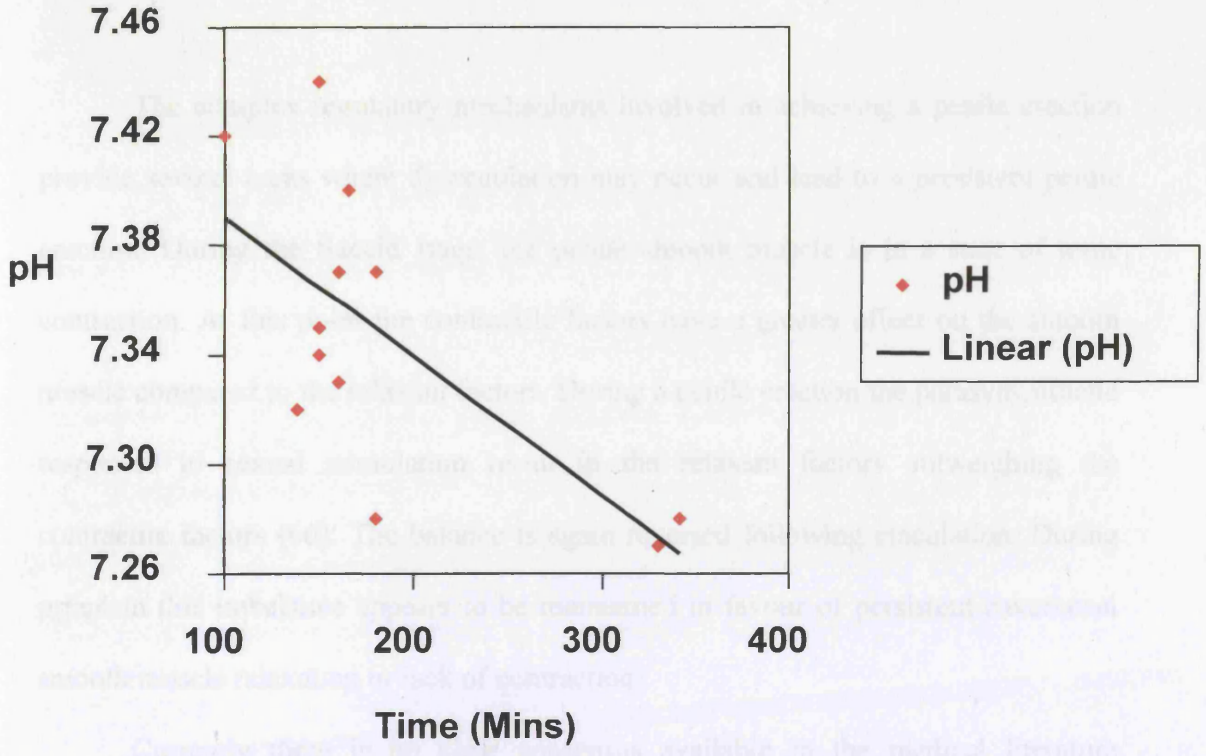


Figure 6: Changes in the corpus cavernosum pH with time following pharmacologically induced erections. Modified from (91).

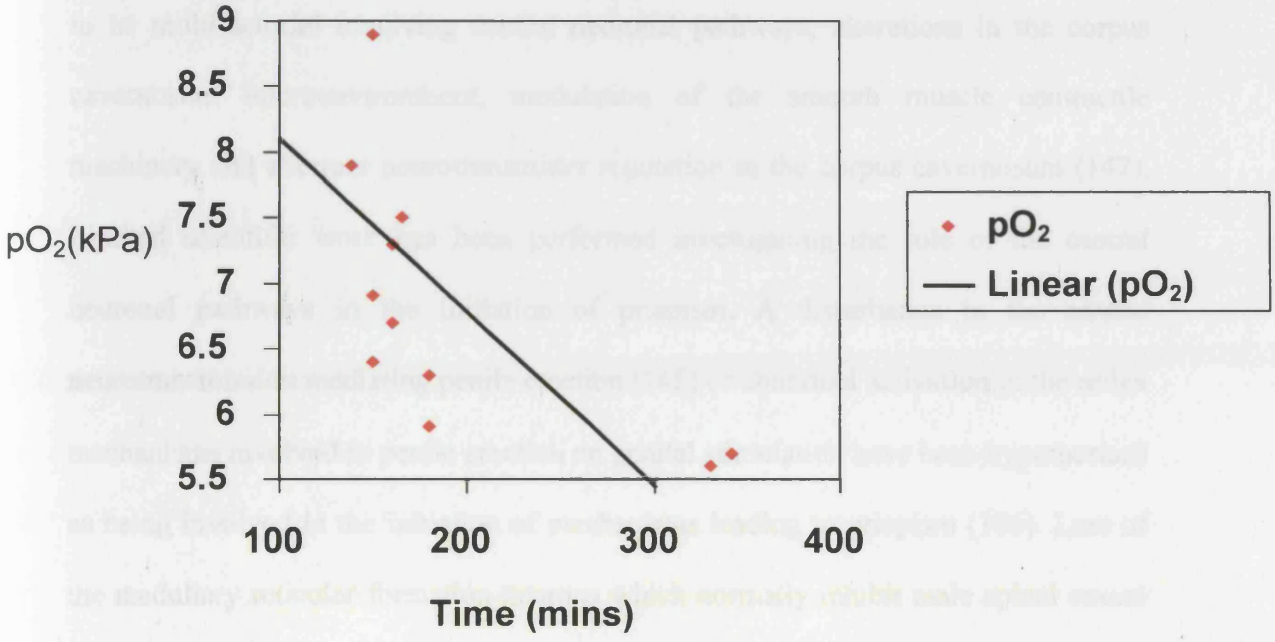


Figure 7: Changes in cavernosal pO_2 following pharmacologically induced penile erection. Modified from (91).

The complex regulatory mechanisms involved in achieving a penile erection provide several areas where dysregulation may occur and lead to a persistent penile erection. During the flaccid stage, the penile smooth muscle is in a state of tonic contraction. At this point the contractile factors have a greater effect on the smooth muscle compared to the relaxant factors. During a penile erection the parasympathetic responses to sexual stimulation result in the relaxant factors outweighing the contractile factors (66). The balance is again reversed following ejaculation. During priapism this imbalance appears to be maintained in favour of persistent cavernosal smooth muscle relaxation or lack of contraction.

Currently there is no clear consensus available in the medical literature relating to the initiating events which leads to a prolonged penile erection. Work has

focussed on the factors which maintain tumescence, in particular the metabolic changes in the corpus cavernosum (88;144-146). The initiating mechanisms are likely to be multifactorial involving central neuronal pathways, alterations in the corpus cavernosum microenvironment, modulation of the smooth muscle contractile machinery and aberrant neurotransmitter regulation in the corpus cavernosum (147). Limited scientific work has been performed investigating the role of the central neuronal pathways in the initiation of priapism. A disturbance in the central neurotransmission mediating penile erection (148) or abnormal activation in the reflex mechanisms involved in penile erection on genital stimulation have been hypothesised as being involved in the initiation of mechanisms leading to priapism (106). Loss of the medullary reticular formation neurons which normally inhibit male spinal sexual reflexes has been proposed using a mouse model (149). The sympathetic nervous system is the major effector system in maintaining detumescence through the release of noradrenaline. Failure of the sympathetic system may also predispose individuals to the development of priapism (87;150).

The main neurotransmitter which is involved in penile smooth muscle relaxation is NO. Whether unregulated amounts of NO initiate the response has been investigated by Kim et al (81). This group found that there is no central role of NO in the maintenance of the erection during priapism. As explained previously, the low pO_2 levels which occur during a prolonged penile erection would be unable to act as a substrate for NOS to maintain the production of NO. Alternatively the failure of endogenous contractile factors such as noradrenaline or endothelin-1 to adequately induce smooth muscle contraction will contribute to the maintenance of corporal smooth muscle relaxation. However, clinically the instillation of α-adrenergic agonists is successful in reversing the condition in a proportion of patients but there is no

indication in the literature as to the success rate of this non surgical mode of treatment. What is more likely is that the smooth muscle dysfunction occurs as a result of the failure of smooth muscle contractile machinery secondary to the alterations in the corporal microenvironment.

The effects of hypoxia on corporal smooth muscle was initially investigated by Broderick et al (144). Strips of rabbit corpus cavernosum were subjected to anoxia by gassing with a mixture of 95% N_2 and 5% CO_2 and the contractile responses were recorded. The corporal smooth muscle tone, contractile activity to α agonists and electrical field stimulation were all reduced under anoxic conditions. Further studies (88) concluded that the reduction in smooth muscle tone is due to increased intracellular calcium levels. However, during prolonged ischaemic priapism, the tissue is subjected not only to hypoxia, but the microenvironment becomes increasingly acidotic (144) . The effects of acidosis *per se* was investigated by Saenz De Tejada et al (145) and showed that the smooth muscle contractility is impaired by acidosis. *In vivo* studies were performed by Moon et al (151) who used anaesthetised cats and hyperventilation to induce metabolic acidosis. In this study the intracavernous pressure measurements indicated that the smooth muscle failed to contract and the overall intracavernous pressure was reduced.

1.4.3 Ischaemia/Reperfusion Injury

Low-flow priapism is analogous to a closed ischaemic compartment for cavernosal smooth muscle. Aspiration or instillation of α-adrenergic agonists can be successful in reversing this state. Whether the perfusion of cavernosal tissue with oxygenated blood results in a reperfusion type injury is not completely understood. Although ischaemia/reperfusion injury has been extensively studied in vascular and

cardiac tissue, the effects on erectile tissue have been investigated in a small number of studies. Evliyaoglu and co-workers (152) examined the effects of allopurinol as a reactive oxygen species scavenger in a reperfusion injury model of veno-occlusive priapism using constriction rings on rat penis. Those animals receiving allopurinol showed reduced lipid peroxidation which is used as a marker of reperfusion injury. A study using anaesthetized rabbits exposed to low oxygen tension found a significant increase in lipid peroxidation and myeloperoxidase activity following ischaemia/reperfusion (146).

1.5 Design and Aim of This Study

Up until now the investigation of low-flow priapism has involved the use of both *in vitro* and *in vivo* models which have attempted to reproduce the change in the corporal microenvironment parameters namely hypoxia and acidosis. Apart from the work of Broderick and Harkaway (91) which documented the temporal change in the corporal microenvironment with regards to the degree of acidosis and hypoxia, there have been no documented studies which have analysed corporal blood aspirated from those patients who have presented with low-flow priapism which is refractory to conventional non surgical management. Therefore the initial part of our study was to document the degree of hypoxia, acidosis and also the glucose level found in the corporal blood of patients with refractory priapism in order to identify whether there is a critical level in these parameters which induces a failure in the contractile machinery.

Previous studies which have utilised an *in vitro* model to investigate the effects of hypoxia and acidosis on corporal smooth muscle did not investigate the

recovery of corporal smooth muscle tone following exposure to these conditions. It is not known whether prolonged exposure to hypoxia or acidosis either alone or in combination can cause irreversible corporal smooth muscle dysfunction. Hypoxia and acidosis coexist in low-flow priapism states and may perpetuate the failure of smooth muscle relaxation in response to α agonist treatment. However, if these conditions were to be reversed, which clinically is achieved by aspiration of blood from the corpus cavernosum, then would the smooth muscle tone recover completely or are these two parameters the predominant mediators of irreversible cavernosal smooth muscle dysfunction.

Currently in the clinical setting the aspiration of blood from the corpus cavernosum is a fundamental step in the initial management of patients presenting with priapism. Aspirates are analysed in a blood gas analyser and the degree of hypoxia and acidosis is used as a gauge in order to classify the priapism into a high-flow or a low-flow subtype. However, despite varying degrees of hypoxia and acidosis, some of these individuals manage to achieve detumescence using aspiration alone whereas others also require the instillation of α-adrenergic agents.

Our institution is a major national referral centre for acute priapisms. Therefore by analysing the results of corporal blood aspirates from those patients who fail to respond to conventional treatment we can investigate the underlying parameters which lead to this clinical scenario by developing a reproducible *in vitro* model of low-flow priapism.

In order to identify the parameters mediating irreversible smooth muscle dysfunction, I have developed a model of ischaemic priapism using rabbit corpus cavernosum. Each parameter was investigated individually in order to assess whether

for instance hypoxia alone or in combination with acidosis can result in irreversible smooth muscle dysfunction.

The limitations of this study are that the availability of human tissue for *in vitro* work is limited. *In vitro* work using human tissue mainly utilised tissue from patients undergoing gender reassignment surgery and those undergoing penectomy operations for penile cancer.

Due to the limited availability of human corpus cavernosum tissue, the majority of the *in vitro* work was carried out using rabbit corpus cavernosum. This tissue has been used extensively in basic science research into erectile dysfunction and is reproducible.

Aims of the study:

1. To identify the cavernosal blood parameters in patients presenting with low-flow priapism which fail to achieve penile detumescence.
2. To assess smooth muscle dysfunction in these patients dynamically.
3. To develop an *in vitro* model of ischaemic priapism
4. To investigate potential pharmacological agents which may reduce the degree of smooth muscle dysfunction and promote detumescence.
5. To investigate the underlying factors responsible for irreversible smooth muscle dysfunction.

CHAPTER 2

MATERIALS AND METHODS

2.1 Analysis of Cavernosal Blood Aspirates from Patients Presenting with Priapism

Patients (n=10) presenting to our department with clinically diagnosed priapism underwent a penile Doppler study. All adult patients with a first presentation of priapism were included. Patients with a history of sickle cell disease and stuttering priapism or those with idiopathic stuttering priapism were excluded from the study. Penile Doppler studies were performed by a radiologist using a 10MHz probe. Those patients diagnosed with a low-flow priapism on Doppler studies underwent aspiration of blood from the corpus cavernosum as part of the initial management of the condition. Samples were transported on ice in a syringe to be analysed in an automated blood gas analysis chamber in order to measure the pH, pO_2 and glucose.

2.2 *In vitro* Experiments Using Human Corpus Cavernosum

Experiments on human corpus cavernosum tissue were approved by the Joint UCL/UCLH Ethics Committees REC Ref 03/0158. Normal human corpus cavernosum was obtained from patients undergoing partial penectomy or total penile amputation for penile carcinoma. At the time of the operation a 2cm long piece of corpus cavernosum was dissected from the surgical specimen and the tissue was immediately placed into ice cold Krebs solution.

Priapic patients that were confirmed as having low-flow priapism on the basis of a clinical history, corporal aspirates indicating ischaemia (pH<7.25 and pO2<6kPa) and penile Doppler studies and who were unable to achieve detumescence following

corporal blood aspiration and α-adrenergic agonist instillation were consented to undergo an open biopsy of the corpus cavernosum under general anaesthesia. They were counselled regarding the purpose of the biopsy and given the option of having a malleable penile prosthesis inserted if the biopsy showed that the penile tissue was non viable. At the same time, a 1cm biopsy of the corpus cavernosum was removed and immediately placed into Krebs solution.

The tissues were dissected into smaller 0.3cm x 0.5cm pieces and mounted in superfusion chambers under 0.6g tension. After a 30 minutes equilibration period, increasing concentrations of phenylephrine (0.1μM-1mM) were added to the perfusion fluid and the isometric tension was recorded simultaneously. At the end of the experiment, scopolamine (10μM) and guanethidine (10μM) were added to block cholinergic and noradrenergic pathways followed by electrical field stimulation (EFS; 50V, 0.3ms pulse duration, 1-50 Hz, for 5 s, every 2 minutes).

2.3 *In vitro* Experiments Using Rabbit Corpus Cavernosum.

Male New Zealand white rabbits were supplied by Harlan, UK and housed in the Biological Services Unit, Wolfson Institute for Biomedical Research between 16-19°C conforming to Home Office regulations. The animals were allowed to settle in for a week before being used. The rabbits were killed using an intravenous overdose of pentobarbitone into the marginal vein of the ear (Figure 8). The penis was identified (Figure 9) and dissected up to the level of the crural attachment to the ischium. Sharp dissection was used to free the penis from the pubic bones and the organ was immediately placed into Krebs solution (Table 2) pre-oxygenated with carbogen (95% O_2 and 5% CO_2) at room temperature. Dissection of the residual adventitial tissue (Figure 10) was followed by a longitudinal incision of the tunica

albuginea which encircles the corpus cavernosum. The cavernosal tissue was dissected free of the tunica albuginea and four strips measuring 0.5cm x 0.3cm were obtained.

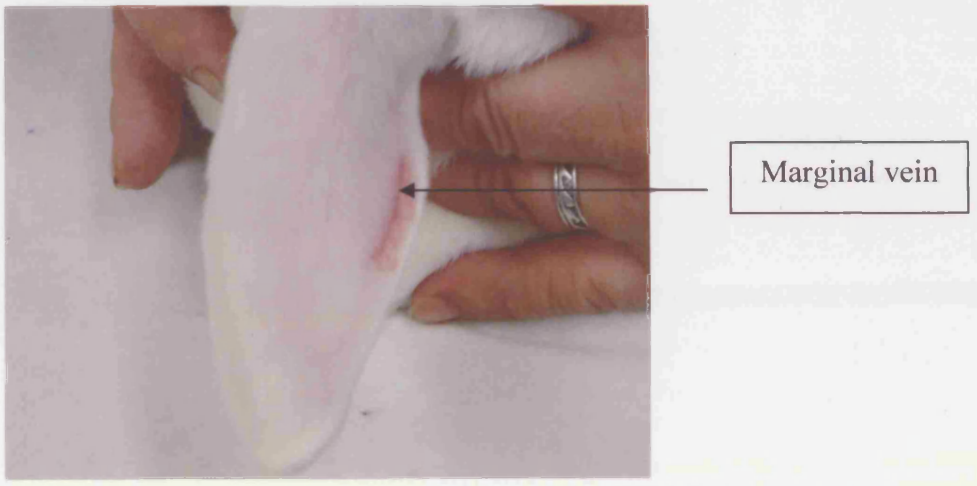


Figure 8: Marginal vein of the ear demonstrated following shaving of the ear fur.

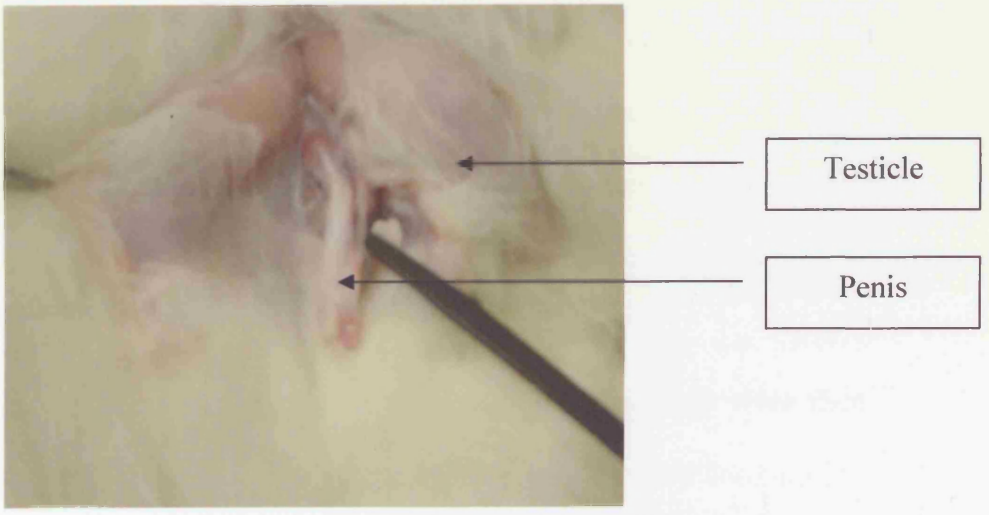


Figure 9: Surrounding tissue dissected to expose the penis.

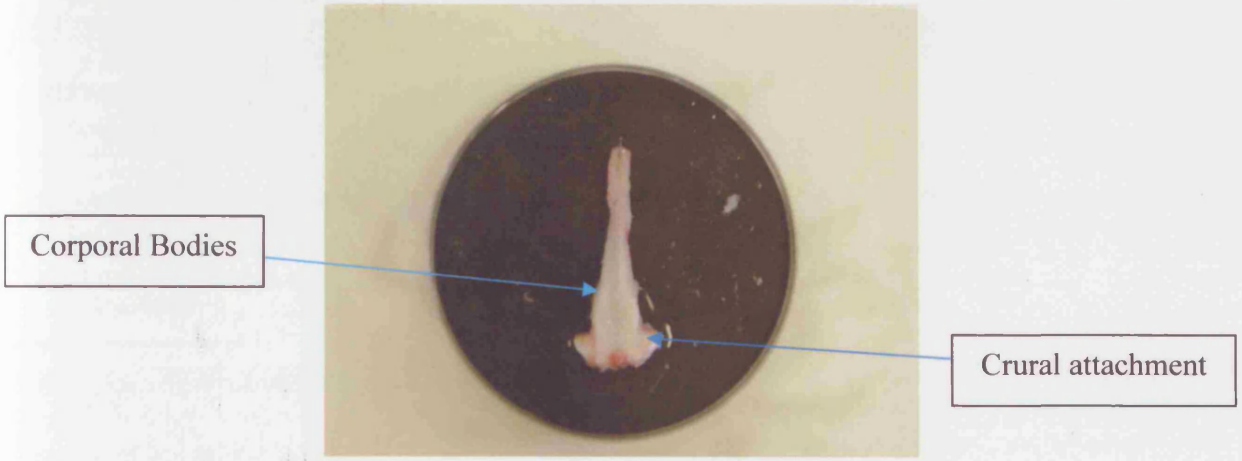


Figure 10: Body of the penis following removal of the urethra

Cavernosal strips were mounted horizontally in superfusion chambers between the two ring electrodes of 4 mm in diameter. Each double jacketed chamber was maintained at 37°C. The chambers were perfused with modified Krebs solution at a constant flow rate of 1ml per minute using a Miniplus 2 (Gilson, Luton, United Kingdom) peristaltic pump. One end of the preparation was tied to an FT03C (Grass Instruments, Quincy, Massachusetts, USA) force displacement transducer connected to a Linearcorder WR 3101 (Graphtec, Tokyo, Japan) and a computer for recording isometric tension (Figure 11). A schematic diagram of the superfusion system is shown in Figure 12.

The preparations were stretched with a tension of 0.6g. They were then allowed to equilibrate for 60 minutes. The test conditions were induced according to the protocol explained below and the experiments were terminated by adding prazosin to the test solution in order to identify the experimental baseline.

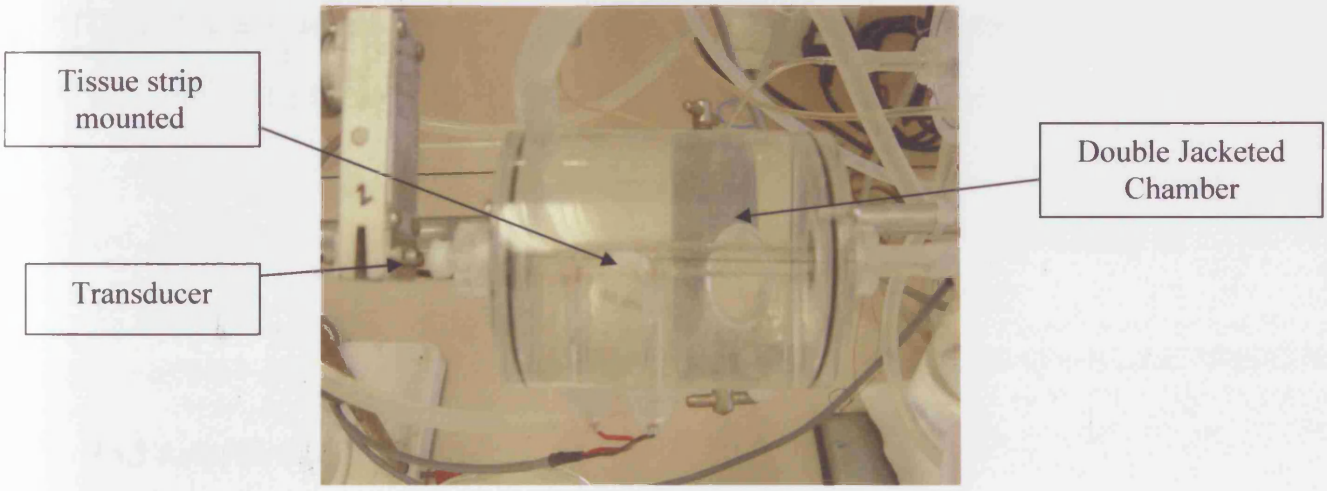


Figure 11: Tissue strip placed in the superfusion chamber with one end fixed and the other end attached to a transducer.

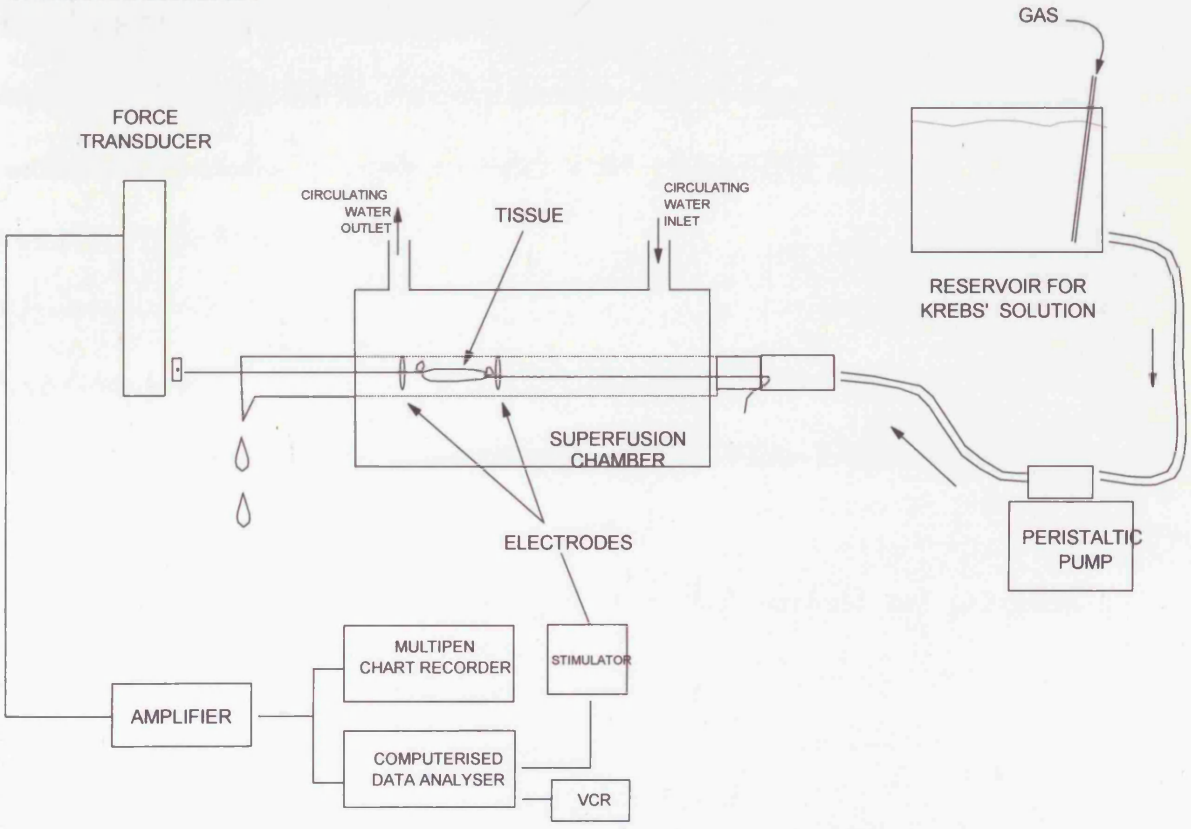


Figure 12 Schematic illustration of the experimental set up

2.3.1 Gas Preparations

Under normal conditions the Krebs solution was gassed with carbogen (95% O_2 and 5% CO_2). In order to induce hypoxia, the gas was changed to 95% N_2 and 5%

CO_2. Measurement of the pO_2 in the reservoir and chamber showed that the pO_2 was over 100mmHg(13.3kPa) when gassed with carbogen. When the gas mixture was changed to 95% N_2 and 5% CO_2, the pO_2 in the reservoir was 5-7mmHg(0.7-0.9kPa) and due to equilibration with the silicone tubing it was 40-50mmHg(5.3-6.7kPa) in the chamber.

2.3.2 Acidotic Krebs Solution

The HCO_3^- composition of the Krebs solution was reduced from 16.3mM to 5mM in order to reduce the pH to 6.9. The osmolarity was maintained by equimolar addition of NaCl such that the final concentration of NaCl was 144mM. The pH of the medium was periodically monitored using a pH meter (AR15 pH meter, Accumet Research, Fisher Scientific, UK).

2.3.3 Glucopenia

Experiments requiring glucopenia used modified Krebs solution in which the glucose was omitted.

The contents of the Krebs solution in normal, acidotic and glucopenic conditions are summarised in Table 2.

	Normal	Hypoxic	Acidotic	Glucopenic
NaCl	133mM	133mM	144mM	133mM
KCl	4.7mM	4.7mM	4.7mM	4.7mM
NaH_2PO_4	16.3mM	16.3mM	16.3mM	16.3mM
$MgSO_4$	0.61mM	0.61mM	0.61mM	0.61mM
$NaHCO_3$	16.3mM	16.3mM	5mM	16.3mM
$CaCl_2$	2mM	2mM	2mM	2mM
Glucose	7.8mM	7.8mM	7.8mM	0mM
Dexamethasone	0.01mM	0.01mM	0.01mM	0.01mM
pH	7.4	7.4	6.9	7.4
pO_2	>100mmHg	40-50 mmHg	>100mmHg	>100mmHg

Table 2: Composition of the modified Krebs solution for each experimental protocol

2.4 Experimental Protocols Using Rabbit Corpus Cavernosum

2.4.1 Calculating the EC_{80} for Phenylephrine in Normal Conditions

Once the tissue preparations were set up in the superfusion chambers as explained above, the tissue was allowed to equilibrate for one hour at 37°C. After the equilibration period, cumulative concentrations of phenylephrine (0.1-300 μM) were added to the reservoir. Isometric tension was recorded simultaneously until the maximum tension was achieved. The EC_{80} value for phenylephrine was calculated by plotting the tension as a percentage of the maximum tension and calculating the

concentration required to achieve 80% of the maximum tension as illustrated in Figure 14. This calculated concentration was then used to pre-contract the tissues in the subsequent experiments.

2.4.2 Eliciting Nitrergic Relaxations

Following a one hour equilibration period, electrical field stimulation (EFS; 50V, 0.3ms pulse duration, 0.5-50 Hz, for 5s, every 2 min) was initiated. Scopolamine (10 μM) and guanethidine (10 μM) were then added to block cholinergic and noradrenergic components respectively. The cavernosal strips were then pre-contracted with 20μM phenylephrine. Under these conditions the strips maintained a tone and EFS elicited reproducible relaxation responses.

2.4.3 Acute Effect of Hypoxia, Acidosis and Glucopenia on the Elevated Tone

Tissue strips were pre-contracted with EC_{80} of phenylephrine following the equilibration period. The isometric tension was recorded simultaneously as conditions of hypoxia, acidosis or glucopenia or their various combinations were instilled.

2.4.4 Time Course for the Recovery of Phenylephrine-Induced Tone after Reversal of Combination of Hypoxia, Acidosis and Glucopenia

Tissue strips were pre-contracted with EC_{80} of phenylephrine following the equilibration period. The tension was recorded simultaneously as the tissue strips were superfused in a combination of hypoxia, acidosis and glucopenia. At various time points between 30 minutes to 6 hours the conditions were reversed using normal Krebs solution containing phenylephrine and gassed with carbogen. The recovered tone was expressed as the percentage of the initial tone. The tone from a control tissue

which was superfused with normal Krebs solution was also recorded for up to 6 hours to assess the spontaneous loss of tone.

2.4.5 Recovery of Phenylephrine-Induced Tone after Reversal of Hypoxia, Acidosis and Glucopenia

Tissue strips were pre-contracted with EC_{80} of phenylephrine following the equilibration period. The isometric tension was recorded simultaneously as conditions of hypoxia, acidosis or glucopenia or their various combinations were instilled. The strips were exposed to these conditions for 4 hours. The tone from a control tissue which was superfused with normal Krebs solution was also recorded to assess the spontaneous loss of tone during this time period. At the end of 4 hours, the conditions were reversed using normal Krebs solution containing phenylephrine and gassed with carbogen. The recovered tone was expressed as the percentage of the initial tone.

2.4.6 Cumulative Concentration Response to Phenylephrine, Endothelin-1 and U46619 with and without Hypoxia, Acidosis and Glucopenia

Tissue strips were divided randomly into two groups. Each group underwent repeat experiments after precontraction with endothelin-1 (33.33nM) or U46619 (50nM) as opposed to phenylephrine. The first group was superfused in normal Krebs solution for 4 hours. The second group was superfused with a combination of hypoxia, acidosis and glucopenia for 4 hours. At the end of 4 hours, both groups were superfused with normal Krebs solution for 30 minutes. Then increasing concentrations of phenylephrine (0.1 - 300 μM), endothelin-1 (33.33nM) or U46619 (50nM) were added.

2.4.7 Effect of Hypoxia, Acidosis and Glucopenia on Nitrergic Responses

Tissue strips were pre-contracted with EC_{80} of phenylephrine and were then subjected to 4 hours of a combination of hypoxia, acidosis and glucopenia. During this period nitrergic relaxation responses were measured and their magnitude was expressed as a percentage of the tone. The nature of the relaxation responses were confirmed by addition of an inhibitor of NO synthase (N^{ω}-nitro-*L*-arginine methyl ester; L-NAME, 500 μM) or the nerve blocker tetrodotoxin (TTX, 3 μM).

2.4.8 Investigation of Putative Therapeutic Agents

Tissue strips were pre-contracted with EC_{80} of phenylephrine and were then subjected to 4 hours of a combination of hypoxia, acidosis and glucopenia. The effects of cumulative concentrations of some agents, which have been suggested to be protective against tissue damage to ischemia or have the ability to reverse smooth muscle relaxation, were then tested on the tone of the strips. These agents were calcium (1-10μM), the direct calcium channel activator Bay K 8644 (1μM), N-acetylcysteine (anti-oxidant, 100μM), N^{ω}-nitro-L-arginine methyl esther (L-NAME, NO synthase inhibitor, 500μM), digoxin (Na/K ATPase inhibitor, 10μM) and glutathione (500μM).

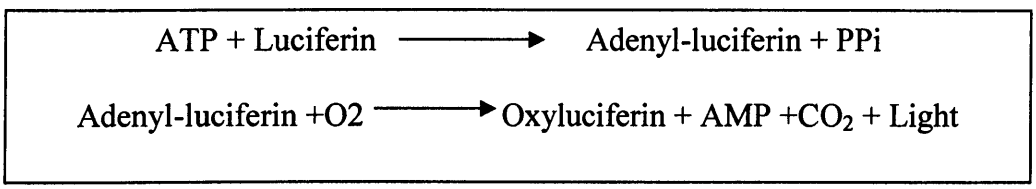
2.4.9 Experiments Utilising Metabolic Inhibition

In order to reproduce the conditions of glucose depletion and hypoxia, iodoacetic acid (500μM) and myxothiazol (500μM) were used to inhibit the glycolytic pathway (interaction with glycerol phosphate dehydrogenase) and the electron transport chain (inhibition of mitochondrial complex III) respectively. The iodoacetic acid was added following the equilibration period and pre-contraction with

phenylephrine (EC_{80}). Similarly in separate experiments myxothiazol was added at the end of the equilibration period and pre-contraction with phenylephrine (EC_{80}) and tone was recorded simultaneously.

2.5 Measurement of ATP Concentrations

Experiments were repeated where tissue strips underwent immediate snap freezing in liquid nitrogen at the following four time points: at the end of 60 minutes equilibration, after pre-contraction with phenylephrine (20 μM), following 4 hours of combination of hypoxia, acidosis and glucopenia and following one hour of reperfusion with normal Krebs solution. Four strips from the same animal were used for each time point and the experiment was repeated four times. The frozen tissues were crushed into a fine powder using a stainless steel pestle and mortar on dry ice. 200 μl of 70% perchloric acid was added to the powder and vortex mixed vigorously. The preparation was then centrifuged for 10 minutes (13,000 g, 4°C). 150 μl of the supernatant was neutralised with 20μl of 5M K_2CO_3. After a further 10 minutes of centrifuging (13,000 g, 4°C) ATP was measured in duplicate in the supernatant using an ATP bioluminescent assay kit (Sigma, Poole, UK) according to the manufacturer's instructions. ATP standards (1nM-10μM; Sigma, UK) were used to construct a standard curve. As ATP is consumed, light is emitted when firefly luciferase catalyses the oxidation of D-luciferin.



Protein concentration in each sample was measured using Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK) based on the dye-binding procedure of Bradford (153). The ATP concentration of each sample was expressed as pmol ATP/μg protein.

2.6 Histochemistry and Immunofluorescence of Tissue Exposed to Hypoxia, Acidosis and Glucopenia in Combination

Tissue strips were fixed in 4% paraformaldehyde in 0.1M phosphate buffer at four different time points: at the end of 60 minutes equilibration, after pre-contraction with phenylephrine, following 4 hours of combination of hypoxia, acidosis and glucopenia and following one hour of reperfusion with normal Krebs solution. The specimens were processed for immunofluorescence and for routine haematoxylin eosin (H&E) staining performed by Dr A. Dogan at the Histopathology Department, University College London. For immunofluorescence; after fixation in 4% paraformaldehyde, the tissues were transferred into 30% sucrose in phosphate buffer and kept at 4°C overnight. The samples were then frozen in OCT compound (BDH, Poole, UK) and serial cryosections at 20μm interval were obtained using a cryostat (18°C; 2800 Frigocut-E; Leica, Bensheim, Germany). The sections were dried on gelatine-coated slides for 2 hours at room temperature and then incubated with phosphate buffered saline (PBS) containing 0.1% TritonX-100 and 5% goat serum (Vector, Burlingame, California). The slides were incubated with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) reaction mixture (Roche, Lewes, UK) overnight at 37°C and then with a monoclonal antibody against smooth muscle specific α-actin (1/250; Sigma, UK) for 2 hours at room temperature followed by fluorescein-conjugated anti-mouse IgG (1/100; raised in goat; Chemicon,

Harrow, UK) for 1 hour at room temperature. The images were obtained using a laser-scanning confocal microscope (Leica TCS-DMRE, Germany). Image analysis was performed using Leica Confocal Software (Version 2.00, build0871, Germany). In order to avoid day-to-day variation in signal intensity, several sections from different experimental groups were immunostained and analysed in the same batch on the same day. The laser intensity and gain functions were set according to the control tissue; thereafter, these settings were applied to all sections from all experimental groups within the same batch. The results from TUNEL staining were expressed as the percentage of smooth muscle cells with TUNEL-positive nuclei.

2.7 Chemicals and Drugs

Drugs utilised and suppliers	
Phenylephrine hydrochloride	Sigma
Scopolamine hydrochloride	Sigma
Guanethidine monosulphate	Sigma
Bay K 8644	Calbiochem
Glutathione	Sigma
N-acetylcysteine	Sigma
Digoxin	Sigma
Prazosin	Sigma
U46619	Calbiochem
N^{ω}-Nitro-L-arginine methyl ester hydrochloride	Sigma
Dexamethasone	Sigma
Myxothiazol	Sigma
Ammonium formate	BDH
Ammonium phosphate	Sigma
Potassium carbonate	Sigma
Endothelin-1	Sigma
ATP,ADP,AMP,β methylene ATP	Sigma

Table 3: Chemicals utilised for the experimental procedures and suppliers.

2.8 Statistical Analysis and Presentation of the Results

All results were expressed as mean ± standard error of mean. Isometric tension was expressed as a percentage of the initial tone. Statistical analysis for pharmacology experiments was performed using Student's t-test for unpaired observations. For the ATP measurements Wilcoxon signed rank test for paired samples was used where the tissues from the same animal were paired. P values less than 0.5 was considered significant. The statistical analysis was performed using GraphPad Prism software (version 3.00, GraphPad Software Inc, San Diego, California, USA).

CHAPTER 3

RESULTS

3.1 Analysis of Blood Aspirates from Patients Presenting with Priapism

Patients presenting with a history suggestive of low-flow priapism which was unresponsive to pharmacological intervention underwent penile Doppler studies and corporal blood aspirations. Of the 10 patients presenting to our department with priapism, a total of 7 patients underwent full investigation with corporal blood aspirations together with the penile Doppler studies performed by one of two radiologists (Table 4). The corporal blood aspirations demonstrated that the blood in the corpus cavernosum is acidotic, hypoxic and also glucopenic.

Patient	Duration of priapism	pH	pO2 (kPa)	Glucose (mmol/l)	Penile Doppler
1	60 Hours	6.9	1.6	0	Not done
2	4 days	7.1	2.3	0.2	Low-flow
3	7 days	7.0	2.1	0.3	Low-flow
4	8 days	6.9	1.7	0	Low-flow
5	9 days	7.05	1.82	0	Low-flow
6	10 days	6.9	1.5	0.1	Low-flow
7	72 Hours	7.19	4.4	2.7	High-flow

Table 4 Results of the corporal blood analysis from patients presenting with prolonged low-flow priapism. Comparison is made with the outcomes from the penile Doppler studies.

3.2 *In Vitro* Experiments Using Human Corpus Cavernosum

Normal human corpus cavernosum using tissue obtained from patients undergoing penectomy operations showed a concentration response to phenylephrine as illustrated in Fig 13. The calculated EC_{80} was 3μM.

Patients who failed conventional management for low-flow priapism underwent an immediate insertion of a penile prostheses. Human corpus cavernosum was obtained from these patients and the tissue was mounted as described (section 2.2) and cumulative concentrations of phenylephrine (0.1-300 μM) were added to the reservoir. In all 6 cases of suspected low-flow priapism there was a failure of contraction of the tissue strips despite using high doses of phenylephrine up to 300μM. Again this is illustrated in Fig 13.

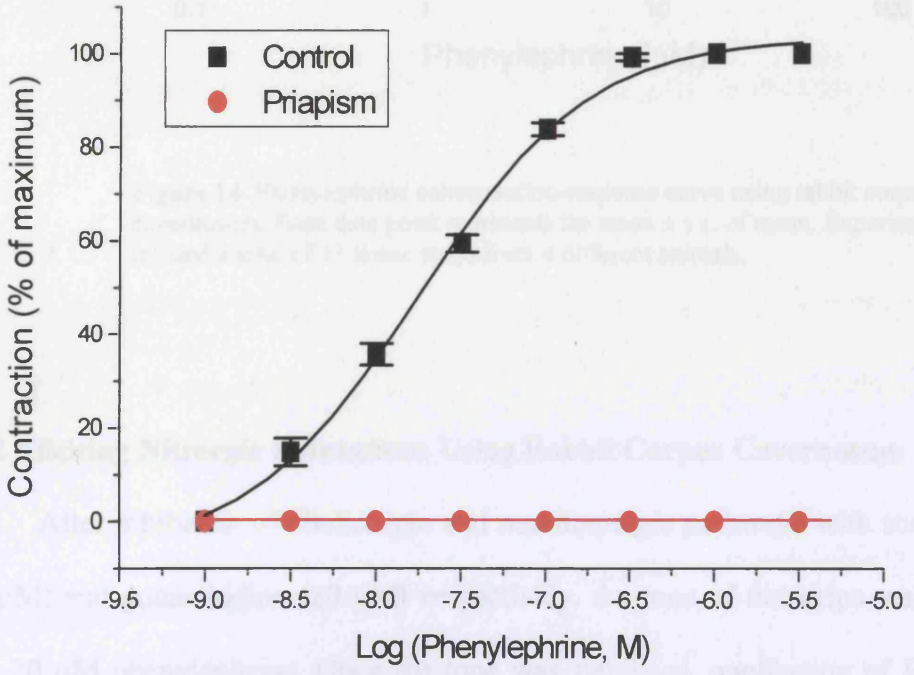


Figure 13. Concentration response curve to phenylephrine in normal human corpus cavernosum tissue (n=4). This is compared to the response of isolated human corpus cavernosum from patients presenting with low-flow priapism (n=6).

3.3 *In vitro* Experiments Using Rabbit Corpus Cavernosum

3.3.1 Calculating the EC_{80} for Phenylephrine in Normal Conditions

In order to establish the EC_{80} value, cumulative concentrations (0.1 – 300μM) of phenylephrine were added to the reservoir. Figure 14 illustrates the concentration response of rabbit corpus cavernosum to phenylephrine. From this curve an EC_{80} of 20 μM was calculated.

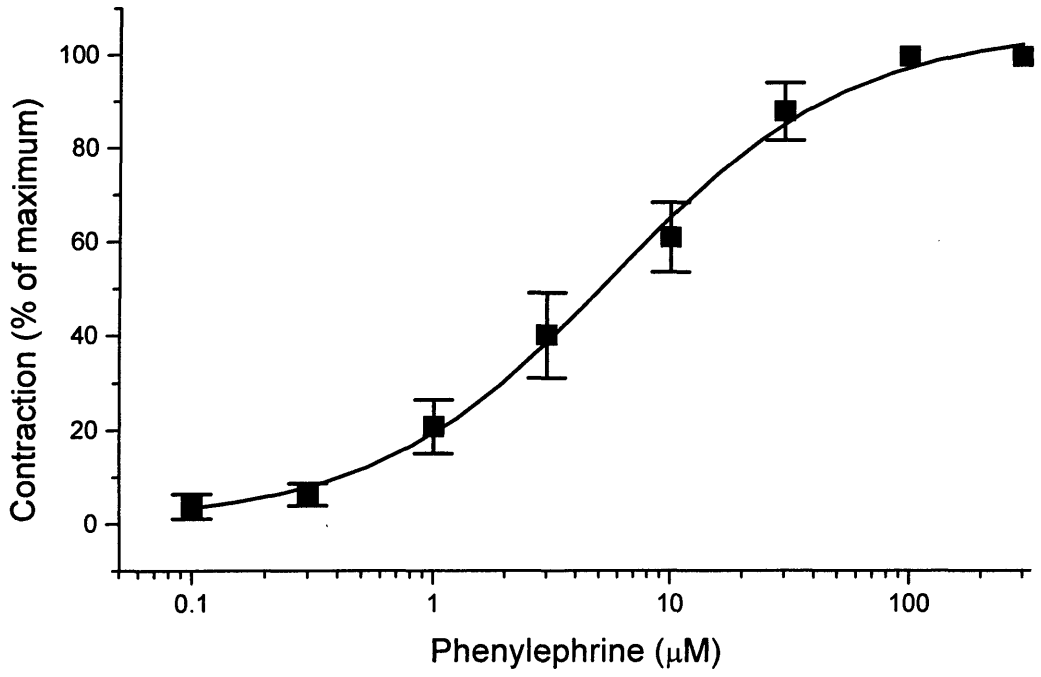


Figure 14. Phenylephrine concentration-response curve using rabbit corpus cavernosum. Each data point represents the mean ± s.e. of mean. Experiments utilized a total of 11 tissue strips from 4 different animals.

3.3.2 Eliciting Nitrergic Relaxations Using Rabbit Corpus Cavernosum

After inhibition of cholinergic and noradrenergic pathways with scopolamine (10 μM) and guanethidine (10 μM) respectively, the tone of the strips was elevated with 20 μM phenylephrine. Once the tone was stabilised, application of EFS (50V, 0.3 ms pulse duration, for 5 s, every 2 min, 0.5-50 Hz) elicited reproducible relaxation

responses (Figure 15). These relaxations were frequency-dependent and were inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME, 500 μM) or tetrodotoxin (TTX, 3 μM).

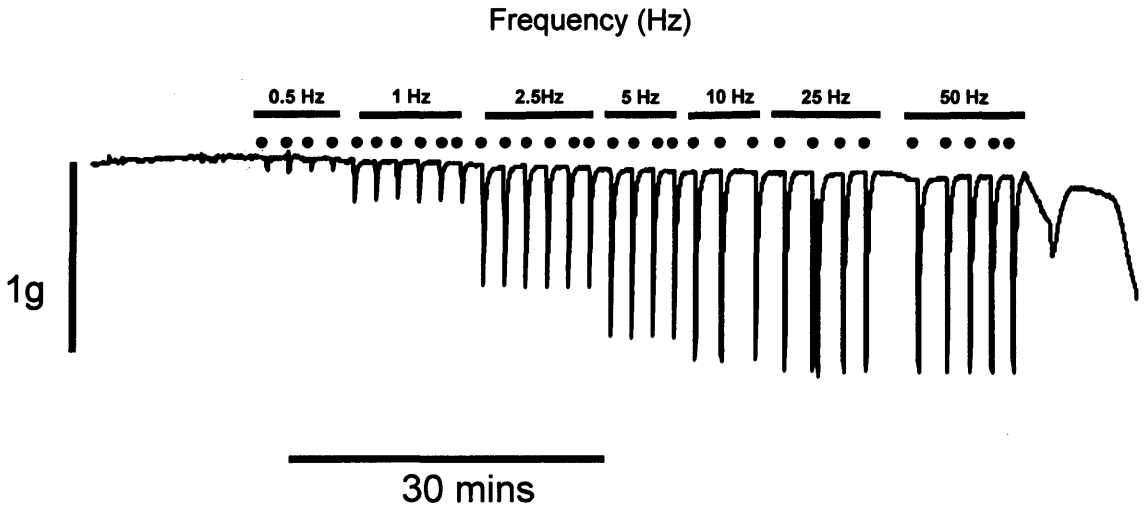


Figure 15. Representative tracing illustrating EFS (50V, 0.3ms pulse, frequency 0.5-50 Hz) induced nitrergic relaxation of tissue strips under control conditions. Each • indicates the point of stimulation.

3.3.3 Acute Effect of Hypoxia, Acidosis and Glucopenia on the Elevated Tone

The constituents of the Krebs solution were altered such that each parameter was investigated either individually or in combination. Hypoxia, acidosis or glucopenia alone reduced the tone of pre-contracted strips to 28.4±2.9%, 59.6±7.5% and 9.4±3.8% of the initial tone respectively. Combinations of glucopenia+acidosis, glucopenia+hypoxia and hypoxia+acidosis reduced the tone to 13.9±2.8%, 8.1±2.5% and 6.4±3.1% of the initial tone respectively. The combination of hypoxia, acidosis and glucopenia resulted in a loss of tone which was 8.2±1.6% of the initial tone. These results are summarised in Figure 16. The time taken to reach the minimum tone is shown later in Table 5 for each condition. Glucopenia resulted in the slowest rate of

loss of tone. Acidosis alone resulted in the most rapid reduction in tone to plateau levels. With regards to recovery following the reversal of each condition, again acidosis alone resulted in the fastest rate of recovery.

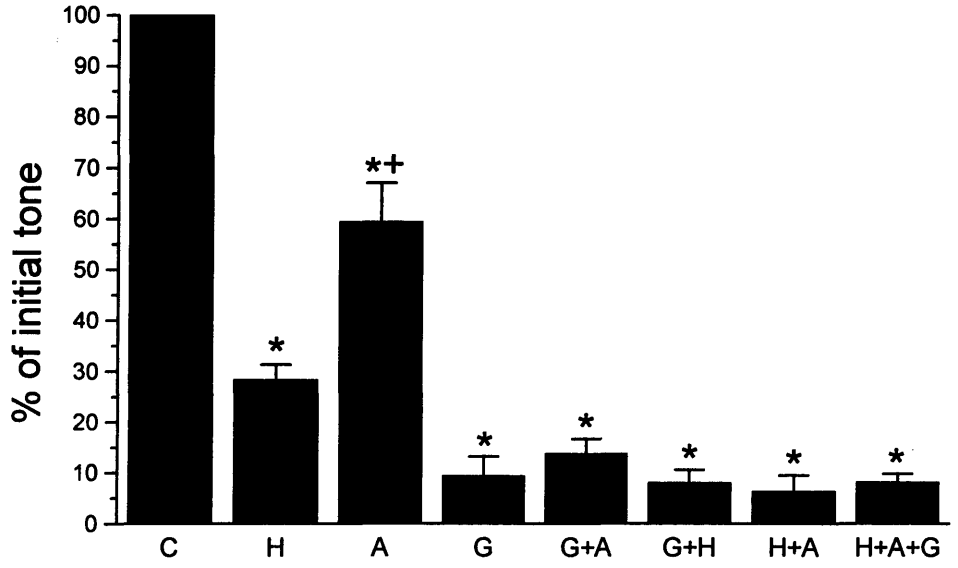


Figure 16: Acute effect of hypoxia (**H**), acidosis (**A**), glucopenia (**G**) and their combinations to phenylephrine-induced tone of the rabbit corpus cavernosum. Control (**C**) tissue was superfused with normal Krebs solution. The tone was measured when the tissue reached a plateau. Each data point represents mean ± s.e. of mean of 4-10 separate tissue strips from different animals. *P<0.05 significantly different from control; +P<0.05 significantly different from other treatments.

3.3.4 Effect of Prolonged Exposure to Hypoxia, Acidosis and Glucopenia on the Elevated Tone

In my initial experiments prolonged exposure to hypoxia or acidosis produced significant loss of tone which was partially reversible even at 48 hours (not shown). However the tone of the control tissue also showed a progressive decline which became significant after 6 hours. Figure 17 shows the progressive reduction in the tone over 12 hours when tissue strips were exposed to hypoxia alone or hypoxia and acidosis in combination.

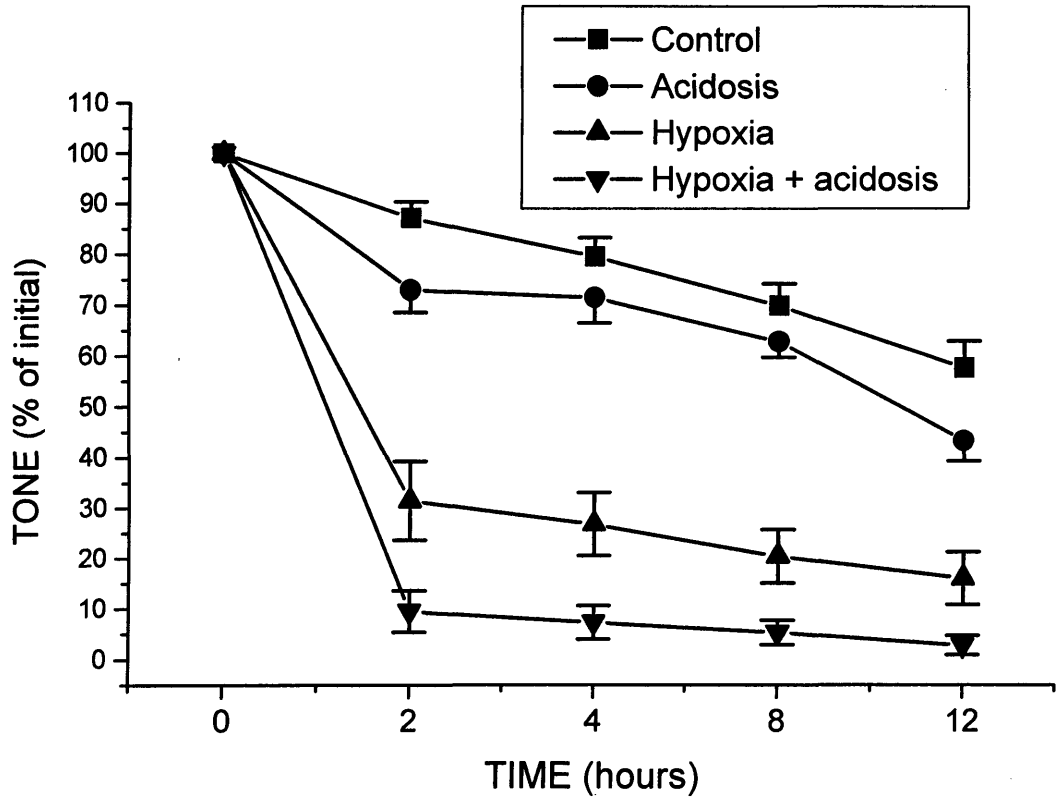


Figure 17: Reduction in corpus cavernosum tone following prolonged exposure to hypoxia and acidosis either alone or in combination. Note that the control tissue lost significant amount of tone especially after 6 hours. Each data point represents mean ± s.e. mean. For each condition 4-8 tissue strips were used from different animals.

3.3.5 Time Course for the Recovery of Phenylephrine-Induced Tone after Reversal of Combination of Hypoxia, Acidosis and Glucopenia

The control tissue lost part of its initial tone during the first 6 hours of the experiment. The combination of hypoxia, acidosis and glucopenia resulted in a loss of tone which progressively became irreversible after reversal of the conditions. A sudden drop in the recovered tone was observed between 1 and 2 hours (Figure 18). Since the maximum irreversibility was reached at 4 hours, this time point was used in the subsequent experiments whereby the conditions were reversed.

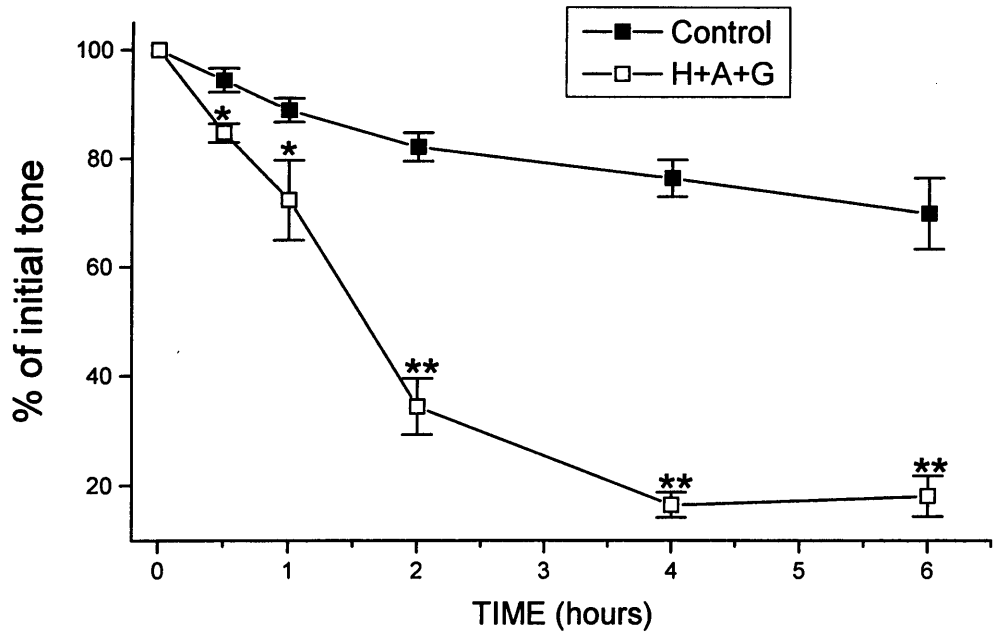


Figure 18: Time course for the recovery of phenylephrine-induced tone after reversal of combination of hypoxia, acidosis and glucopenia (**H+A+G**; open squares). The control tissue (**Control**; solid squares) lost some of its tone during 6 hours. The loss of tone became progressively irreversible after exposure to H+A+G reaching a maximum at 4 hours. The tone was measured when the tissue reached a plateau after reversal of the conditions. Each data point represents mean ± s.e. of mean of 4-10 separate tissue strips from different animals. *P<0.05; **P<0.0001 significantly different from control at the same time point.

3.3.6 Recovery of Phenylephrine-Induced Tone after Reversal of Hypoxia, Acidosis and Glucopenia

In order to ascertain which parameter caused irreversible tone loss, the experiments were repeated using each individual parameter either alone or in combination during a 4 hour superfusion period.

At the end of 4 hours, by reintroducing each parameter, the recovery of smooth muscle tone was recorded. Expressed as a percentage of the initial tone, the results showed almost complete recovery following the reversal of all the parameters except when glucopenia was combined with hypoxia or in the presence of a combination of hypoxia, glucopenia and acidosis (Figures 20). The time taken to recover and to reach a plateau is summarised in Table 5 for each condition. The fastest recovery was observed when acidosis was used alone.

Table 5: Time in minutes to reach the minimum recorded tone upon exposure to different conditions (T1) and to recover (to reach a plateau) after 4 hours of exposure (T2). The measurement of T1 and T2 are explained below in a sample tracing. The results are expressed as mean ± s.e.m. *P<0.05 significantly different from hypoxia T1 or acidosis T1. **P<0.05 significantly different from hypoxia T2 or glucopenia T2.

Condition	T1	T2	n
Hypoxia	29.1 ± 2.2	20.8 ± 1.5	6
Acidosis	13.7 ± 1.1	11.5 ± 1.0**	4
Glucopenia	74.1 ± 15.0*	31.1 ± 4.1	5
Glucopenia + Acidosis	70.4 ± 7.1	22.9 ± 1.1	5
Glucopenia + Hypoxia	45.6 ± 4.4	32.7 ± 3.3	5
Hypoxia + Acidosis	52.7 ± 4.6	42.1 ± 4.8	5
Hypoxia + Acidosis + Glucopenia	39.1 ± 1.9	24.1 ± 2.5	10

T1 – Time to reach minimum recorded tone
T2 – Time to recovery

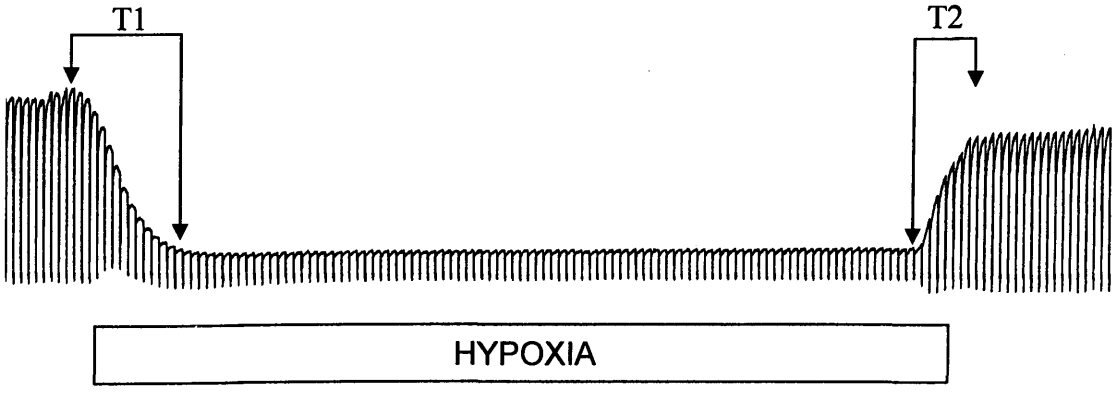


Figure 19. Representative trace showing the effects of hypoxia alone on the tone of precontracted tissue strips and the recovery following the reversal of hypoxia.

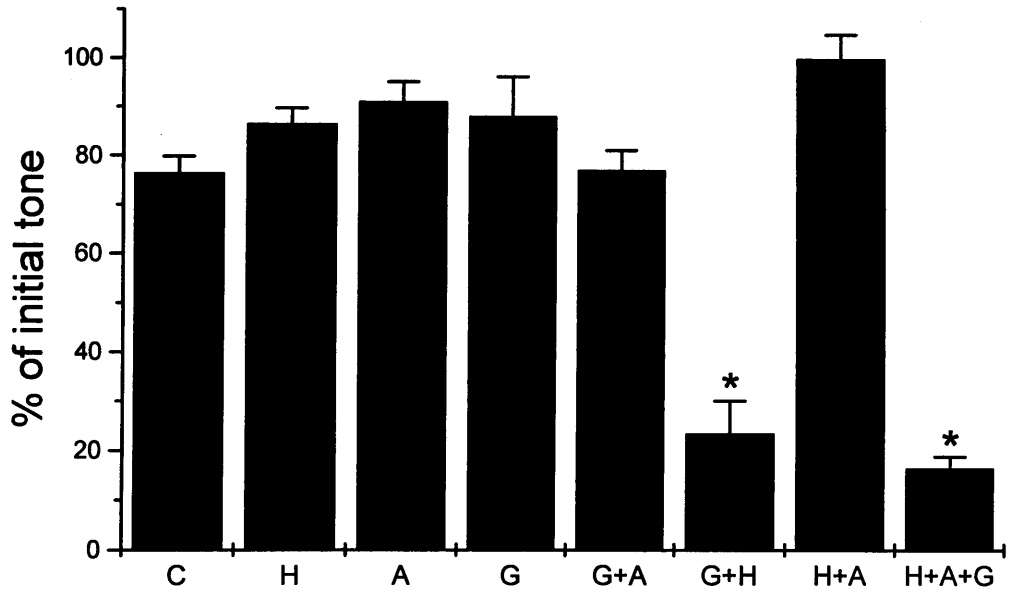


Figure 20: The recovery of phenylephrine-induced tone after reversal of hypoxia (**H**), acidosis (**A**), glucopenia (**G**) or their combinations after 4 hours. The control tissue (**C**) lost some of its tone during this time period. The loss of tone was irreversible only in **G+H** and **H+A+G** groups. The tone was measured when the tissue reached a plateau after reversal of the conditions. Each data point represents mean ± s.e. of mean of 4-10 separate tissue strips from different animals. *P<0.05 significantly different from control.

Representative traces from each experimental condition showing the effects of the tone of tissue strips and the recovery once the conditions are reversed are illustrated in Figure 19 for hypoxia and Figures 21 to 27. For the sake of clarity, EFS induced relaxations are shown although the point of application of EFS has been omitted.

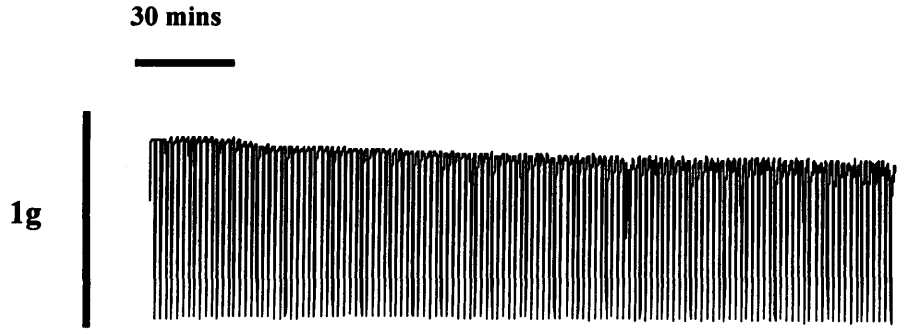


Figure 21. Representative trace showing control tissue strips subjected to superfusion for 4 hours. There was no significant change in the tone throughout this period.

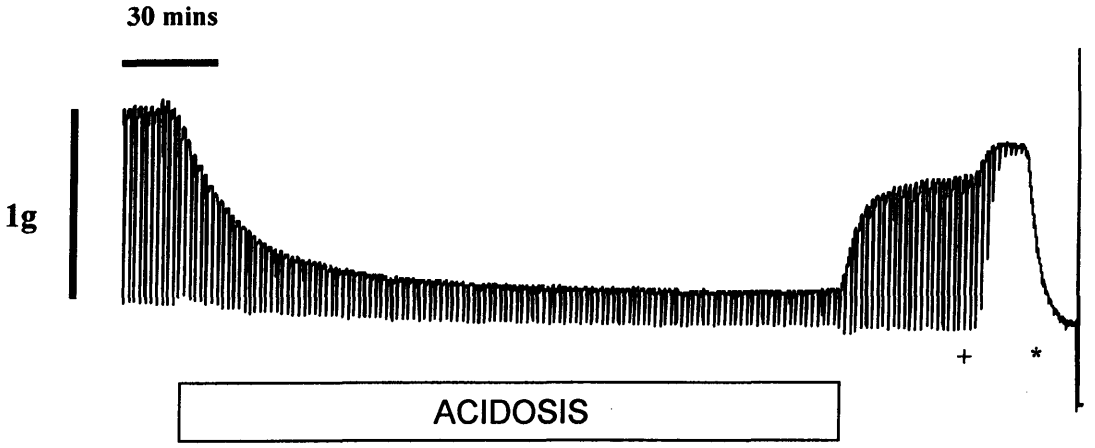


Figure 22. Representative tracing illustrating the effects of acidosis alone on precontracted tissue strips. EFS induced nitrergic relaxations are abolished by the addition of L-NAME (illustrated by +) and the precontraction with phenylephrine is reversed using prazosin (illustrated by *)

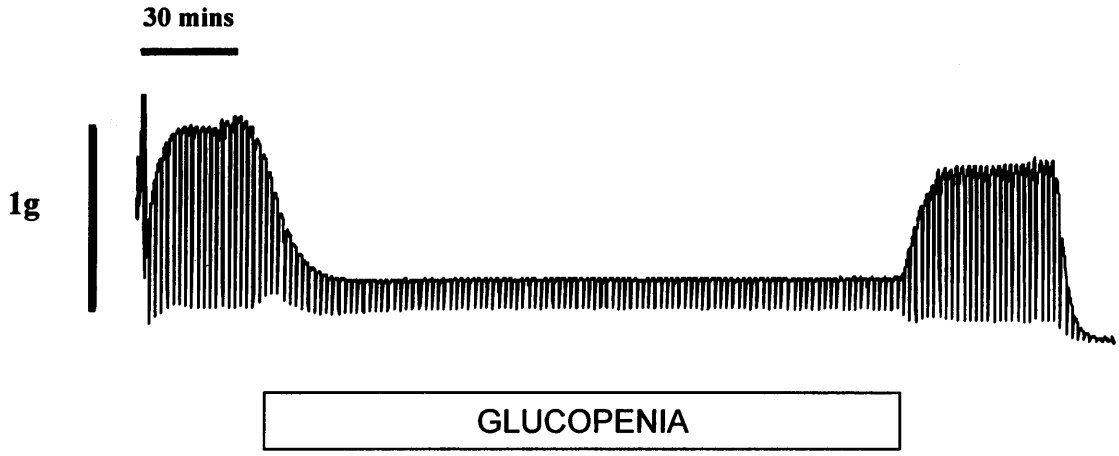


Figure 23. Representative tracing illustrating the effects of glucopenia alone on the tone of precontracted tissue strips and the effects of reversing the conditions.

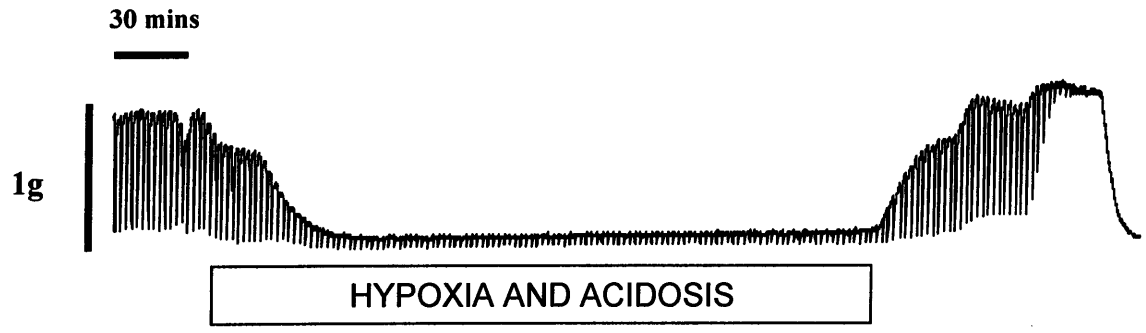


Figure 24. Representative trace illustrating the effects of hypoxia and acidosis in combination on the tone of precontracted tissue strips and also the effects of reversing the conditions. At the end of the recovery period L-NAME and prazosin were added.

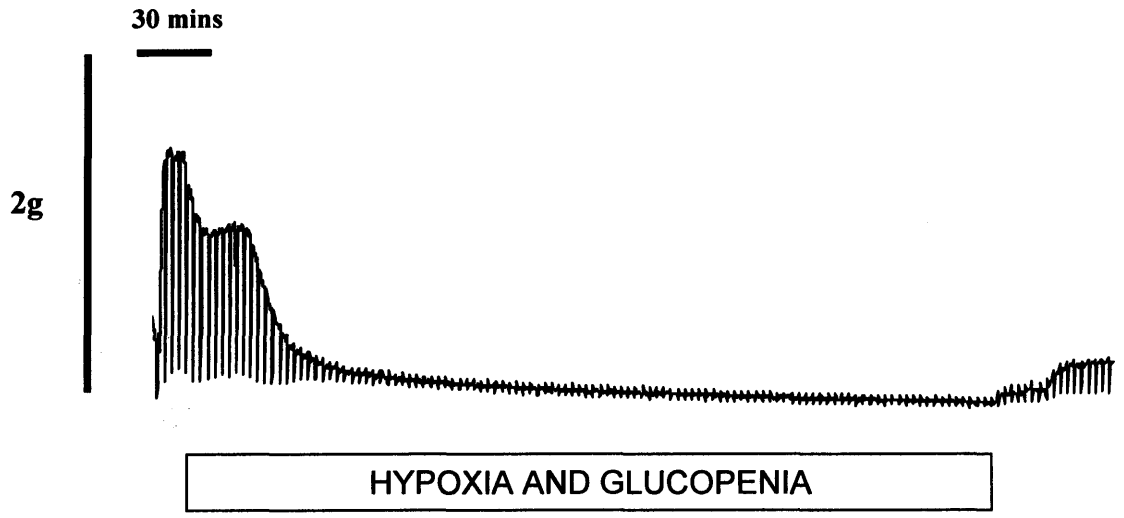


Figure 25. Representative trace illustrating the effects of hypoxia and glucopenia in combination on the tone of precontracted tissue strips and also the effects of reversing the conditions.

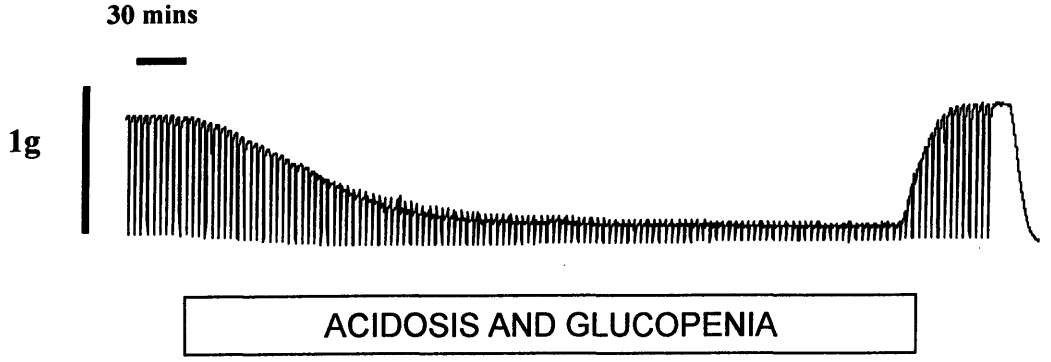


Figure 26. Representative trace illustrating the effects of acidosis and glucopenia in combination on the tone of precontracted tissue and the effects of reversing the conditions.

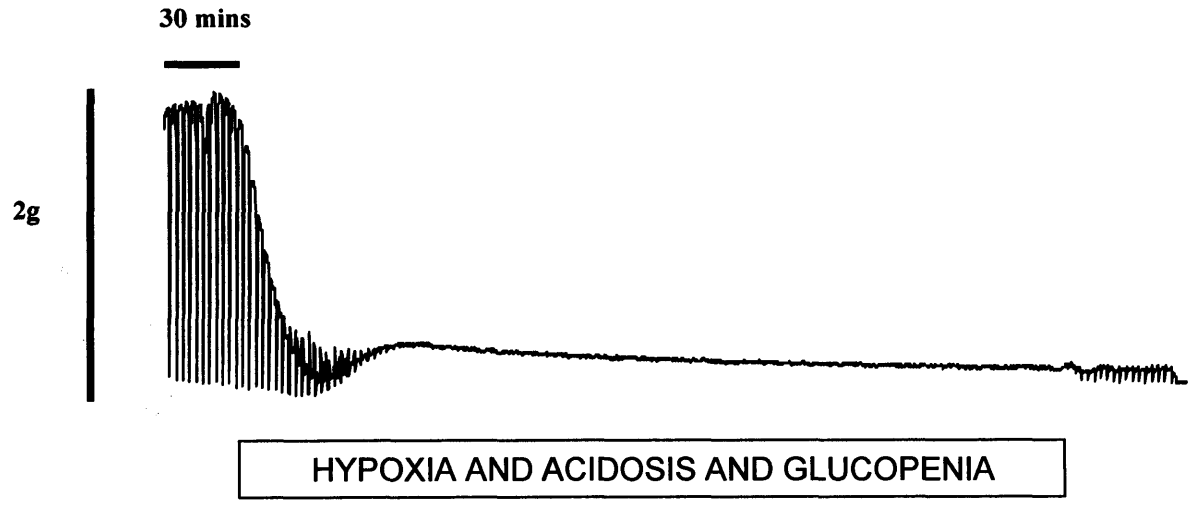


Figure 27. Representative trace illustrating the effects of hypoxia, acidosis and glucopenia in combination on the tone of precontracted tissue and the effects of reversing the conditions.

3.3.7 Cumulative Concentration Response to Phenylephrine with and without Hypoxia, Acidosis and Glucopenia

In order to assess the change in sensitivity of the corpus cavernosum smooth muscle to adrenergic stimulation under ischaemic conditions, concentration response curves to phenylephrine were constructed before and after exposure to a combination of hypoxia, acidosis and glucopenia for 4 hours. Cumulative addition of phenylephrine (0.1-300μM) to tissue strips in normal Krebs solution resulted in a contraction with EC_{50}~ 10μM and a maximum tone of 3.1 ± 0.7g. Tissue strips were then washed and superfused in conditions of hypoxia, glucopenia and acidosis for a 4 hour period which was followed by superfusion in normal Krebs solution. A further phenylephrine concentration response experiment gave an EC_{50}~ 3μM and a maximum tone of 0.9±0.2 g which were both significantly different from that obtained in control tissue ($p<0.05$) (Figure 28).

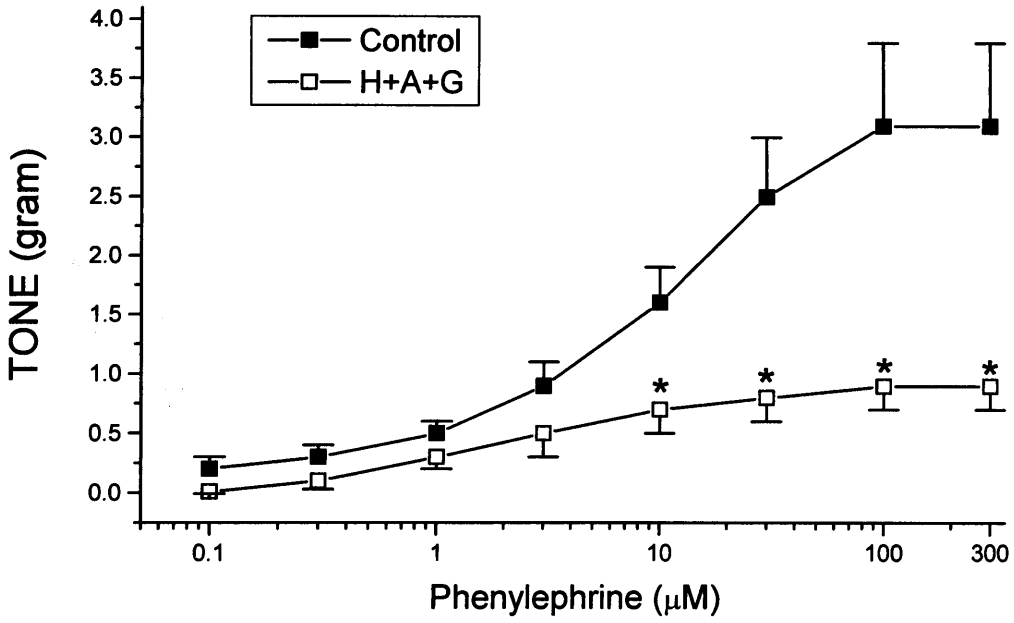


Figure 28: Phenylephrine concentration-response curves in control tissues which were superfused with normal Krebs solution throughout the experiment (**Control**; solid squares) and in tissues which were exposed to the combination of hypoxia, acidosis and glucopenia (**H+A+G**; open squares) for 4 hours and then superfused with normal Krebs solution. Each data point represents mean ± s.e. of mean of 4-6 separate tissue strips from different animals. *P<0.05 significantly different from control at the same concentration of phenylephrine.

3.3.8 Effect of Hypoxia, Acidosis and Glucopenia on the Tone Elevated by Endothelin-1 and U-46619

Experiments were repeated by pre-contracting the tissue strips with either endothelin-1 (33.33nM) or U-46619(50nM) instead of phenylephrine. The tissue was superfused in conditions of hypoxia, acidosis and glucopenia for a 4 hour period following which the conditions were reversed. Table 6 illustrates the minimum tone recorded during ischaemic conditions and the final tone recorded following the reversal of ischaemia.

	Minimum tone	Reversal
Phenylephrine (n=10)	**8.19±1.64**	**16.46±2.32**
Endothelin-1 (n=4)	**14.25±2.28**	**23.51±4.38**
U-46619 (n=4)	**22.48±3.04**	**28.38±2.68**

Table 6. Comparison of the effects of hypoxia, acidosis and glucopenia on elevated tone and recovery using tissue precontracted with either phenylephrine, Endothelin-1 or U-46619.

3.3.9 Effect of Hypoxia, Acidosis and Glucopenia on Nitrergic Responses

EFS elicited reproducible and frequency-dependent relaxation responses after inhibition of noradrenergic and cholinergic pathways and elevation of the tone. These EFS-induced relaxation responses were completely inhibited with L-NAME (500 μM) or TTX (3μM) (refer Figure 22) confirming that they are nitrergic in nature and neuronal in origin. 4 hours of incubation with a combination of hypoxia, acidosis and glucopenia reduced the tone of the strips as mentioned above. During this period nitrergic responses remained unchanged when expressed as the percentage of the tone (Figure 30). Following the reversal of hypoxia, acidosis or glucopenia, the tone partially recovered and nitrergic relaxations appeared to be preserved (Figures 29 & 30).

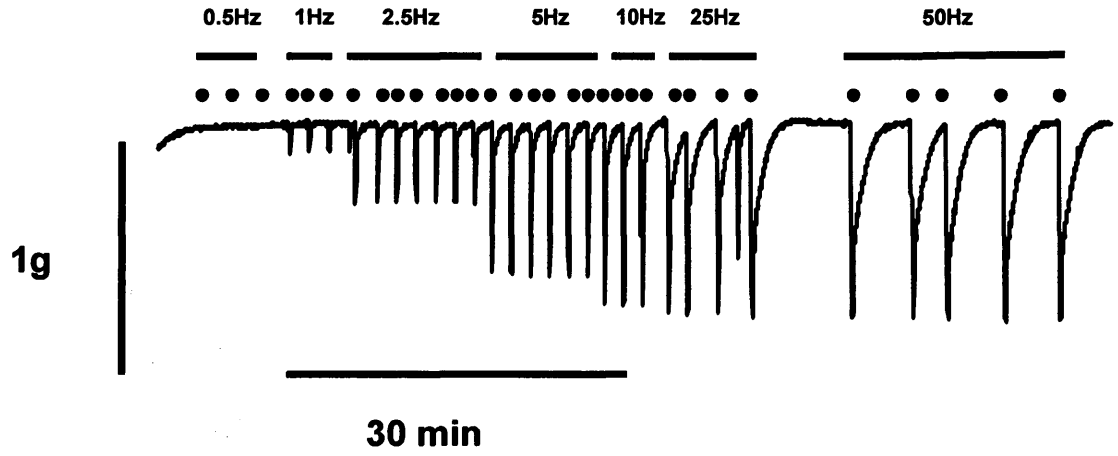


Figure 29. Representative tracing illustrating EFS((50V, 0.3ms pulse, frequency 0.5-50 Hz) induced nitrergic relaxations following exposure of tissue strips to 4 hours of hypoxia, acidosis and glucopenia in combination.

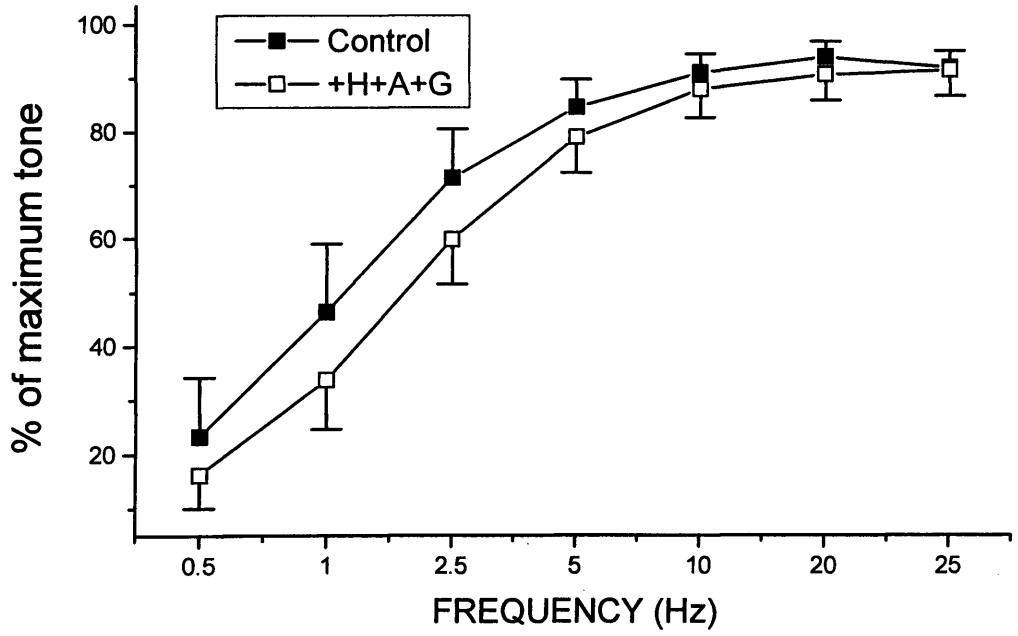


Figure 30: Nitrergic relaxations were not affected by hypoxia, acidosis and glucopenia. Frequency-response curves of nitrergic relaxations expressed as the percentage of the maximum tone in control tissues which were superfused with normal Krebs solution throughout the experiment (**Control**; solid squares) and in tissues which were exposed to the combination of hypoxia, acidosis and glucopenia (**H+A+G**; open squares) for 4 hours and then superfused with normal Krebs solution. Each data point represents mean ± s.e. of mean of 10 separate tissue strips from 5 different animals for the control group and 22 tissue strips from 6 different animals for the experiments involving hypoxia,acidosis and glucopenia.

3.3.10 Investigation of Putative Therapeutic Agents

Several putative agents were tested to assess their potency to protect the tissues against irreversible tone loss in ischemic conditions. These agents [calcium (3μM-10μM), Bay K 8644 (direct Ca2+ channel activator, 1μM), N-acetylcysteine (anti-oxidant, 100μM), N^{ω}-nitro-L-arginine methyl esther (L-NAME, NO synthase inhibitor, 500μM), digoxin (Na/K ATPase inhibitor, 10μM) and glutathione (500μM)] were added to the reservoir after the combination of hypoxia, acidosis and glucopenia was introduced. The agents were in the medium after normal Krebs solution was introduced at the end of 4 hours. None of the agents altered the tone during 4 hours or after reversal of the conditions. As an example the effect of N-acetylcysteine is shown in Figure 31. The effect of the agents at the initial tone and the reversal phase are shown in Table 7.

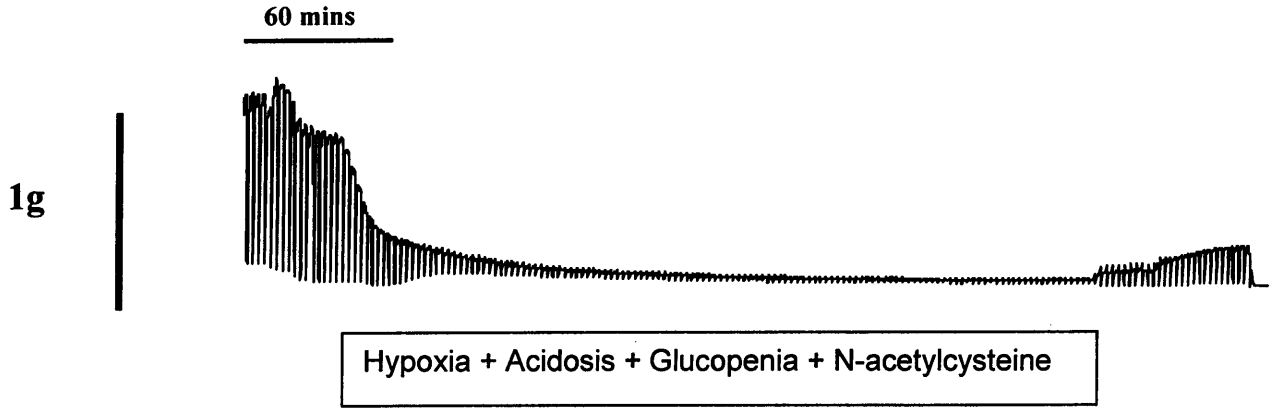


Figure 31. The effects of adding N-acetylcysteine on corpus cavernosum smooth muscle tone and recovery during 4 hours superfusion in conditions of hypoxia, acidosis and glucopenia. Total of 8 tissue strips from 4 separate animals.

Putative Agent	*Minimum Tone*	*Reversal*
Hypoxia+Acidosis+Glucopenia	**8.19±1.64**	**16.46±2.33**
N-acetylcysteine	5.4±2.35	15.46±1.75
Bay K 8644	5.93±1.35	16.8±4.5
Glutathione	10.01±1.92	15.23±3.98
Digoxin	9.48±3.62	24.29±5.74
Calcium	8.03±3.49	31.7±9.06
L-NAME added t=0	11.67±2.12	13.58±1.9
L-NAME t=4 hours	16.44±3.09	18.63±3.11

Table 7. The effects of putative agents on corpus cavernosum smooth muscle tone during 4 hours superfusion in conditions of hypoxia, acidosis and glucopenia. Results expressed as a % of the initial tone. All results represent mean±SEM. n=4 for each agent (4-8 strips). There was no significant difference in the recovery of tissue tone in the presence of putative agents compared to hypoxia, acidosis and glucopenia used in combination.

3.3.11 Experiments Utilising Metabolic Inhibition

In order to assess the role of oxidative phosphorylation and glycolysis in smooth muscle contraction mechanisms, experiments were repeated using 500μM iodoacetic acid to inhibit glycolysis or 500μM myxothiazol in order to inhibit oxidative phosphorylation. Experiments utilising iodoacetic acid showed a rapid reduction in the smooth muscle tone to the baseline within 30 minutes. This was completely irreversible (Figure 33). Myxothiazol showed a more gradual reduction in the tone to 20% of the initial tone and took up to 2 hours to reach this point (Figure 32). Washout of myxothiazol resulted in a recovery of tone to 66% of the initial tone.

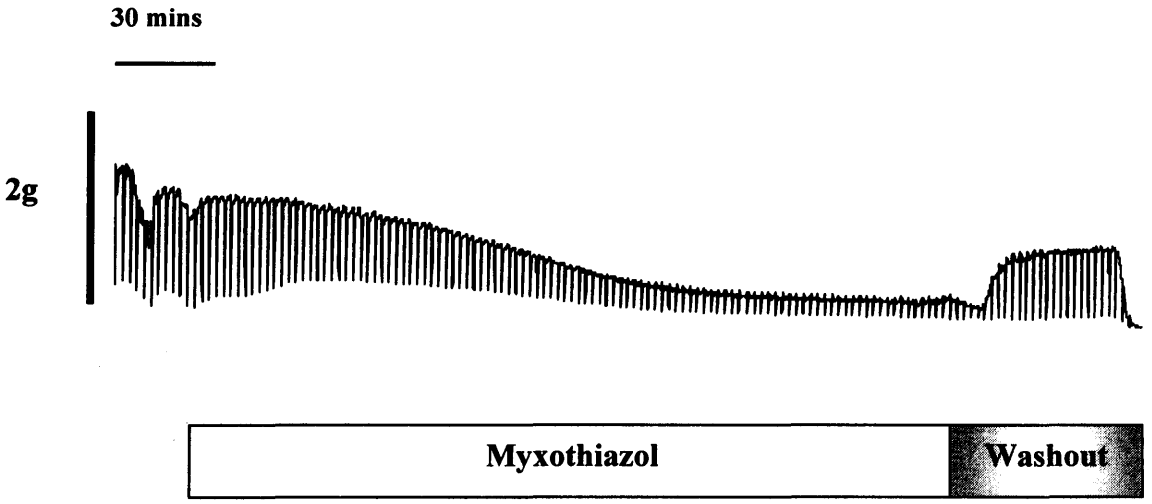


Figure 32: The effects of adding myxothiazol on smooth muscle tone. EFS(50V, 0.3ms pulse, frequency 5Hz) was continued throughout the experimental period. For the purpose of clarity the points for EFS have not been illustrated. Recovery of tone following the washout of myxothiazol is also demonstrated.

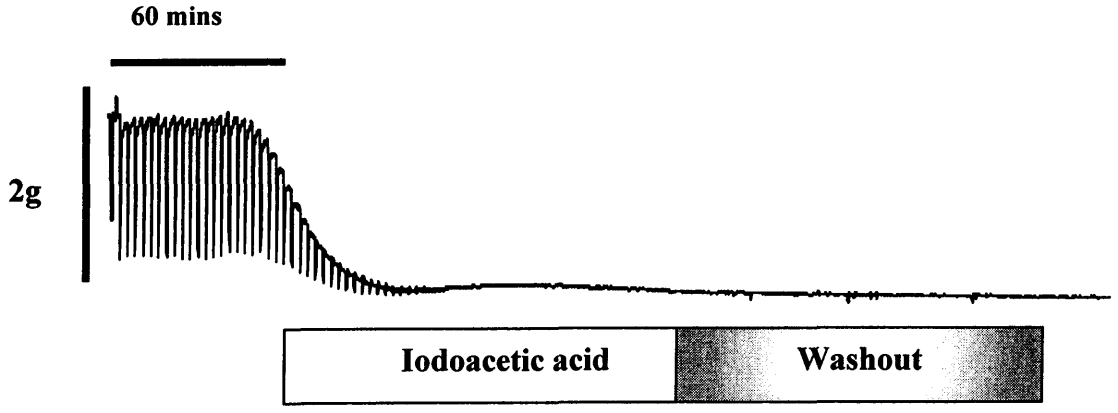


Figure 33: The effects of adding IAA on smooth muscle tone and recovery. Tissue strips were precontracted and EFS (50V, 0.3ms pulse, frequency 0.5Hz) continued throughout the perfusion period. For the purpose of clarity the EFS points have not been illustrated. The lack of recovery of tone following the washout of iodoacetic acid is also demonstarated.

3.4 The measurement of ATP concentrations in the corpus cavernosum

In order to investigate the effects of ischaemic conditions on tissue ATP levels, the tissue ATP levels were measured at 4 time points:

- Following the equilibration period
- Following pre-contraction with phenylephrine
- Following 4 hours superfusion with a combination of hypoxia, acidosis and glucopenia
- Following 1 hour reperfusion in normal Krebs solution.

A typical standard curve constructed from ATP standards is illustrated in Figure 34. The ATP concentration was expressed as pmol/μg protein. The results showed a significant reduction in ATP concentration following 4 hours incubation in conditions of hypoxia, acidosis and glucopenia. Tissue ATP failed to return to basal levels following the reintroduction of oxygen, normal pH and glucose (Table 8).

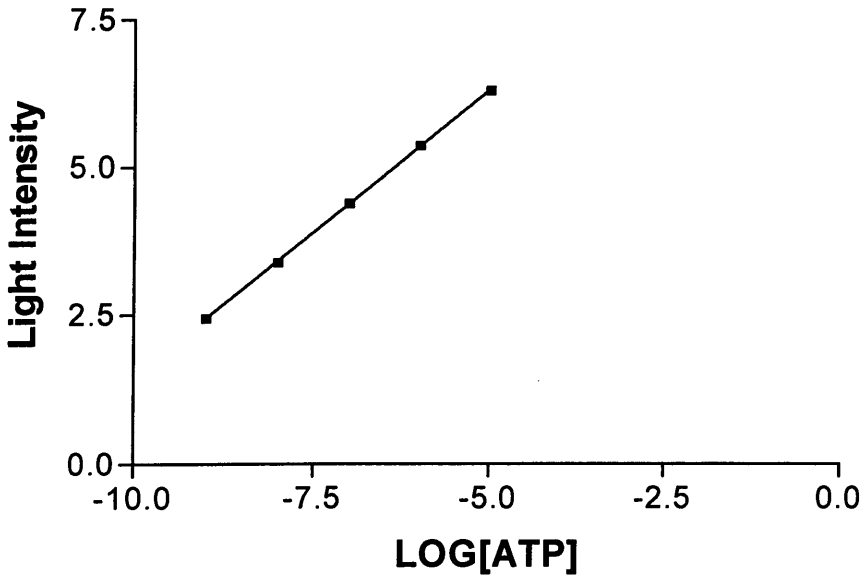


Figure 34. Example of a typical ATP standard curve.

Time Point	*ATP concentration (pmol/μg)*
Equilibration	**60.2±9.3**
Precontraction with phenylephrine	**65.3±5.2**
Following 4 hours hypoxia,acidosis and glucopenia	**46.3±4.3***
Following reperfusion	**48.7±2.4***

Table 8: Changes in the tissue ATP concentration following 4 hours superfusion in conditions of hypoxia, acidosis and glucopenia followed by reversal. (* $p<0.05$)

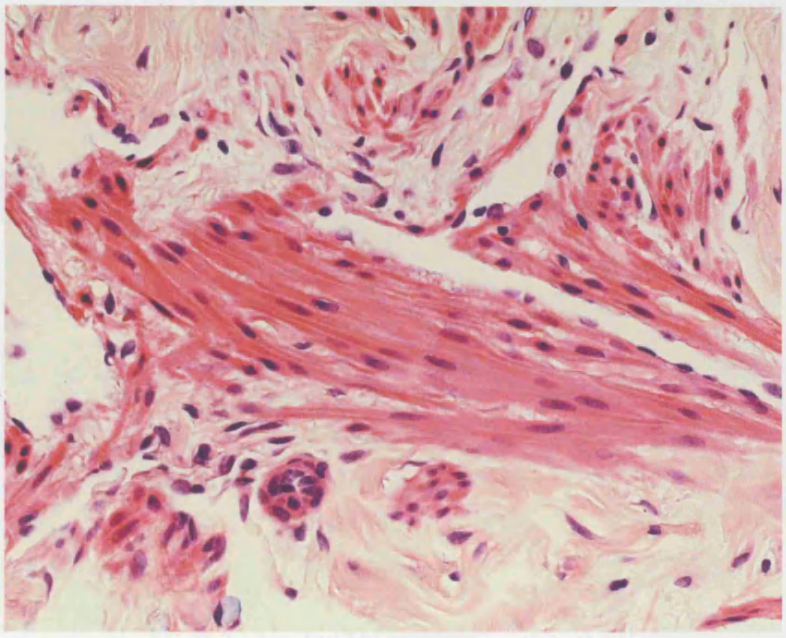
3.5 Microscopic changes in the corpus cavernosum following exposure to hypoxia, acidosis and glucopenia in combination.

Histological analysis of the strips using H& E staining showed no significant change in the smooth muscle and endothelial structures after incubation with a combination of hypoxia, acidosis and glucopenia for 4 hours (Figure 35). However immunofluorescence studies showed a significant increase in the number of TUNEL-positive nuclei in the smooth muscle cells under these conditions. Reperfusion with normal Krebs solution failed to reverse the increase in TUNEL staining. Hypoxia alone caused an increase in TUNEL-positive nuclei although this was not to the same degree as that due to a combination of hypoxia, acidosis and glucopenia (Figure 36). Under control conditions less than 1% of smooth muscle cells were TUNEL-positive. TUNEL positive-smooth muscle cells were 66.5±2.5% of the total smooth muscle cells after 4 hours of hypoxia ($P<0.05$ vs control). After 4 hours of a combination of hypoxia, acidosis and glucopenia, this percentage was 88.6±6.9% ($P<0.05$ vs control and vs hypoxia). Reperfusion of the tissue with normal Krebs solution for 1 hour after

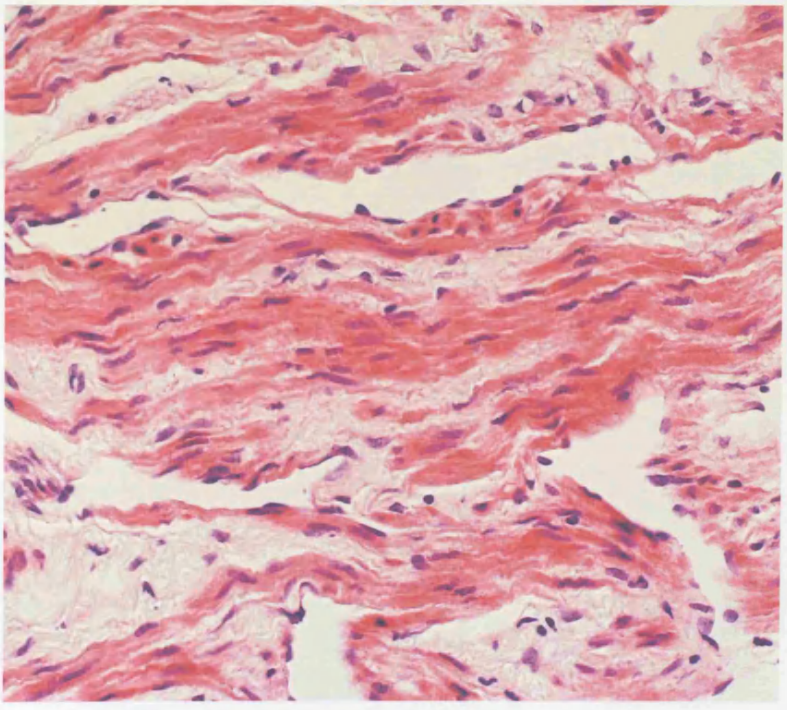
4 hours of hypoxia, acidosis and glucopenia did not alter the TUNEL staining (not shown).

3.6 Microscopic Changes in Human Corpus Cavernosum Following Prolonged Low-flow Priapism

TUNEL staining was also performed using human corpus cavernosum obtained from patients undergoing penectomy operations. This was compared to human corpus cavernosum obtained from a patient who presented with prolonged low-flow priapism and showed a significant increase in TUNEL-positive smooth muscle cells (Figure 37).

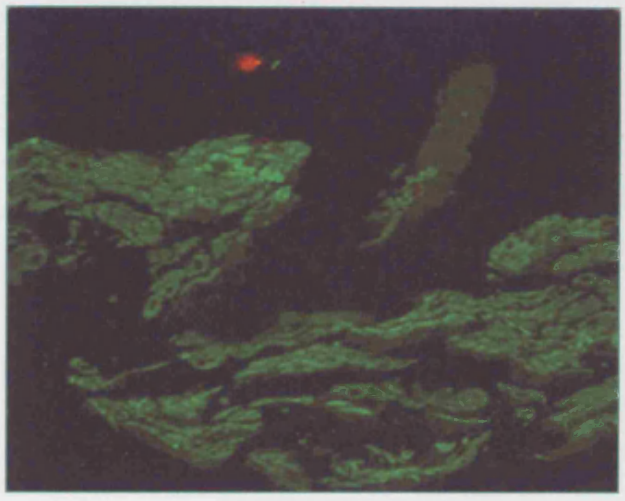


(a)

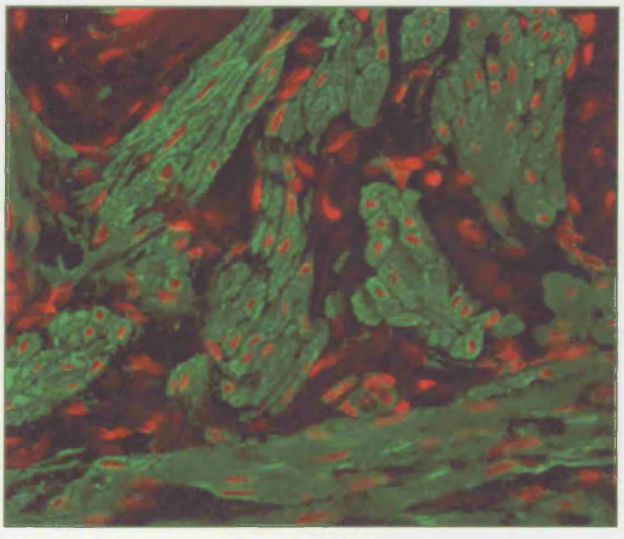


(b)

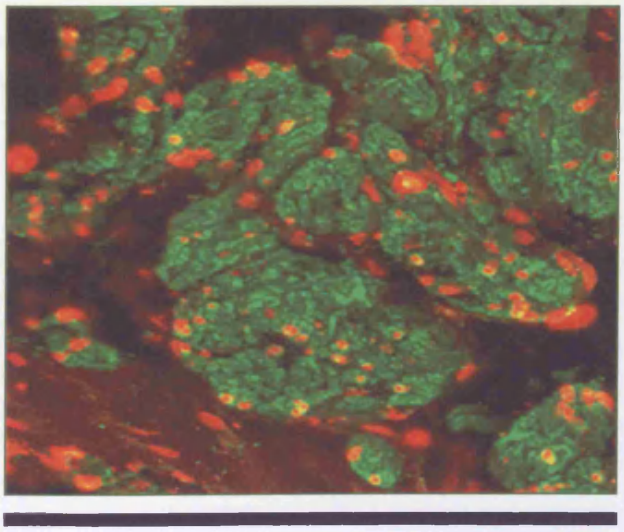
Figure 35. Histological analysis of rabbit corpus cavernosum using H&E staining. Magnification x250 (a) Control tissue. (b) Corpus cavernosum following 4 hours superfusion in conditions of hypoxia, glucopenia and acidosis followed by immediate fixation in formalin.



Control



Hypoxia



Hypoxia + Acidosis + Glucopenia

225μm

Figure 36. Representative pictures comparing TUNEL staining of control tissue with tissue subjected to 4 hours of hypoxia or 4 hours hypoxia, acidosis and glucopenia.

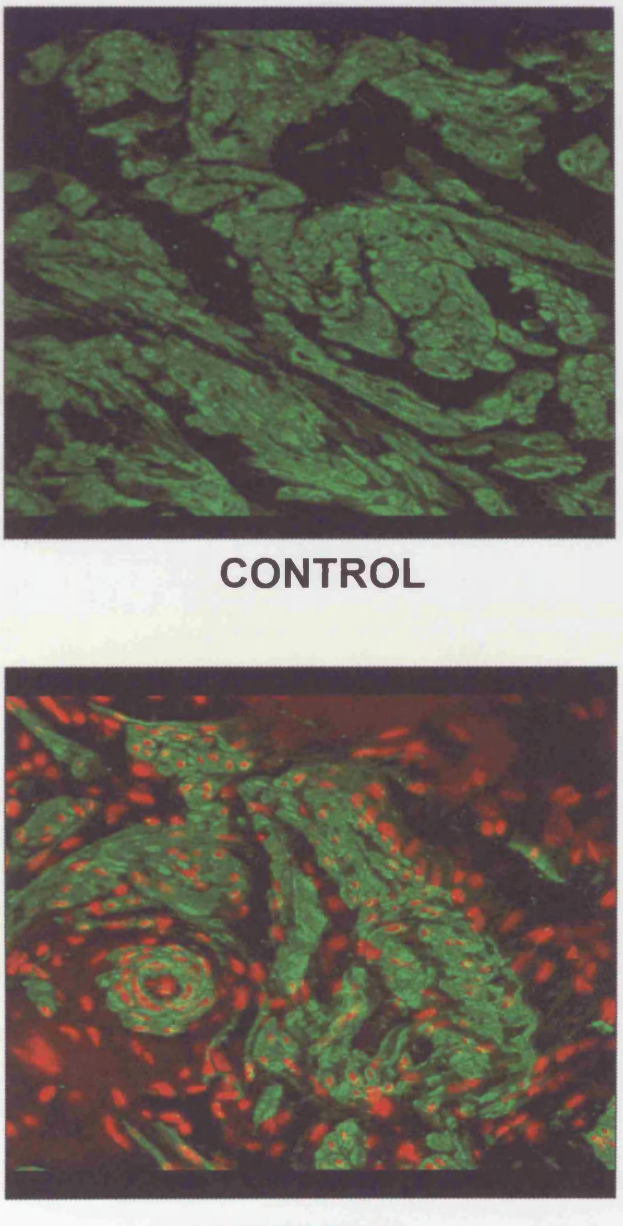


Figure 37. TUNEL staining of normal human corpus cavernosum with comparison to corpus cavernosum obtained from patients presenting with low-flow priapism.

Chapter 4

Discussion

4.1 Aims of the thesis

Low-flow priapism is a rare medical emergency and therefore very limited research has been undertaken in order to understand the underlying pathophysiology leading to this condition. Even when medical interventions are successful in achieving penile detumescence, the incidence of long term erectile dysfunction is still reported at approximately 50% (86). Where patients have presented with a prolonged low-flow priapism, successful medical intervention either using pharmacological agents or surgical procedures are generally unrewarding leading to a high probability of erectile dysfunction in these individuals (86). In order to achieve full penile detumescence the smooth muscle contractile mechanisms must outweigh the effects of relaxation factors such as NO and prostaglandins. The contraction of cavernosal smooth muscle is altered by changes in the local smooth muscle microenvironment (pO_2 and pH) together with the availability of substrates (i.e. glucose) required for glycolysis and oxidative phosphorylation. Although the effects of hypoxia and acidosis on cavernosal smooth muscle tone have been investigated both *in vitro* and *in vivo*, (88;144;145) these studies have not investigated the effects of prolonged exposure to each of these conditions and also the effects upon the recovery of smooth muscle tone. In addition to this, the effects of glucopenia upon cavernosal smooth muscle tone and recovery of tone following the reversal of glucopenia has not been investigated using *in vitro* models. The aim of this thesis was to investigate the factors which cause irreversible smooth muscle dysfunction in low-flow priapism. By developing an *in vitro* model of low-flow priapism, I set out to investigate the effects

of prolonged exposure of cavernosal smooth muscle to hypoxia, acidosis and glucopenia and also the capacity of this tissue to contract once these conditions were reversed.

4.2 General Discussion

In order to achieve penile detumescence, the contractile mechanisms in the corpus cavernosum must overcome the factors mediating relaxation. The development of stagnant ischaemia in a closed compartment such as the corpus cavernosum during a prolonged penile erection results in progressive hypoxia and acidosis. The analogy in an *in vitro* model would be that pre-contracted smooth muscle represents penile detumescence and a reduction in this basal tone represents smooth muscle relaxation corresponding to penile tumescence. By using such a model, I planned to investigate the effects of these conditions on smooth muscle tone using corpus cavernosum. My initial hypothesis was that the development of hypoxia *per se* would be the most important parameter involved in mediating irreversible smooth muscle dysfunction. Although exposure of smooth muscle strips to prolonged periods of hypoxia resulted in a reduction of the cavernosal smooth muscle tone, I found that the smooth muscle tone recovered well upon re-oxygenation.

The second hypothesis proposed that a combination of acidosis and hypoxia would result in irreversible recovery of smooth muscle tone following prolonged periods of perfusion. Again I established that despite superfusing the tissue strips for continuous prolonged periods of up to 48 hours, on reversal of these conditions to normoxia and pH 7.4, the smooth muscle tone would still recover to approximately 20% of the initial tone. In this experiment the control tissue strips demonstrated a

progressive reduction in tone as the perfusion period increased. At the end of the perfusion period the tone of the control tissue strips was completely lost and there was no recovery of tone when the conditions were reversed back to normoxia and pH 7.4.

In order to establish whether another common parameter exists in the corpus cavernosum microenvironment which would mediate the changes of irreversible smooth muscle dysfunction, I studied the results of cavernosal blood aspirates from patients who presented with prolonged low-flow priapism which was refractory to conventional treatments of corporal blood aspiration and instillation of α-adrenergic agonists. From the results obtained, I established that biochemical analysis of the aspirates consistently showed the presence of either a very low or absent glucose concentration coexisting with hypoxia and acidosis. Whether the irreversible nature of priapism is due to substrate depletion was still unknown. The effects of glucopenia on smooth muscle tone and recovery had not been previously investigated. Therefore experiments were repeated to incorporate a shorter perfusion period to maintain the integrity of the control strips and also investigate the effects of substrate depletion on the smooth muscle tone and recovery following reversal of these conditions. These experiments established that the two common parameters which mediate irreversible corpus cavernosum smooth muscle dysfunction were hypoxia and glucopenia in combination. I established that the degree of hypoxia and glucopenia in the corporal aspirates was a better predictor for smooth muscle recovery in individuals who presented with prolonged episodes of priapism.

Although several putative agents have been proposed for the pharmacological management of priapism (146) the clinical evidence is limited and therefore the

instillation of α-adrenergic agonists remains the most established treatment option. Therefore, I investigated the role of alternative pharmacological agents which could potentially reduce the degree of irreversible smooth muscle dysfunction. I had already confirmed that in conditions of hypoxia and acidosis, the cavernosal smooth muscle tone is significantly reduced in keeping with previous observations (88;144). Glucopenia was also found to reduce the smooth muscle tone. These components which constitute an ischaemic microenvironment precipitate smooth muscle relaxation and hence prolong the penile tumescence. Thus in the clinical scenario, for a particular pharmacological agent to be efficacious, the degree of ischaemia present in the corpus cavernosum must be reduced. Clinically this is achieved by the technique of corporal blood aspiration.

I hypothesised that the irreversible recovery of smooth muscle tone following superfusion in ischaemic conditions may be due to the reduction in the availability for intracellular Ca^{2+} required for smooth muscle contraction. On the basis of this hypothesis, pharmacotherapies were used to increase the intracellular Ca^{2+} levels by

1. Increasing extracellular Ca^{2+} $[Ca^{2+}]_e$
2. Direct activation of Ca^{2+} channels using the compound Bay K 8644
3. Use of digoxin to inhibit Na^+/K^+ ATPase and therefore increase intracellular Ca^{2+} $[Ca^{2+}]_i$

None of these methods were effective in completely restoring the smooth muscle tone and so I hypothesised that there may be significant smooth muscle cell damage during tissue reperfusion which immediately follows the ischaemic period. In the *in vivo* situation one would expect endogenous antioxidants to accumulate during the

period of stagnant ischaemia in the corpus cavernosum in order to prevent significant tissue injury during the reperfusion period. In order to prevent reperfusion tissue injury, experiments were conducted in the presence of the antioxidant compounds N-acetylcysteine and glutathione. However, incubating the tissue strips in the presence of these compounds throughout the ischaemic period did not significantly alter the degree of irreversible cavernosal smooth muscle dysfunction.

My final hypothesis was that the establishment of a hypoxic and glucopenic microenvironment in the corpus cavernosum would result in significant intracellular metabolic inhibition to eventually result in smooth muscle apoptosis. Substrate depletion during the period of hypoxia and glucopenia would inhibit both the oxidative phosphorylation pathway and the glycolytic pathway necessary for the production of ATP. Smooth muscle contraction requires the generation of ATP predominantly from the oxidative phosphorylation pathway. ATP is also required for normal cellular homeostasis. My experiments showed that in the presence of ischaemia, the tissue ATP levels were significantly reduced. Following reperfusion after 4 hours the tissue levels of ATP failed to recover back to basal cellular levels. Coinciding with this irreversible fall in tissue ATP levels, TUNEL staining of the cells showed irreversible DNA fragmentation which is a marker of cellular death. However, the histological analysis of the same tissue utilising H&E staining demonstrated that there was no significant morphological difference in the tissue before and after the period of ischaemia. In the long term cellular death is associated with the recruitment of inflammatory mediators and fibroblasts which ultimately lead to the replacement of myocytes with fibrous tissue. The disproportional replacement of corpus cavernosum myocytes which would normally mediate smooth muscle

relaxation is ultimately responsible for the development of long term erectile dysfunction.

4.3 The Characterisitic Features of Patients Presenting with Low-flow Priapism

The investigation of patients with refractory low-flow priapism is the first study to establish the common factor of glucopenia in the corporal blood aspirates of these patients. It also confirmed the presence of significant hypoxia and acidosis in keeping with the previous study by Broderick and Harkaway (91). Prolonged low-flow priapism results in the development of vascular stasis in a closed compartment. Therefore with prolonged stasis the utilisation of substrates such as glucose continues with very little replenishment. The intracellular stores of glycogen can be an additional source of substrate for glycolysis in order to maintain cellular homeostasis during periods of hypoxia. The failure of replenishment of these intracellular stores results in the progressive development of glucopenia.

Doppler studies are utilised as a diagnostic radiological investigation in order to differentiate between a high-flow priapism and low-flow priapism. Although my series of patients clinically presented with a low-flow priapism, confirmed by the results of corpus cavernosum aspirations, there were two cases whereby the Doppler studies suggested a high-flow picture. The use of Doppler studies are not entirely specific for a diagnosis of low-flow priapism as the Doppler picture can be misinterpreted following multiple cavernous needle aspirations or shunt surgery which may result in local areas of cavernosal reperfusion despite the overall picture remaining as one of a low-flow priapism. Therefore the results of Doppler studies must be used with caution as false negative results are also possible.

The effects of high dose phenylephrine on tissue strips from priapic patients were compared with the effects on strips obtained from penile cancer patients. Although the phallus in penile cancer patients is pathological, all the tumours were confined distally and the pre-operative erectile function was preserved. Therefore I conducted this study with the assumption that these tissue strips are representative of normal corpus cavernosum. An alternative source of tissue obtained from patients undergoing gender reassignment surgery showed that the EC_{50} was comparable to that of tissue obtained from my series of penile cancer patients (154).

Histological analysis conducted by the UCLH histopathology department of tissue biopsied from patients presenting with refractory low-flow priapism demonstrated changes in the intracellular organelles which would be incompatible with cellular viability. These tissue strips were reperfused *in vitro*. Upon reperfusion in normoxic conditions, there was still a complete failure of smooth muscle contraction to high doses of phenylephrine. Subsequent TUNEL staining of this tissue confirmed the presence of widespread cellular death. Therefore in this clinical situation the duration of priapism is perceived as being sufficiently prolonged to induce cell death in the corpus cavernosum. The eventual long term sequel would be that inflammatory cells and fibroblast recruitment into the tissue leads to corporal fibrosis which ultimately leads to erectile dysfunction.

4.4 The Factors Involved in Cavernosal Smooth Muscle Dysfunction.

An *in vitro* model of ischaemic priapism was developed in order to reproduce the clinical scenario and investigate the factors which are responsible for reducing the corporal smooth muscle tone and mediating irreversible smooth muscle dysfunction.

Tissue strips were pre-contracted with phenylephrine as we know that penile flaccidity is maintained by a tonic sympathetic discharge.

4.4.1 The Effect of Hypoxia on Cavernosal Smooth Muscle Tone

I established that hypoxia alone reduced the smooth muscle tone in keeping with previous studies (88;144). However, upon re-oxygenation, the cavernosal smooth muscle tone recovered completely. This is the first *in vitro* study which has investigated the effects of re-oxygenation following prolonged hypoxia on the recovery of corporal smooth muscle function. The findings suggested that although the development of hypoxia during priapism may have a role in maintaining penile tumescence by causing smooth muscle relaxation, hypoxia alone can not account for the irreversible smooth muscle dysfunction.

My initial experimental studies showed that the control tissue strips progressively lost smooth muscle tone to almost unrecordable levels as the duration of the perfusion period increased upto 48 hours. This phenomenon was irreversible and occurred well within the 48 hour period, normally between 16 to 20 hours. As carbogen was used which is a mixture of 95% O_2 and 5% CO_2 in the perfusion medium this may have resulted in oxidative stress. The formation of NO_2, NO^- and $ONOO^-$ in the presence of O_2 is known to cause tissue injury (155). Therefore in order to allow a valid comparison between the control and experimental tissues, the perfusion periods were reduced to 4 hours so that the control tissue maintained smooth muscle tone throughout the complete experimental period. It is unlikely that the progressive reduction in the tone was due to the induction of iNOS as all experiments were performed in the presence of dexamethasone to prevent iNOS

induction (156). Even in the presence of an inhibitor of NO synthase (L-NAME), I found that the smooth muscle tone of the control tissue progressively declined with time.

Experiments utilising the shorter perfusion periods demonstrated that all the parameters namely hypoxia, acidosis and glucopenia reduced the corporal smooth muscle tone either alone or in combination with the greatest magnitude occurring when hypoxia and glucopenia were used in combination. Although acidosis reduced tone, this was not as significant as when the other parameters were investigated individually. When acidosis was used in combination with hypoxia, the percentage recovery of smooth muscle tone was better than if hypoxia was used in isolation. This suggests that the presence of acidosis in a hypoxic microenvironment may have a protective effect on the corpus cavernosum smooth muscle. A possible explanation for this phenomenon is given later when I discuss the effects of acidosis on smooth muscle tone.

In the presence of hypoxia alone, the tone of the cavernosal smooth muscle is decreased. This confirms the findings from previous studies (88;144). During the perfusion period which utilized only hypoxic conditions, the reduced tone was sustained. When the hypoxia was reversed, the tissue regained the original tone indicating that hypoxia alone does not result in irreversible smooth muscle dysfunction. Only one previous study has demonstrated a similar finding using hypoxia alone. However the perfusion period in this study was restricted to a maximum of 2.5 hours (88). The purpose of my *in vitro* model was to investigate the changes in smooth muscle tone occurring in prolonged low-flow priapism and

therefore progressively longer perfusion periods were employed. The contraction of cavernosal smooth muscle depends on the mobilisation of Ca^{2+} from the sarcoplasmic reticulum and also the transmembrane Ca^{2+} influx. One would expect that the reduction in the $[Ca^{2+}]_i$ results in smooth muscle relaxation by MLC_{20} dephosphorylation. However, studies on alternative smooth muscle sources have shown that relaxation in the presence of hypoxia, the $[Ca^{2+}]_i$ may increase in ileum (157), aorta (158), portal vein (159) and pulmonary tissue (160). The $[Ca^{2+}]_i$ has been shown to remain unaltered in cerebral (161) and taenia coli (162) despite concurrent smooth muscle relaxation. Therefore the smooth muscle relaxation due to hypoxia is likely to be multifactorial. Kim et al (88) demonstrated that the $[Ca^{2+}]_i$ levels increased during hypoxia by loading the tissue with the intracellular fluorescent dye FURA-2. When tissue strips were exposed to hypoxic buffer in this study, there was a transient decrease in the $[Ca^{2+}]_i$ which was followed by a large increase in the $[Ca^{2+}]_i$ within 4 minutes of exposure to hypoxia. This study suggests that there is a dissociation between the $[Ca^{2+}]_i$ levels and generation of force during hypoxia. However, the Ca^{2+} transients which rely on calcium influx through Ca^{2+} channels are reduced during hypoxia (163). This may be secondary to the direct effect of hypoxia on oxygen sensitive Ca^{2+} channels, a reduction in the ATP concentration or the raised $[Ca^{2+}]_i$ inhibiting the channel directly. Therefore hypoxia-induced loss of tone may be secondary to the altered sensitivity of the contractile apparatus to intracellular Ca^{2+} as well as a reduction in the Ca^{2+} transient. Alternatively the contractile apparatus becomes unresponsive to increased Ca^{2+} levels due to a reduction in the intracellular ATP levels. The reduction in cytosolic ATP may reduce the MLC_{20} phosphorylation and account for the reduction in tone. However, previous studies have shown that

there are no significant changes in the MLC_{20} phosphorylation during hypoxia and therefore this alone is unlikely to account for the reduction in force (164).

The discussion so far has failed to convincingly show that there is a straight forward relationship of $[Ca^{2+}]_i$ –force or MLC_{20}-force. Alternative pathways of force reduction during hypoxia must account for the observations. Another possible pathway involves the ATP-gated K^+ channels present in smooth muscle (K_{ATP}). A number of studies have proposed that this channel is involved in the dilatation of coronary and cerebral arterioles during hypoxia (165-167). This channel is inhibited by intracellular ATP and therefore during hypoxia the fall in cytosolic ATP and decrease in intracellular pH will lead to the opening of this channel and subsequent hyperpolarisation and smooth muscle relaxation. Although the K_{ATP} channel may have a partial contribution to the hypoxia-induced relaxation, some studies have shown that hypoxia produces a small membrane depolarisation rather than a hyperpolarisation (168;169). The K_{ATP} channel blocker, glibenclamide, is also unable to reverse the effects of hypoxia in these studies (170). Therefore it is unlikely that the effects of hypoxia on force are solely mediated by the depolarisation due to K_{ATP} channel opening.

Hypoxia has been shown to induce elevations of inorganic phosphate, [Pi], both *in vivo* and *in vitro* (164;171). Pi inhibits Ca^{2+} activated force of permeabilised smooth muscle fibres (172). This occurs independently of MLC_{20} phosphorylation (173;174). Therefore the increase in [Pi] may partly account for the reduction in force during hypoxia without altering MLC_{20} phosphorylation. Studies have reduced the increases in [Pi] during altered metabolism and found that the rates of relaxation and

inhibition of force were also less (164). However, the increase in [Pi] alone can not account for the reduced tone during hypoxia or metabolic inhibition. Therefore, changes in [Pi] may partly explain the change in smooth muscle tone seen in my experiments but the overall contribution is still uncertain.

Another mechanism which may partly account for the effects of hypoxia and metabolic inhibition is the activation of Ca^{2+} dependent actin-capping proteins and proteases (175). These have been suggested to contribute to attenuated cardiac contractility following ischaemic reperfusion (176). The actin capping agents inhibit tonic smooth muscle contraction independently of $[Ca^{2+}]$ and MLC_{20} phosphorylation (177;178). The extent of this inhibition of force is similar to that observed in studies utilising mitochondrial inhibition (179). Therefore it appears that in the presence of hypoxia, the reduction in oxidative phosphorylation combined with a reduction in the Ca^{2+} transients independent of cytosolic Ca^{2+} levels together with elevated [Pi] and changes in the cytoskeleton account for the smooth muscle relaxation. Finally, the fall in ATP levels within the smooth muscle cells during hypoxia would be expected to reduce myosin ATPase activity and reduce phosphorylation of MLC_{20}. However, several studies have proposed that in the presence of hypoxia alone, the change in the ATP levels surrounding the myofilaments is unlikely to limit myosin ATPase and hence contraction (171;180). The fact that the smooth muscle tone recovered following reoxygenation and that the TUNEL staining experiments showed very little in the way of smooth muscle cell death indicates that the ATP levels were not significantly depleted enough to result in significant smooth muscle cell death.

The effects of hypoxia were found to be reversible as the re-introduction of oxygen resulted in a complete recovery of the smooth muscle tone. This suggests that the corpus cavernosum is highly dependent on energy yields from oxidative phosphorylation but can still function in variable degrees of hypoxia without undergoing permanent smooth muscle dysfunction. This would of course be an important consideration bearing in mind the wide range of pO_2 levels that the human corpus cavernosum is subjected to in the stages from flaccidity to full tumescence.

4.4.2 The Effect of Acidosis on Cavernosal Smooth Muscle Function

Although it is postulated that alterations in pH alone can induce cellular injury, our experiments investigating the role of acidosis alone showed that the loss in smooth muscle tone is not of the same magnitude compared to when hypoxia is used alone. Reversing the pH back to pH 7.4 did not result in significant smooth muscle dysfunction. This is the first study which has demonstrated that prolonged exposure to acidosis alone does not result in irreversible corporal smooth muscle dysfunction. Previous studies have investigated the effects of acidosis on smooth muscle tone without looking at the effects of reversing the pH back to physiological levels (145;151). My findings also confirmed that superfusion of corpus cavernosum tissue strips in acidotic conditions results in a reduction in the smooth muscle tone in keeping with previous studies (145;151).

My experiments reduced the pH to 6.9 by reducing the HCO_3 content of the Krebs solution as described by Ralevic (181). An alternative technique involves the use of 10% CO_2 mixtures and is proposed to reduce intracellular pH(pH_i) directly

(182). Although our technique alters extracellular pH(pH_o), studies have shown that a unit change in the pH_o induces a ratio of 0.73 change in pH_i (183). The reduction in the smooth muscle tone following acidification shows that the corpus cavernosum behaves similarly to other vascular smooth muscle as similar findings have been shown in rat mesenteric smooth muscle (183), rat aorta (184) and rat cerebral artery (185). The pH_o induced change in the tone may be accounted for by changes in the $[Ca^{2+}]_i$. The modulation of $[Ca^{2+}]_i$ by changes in the pH may be a result of the direct effects on ion channels, Ca^{2+} stores or Ca^{2+} pumps. Previous studies on different smooth muscle have shown that a reduction in the pH_i reduces Ca^{2+} influx through L-type voltage activated Ca^{2+} channels (186;187). The effect may be mediated by the β-2a subunit of the channel which activates or deactivates the L-type channel according to the degree of protonation or deprotonation (187). The effects of changes in pH_o may be due to the transduction changes in the pH_i although direct effects of pH_o on the L-type Ca^{2+} channel conductance have also been postulated (188). Although the effects on T-type channels has not been extensively studied in smooth muscle, in cardiac cells pH_o had similar effects on the T-type channel as those seen in the L-type Ca^{2+} channel although the T-type channel was insensitive to changes in pH_i (189). The effects of pH changes on smooth muscle tone may also affect the release of Ca^{2+} from intracellular stores. Although in phasic smooth muscle such as the portal vein, the main source of Ca^{2+} is from extracellular influx, in tonic smooth muscle the intracellular stores play a more important role and these tissues are more dependent on agonist induced Ca^{2+} release. The Ca^{2+} release from these stores can occur via Ca^{2+} induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum or IP_3 induced Ca^{2+} release.

The influx of Ca^{2+} through Ca^{2+} channels is the trigger for CICR. Studies on vascular smooth muscle have demonstrated that both acidification or alkalinisation can release Ca^{2+} from the sarcoplasmic reticulum although the effects of acidification are less (190). Therefore the effects of acidosis on reducing the smooth muscle tone are not necessarily due to a direct reduction in the CICR from the sarcoplasmic reticulum. However IP_3-induced release of Ca^{2+} is also pH sensitive and could be modulated by either modulation of IP_3 to its binding site or modulation of the IP_3 sensitive release channel. Studies on alternative tissue have shown the pH dependence of IP_3-induced Ca^{2+} release (191;192). A further mechanism involves changes in the sensitivity of the contractile machinery in the absence of changes in the $[Ca^{2+}]_i$. The mechanism by which this may occur includes modulation of myosin light chain kinase, myosin light-chain phosphatase and Rho kinase. Finally the role of the sarcoplamic reticulum Ca^{2+} ATPase which refills the Ca^{2+} store after CICR and the plasma membrane Ca^{2+} ATPase which maintains resting $[Ca^{2+}]_i$ have not been fully evaluated in smooth muscle. In my model I found that the magnitude of the contribution to the overall reduction in tone induced by alterations in the pH_o is not as great as the effects of hypoxia. I found that the minimum sustained tone during perfusion with acidified Krebs solution was only reduced to 60% of the initial tone. Therefore, although acidosis reduces the smooth muscle tone, the underlying mechanism is likely to be different to that of hypoxia.

A combination of hypoxia with acidosis again showed a reduction in smooth muscle tone which was sustained throughout the perfusion period in these conditions. These findings confirm those of previous studies (151). Again the prolonged periods of perfusion up to 48 hours were not conclusive due to the spontaneous loss of tone in

the control tissue. Once the perfusion period had been shortened the results showed that on reversal of both hypoxia and acidosis, the smooth muscle tone not only recovered but actually increased compared to control tissue. Although there was a gradual loss in tone of the control tissue, the recovery to 100% of the initial tone of tissue strips suggested a protective role for an acidotic microenvironment. Although acidosis does not prevent ATP depletion during ischaemia(193;194), the acidotic pH suppresses degradative enzymes activated by hypoxia such as phospholipases and proteases (195). This affords cytoprotection and delays cellular death in ischaemia reperfusion conditions (196) This phenomenon has been reported in rat hepatocytes using both intracellular and extracellular acidosis (194;197).

The initial experiments did not establish the underlying mechanism mediating irreversible smooth muscle dysfunction. However, our analysis of corporal blood aspirations did provide further data which has not been previously reported. Patients presenting with a failure of detumescence were consistently found to have very low levels or no glucose present in the corporal blood aspirates in addition to hypoxia and acidosis. Whether glucopenia *per se* is responsible for irreversible corporal smooth muscle dysfunction has not been previously investigated.

4.5 The Use of In Vitro Models of Ischaemia to Investigate Low-flow Priapism.

As prolonged ischaemic priapism establishes a closed system of blood engorgement, substrate utilisation would expect to reduce the availability of glucose for normal cellular metabolism. *In vitro* models of ischaemia have utilized hypoxia and glucopenia in combination to reproduce the conditions (198;199). As yet there have been no similar studies investigating the effects of glucopenia per se on the

corpus cavernosum. Whether this would have a significantly greater bearing on reducing corporal smooth muscle tone and the subsequent recovery of smooth muscle tone would be important in the management of patients with prolonged ischaemic priapism.

Our investigations showed that glucopenia reduces corporal smooth muscle tone although the time period taken in order to reach the minimum sustained tone was much longer compared to experiments utilising hypoxia alone. This would suggest that significant stores of glycogen are present in the corporal smooth muscle cells in order to maintain cellular homeostasis. On the reintroduction of glucose, smooth muscle tone recovers fully. Therefore extracellular glucose has a role in maintaining smooth muscle contractility. Intracellular stores of glycogen can maintain cell homeostasis but are inadequate to maintain smooth muscle contraction. This is the first study which has investigated the effects of glucopenia on corporal smooth muscle contraction. However, this still did not explain the basis for the irreversible smooth muscle dysfunction.

When hypoxia and glucopenia were combined, the corporal smooth muscle tone was reduced to 23% of the initial tone. The minimum tone recorded was lower than that for all the other combinations except when hypoxia, acidosis and glucopenia were combined. When the conditions were reversed, the cavernosal smooth muscle only partially recovered the contractile function. This indicates irreversible smooth muscle recovery in these conditions. Similar work utilising guinea pig bladder also demonstrated a greater rate of contractile failure in conditions of hypoxia and glucopenia compared to hypoxia alone (198). Combining hypoxia and glucopenia is

analogous to simulating ischaemia and has been utilised in several studies using a variety of tissue types and locations. This combination was found to be the most important in mediating irreversible smooth muscle dysfunction. It indicates that oxidative glucose metabolism is of central importance in maintaining the smooth muscle contraction.

The effects of glucopenia alone showed that glucose deprivation does not induce irreversible smooth muscle dysfunction. During the four hour period, oxidative phosphorylation utilises stored glycogen and maintains cellular homeostasis with good recovery of tone upon the reintroduction of glucose. Therefore both parameters of hypoxia and glucopenia must coexist in order to mediate irreversible smooth muscle dysfunction. When all three parameters coexist, the presence of acidosis no longer offers protection to ischaemic damage. There is still irreversible recovery with the smooth muscle recovering to 16% of the initial tone.

In order to ascertain whether this phenomenon was due to a conformational change in the phenylephrine/α receptor interaction at the cell membrane level, alternative agents were used to pre-contract the tissue strips. Our results using U-46619 and endothelin-1 which both utilise the IP_3 second messenger intracellular pathway, showed that the phenomenon of irreversible recovery was mediated intracellularly. Therefore when applied to the clinical picture of low-flow priapism, we can say that in the presence of hypoxia and glucopenia failure of detumescence following drainage and α agonist instillation signifies irreversible smooth muscle dysfunction.

4.6 The Role of Putative Agents in Preventing Smooth Muscle Dysfunction

In an attempt to accurately mimic the conditions occurring in refractory low-flow priapism, a model representing irreversible smooth muscle dysfunction was developed in order to investigate the role of potential pharmacotherapies which may have a therapeutic role in preventing irreversible smooth muscle dysfunction which would manifest in the long term as erectile dysfunction. Clinical observations in this study have shown that conventional α agonist instillation into the corpus cavernosum of patients with prolonged low-flow priapism has limited efficacy. By utilising my *in vitro* model of low-flow priapism with irreversible smooth muscle dysfunction, potentially novel therapeutic options were evaluated. The main objective of these agents was to try and limit the smooth muscle dysfunction which followed the ischaemic period.

4.6.1 The Effect of NO Synthase Inhibition on Smooth Muscle Recovery

Nitric oxide is the most important mediator of smooth muscle relaxation in the corpus cavernosum during penile erection. Whether NO was involved in mediating the loss of smooth muscle tone in my model was investigated by adding the NO synthase inhibitor L-NAME to the superfusate. When L-NAME was added at the end of the ischaemic perfusion period and the ischaemic conditions reversed, there was no significant recovery of smooth muscle function. This indicated that the failure of recovery was not mediated by NO. When L-NAME was added at the start of the ischemic perfusion period, the EFS induced nitrergic relaxations were abolished. However, corporal smooth muscle tone continued to fall at the same rate and to the same minimum sustained tone as when ischaemic conditions were utilised on their own. This indicates that the reduction in smooth muscle tone during ischaemia is not

mediated by upregulation of NO. There has been one previous study which has investigated the role of NO in mediating the effects of hypoxia on corpus cavernosum (200). This *in vivo* study used anaesthetised cats and measured the intracavernosal pressure (ICP) in conditions of normoxia and hypoxia. Although there was no change in the ICP when conditions were changed from normoxia to hypoxia, the responses to L-arginine, S-nitroso-N-acetyl-penicillamine (SNAP) which is a NO donor showed that the increase in ICP seen during normoxic conditions were attenuated during hypoxia. This study confirmed that during normoxia cavernosal smooth muscle relaxation is mediated by NO. However this effect is abolished in conditions of definitive hypoxia (pO_2<30mmHg). Therefore in keeping with the findings of my experiments whereby L-NAME failed to prevent the loss of smooth muscle tone in ischaemic conditions and failed to reverse the smooth muscle dysfunction, we can conclude that the NO pathway does not significantly contribute to the loss of tone during prolonged low-flow priapism. Following on from this, NO inhibition does not provide a potential pharmacotherapeutic route to reverse low-flow priapism or prevent irreversible smooth muscle dysfunction. Although the NO pathway may not have a role in sustaining the penile erection during prolonged priapism, it may still have a role in initiating the condition. This area requires further research and investigation.

4.6.2 The Use of Antioxidants in Preventing Irreversible Smooth Muscle Dysfunction

Ischaemia followed by reperfusion is known to generate free radicals and cause tissue injury (see section 1.4.3). We postulated that the reperfusion of the tissue strips following four hours of ischaemia may cause a significant reperfusion tissue injury and mediate the subsequent failure of smooth muscle contraction. By using the

membrane permeable thiol, N-acetylcysteine, throughout the experimental period as a means of cytoprotection we found no significant recovery of smooth muscle function when ischaemia was reversed after 4 hours.

Glutathione is a free radical scavenger and therefore I proposed that the reduction in free radical generation on reperfusion of ischaemic corporal tissue may result in a reduction in the smooth muscle dysfunction by preventing reperfusion injury. However, my experiments utilising glutathione again showed no significant cytoprotection for the corpus cavernosum smooth muscle. Therefore the use of free radical scavengers in prolonged ischaemic priapism is unlikely to prevent cavernosal tissue injury and subsequent long term erectile dysfunction.

One previous study has investigated the physiologic and biochemical changes which occur during reperfusion of ischaemic tissue (146). In this study, rabbit pelvic nerves were stimulated to induce a penile erection followed by clamping of the penis to induce ischaemia. On reperfusion the researchers found a significant increase in myeloperoxidase activity and lipid peroxidation which indicate oxidative stress. It is likely that during the stage of reoxygenation the univalent reductions of molecular oxygen to superoxide anion, hydrogen peroxide and hydroxyl radical occurs (201). It has also been proposed that the reaction of NO with superoxide radicals forming peroxynitrite generates more hydroxyl radicals (202). However, we have previously shown that the NO pathway may have very little role during prolonged low-flow priapism and therefore peroxynitrite generation is unlikely to contribute significantly. Even so, the generation of ROS can lead to significant tissue injury as demonstrated in other organs such as cardiac muscle and intestinal tissue (201). Specifically in the

corpus cavernosum, studies have proposed that prostanoids provide protection against hypoxia-reperfusion injury (84;203;204). The presence of a ROS scavenger during reoxygenation has been shown to increase prostanoid production more quickly (204). This was part of the basis for using glutathione in my experiments. However, both N-acetylcysteine and glutathione failed to prevent smooth muscle dysfunction in my experimental set up. Alternative agents such as allopurinol may have a role. Previous work has shown that allopurinol inhibits the generation of free radicals and has previously been shown to reduce lipid peroxidation in corpus cavernosum (152).

4.6.3 The Use of Digoxin to Reverse Cavernosal Smooth Muscle Relaxation.

Digoxin is a cardiac glycoside associated with erectile dysfunction thought to be mediated via the inhibition of the sodium pump in the corpus cavernosum (135;136). Previous work has shown that digoxin at high concentration (1μM) causes contraction of human corpus cavernosum (135). This has formed the basis for the potential use of digoxin in stuttering priapism although there have been no reports of the potential use of digoxin in acute low-flow priapism. Therefore in my experiments I investigated the effects of a high concentration of digoxin (10μM) in order to reverse the smooth muscle relaxation induced by ischaemia. The results showed that there was no significant increase in the smooth muscle recovery following four hours ischaemia. Digoxin is a Na^+/K^+ ATPase inhibitor which increases the intracellular Ca^{2+} concentration. In cardiac myocytes this leads to a positive inotropic effect. However, despite one previous study reporting digoxin-mediated cavernosal smooth muscle contraction, my study failed to elicit a potential role in reversing smooth muscle dysfunction. The study by Gupta et al (136) utilised the NO donor S-nitroso-glutathione to induce smooth muscle relaxation which was subsequently inhibited by

the use of digoxin. As I have already discussed in section 4.4.1, the smooth muscle relaxation in my model was not mediated by the NO pathway. Therefore digoxin may have a role in preventing NO-mediated smooth muscle relaxation as opposed to smooth muscle relaxation induced by ischaemia.

4.6.4 Modulation of Intracellular Ca^{2+} levels

The final set of experiments involved altering the intracellular Ca^{2+} levels by using the direct L-type Ca^{2+} channel opener Bay K 8644 or by elevating the extracellular Ca^{2+} levels. Previous studies using bronchial rings have shown that hypoxia-induced relaxation of pre-contracted smooth muscle is prevented by the use of Bay K 8644 (205). Herrara and Walker concluded that hypoxia induced relaxation of vascular smooth muscle was due to inhibition of L-type Ca^{2+} channels by using Bay K 8644 to pre-contract the tissue (206). Therefore I proposed that using the direct Ca^{2+} channel activator, Bay K 8644, I may be able to reverse the smooth muscle relaxation induced by ischaemia. I found that following the ischaemic period of tissue perfusion, Bay K 8644 did not significantly increase smooth muscle tone. This is the first study which has utilised this compound in order to reverse the effects of ischaemia on corpus cavernosum smooth muscle. The failure of Ca^{2+} influx via L-Type channels to increase the corpus cavernosum smooth muscle tone suggests that Ca^{2+} transients do not have a significant role in modulating smooth muscle tone following prolonged ischaemia. Increasing the extracellular Ca^{2+} concentration did however increase the smooth muscle tone following the period of ischaemia but the recovery was incomplete. There has only been one previous study utilising guinea pig taenia coli which has also shown that an increase in the extracellular Ca^{2+} levels can

result in an increase in the smooth muscle tone following hypoxia and glucopenia (207).

The increase in Ca^{2+} levels may result in changes in the Ca^{2+} transients or increase CICR from alternative intracellular Ca^{2+} stores. As there is conflicting evidence on changes in the cytosolic Ca^{2+} levels in response to hypoxia depending on the tissue type utilised in the study, further work is required to quantify the changes in the intracellular Ca^{2+} levels in response to ischaemia. Up until now there is only one study which has specifically investigated the changes in the intracellular Ca^{2+} levels in response to hypoxia (88). This study found that in corpus cavernosum the intracellular Ca^{2+} increased in response to hypoxia. However, the experiments utilised FURA-2 loaded tissue as opposed to single cells which would be a more accurate measure of the changes in the Ca^{2+} levels. The small increase in smooth muscle tone in response to increasing the extracellular Ca^{2+} levels may provide a potential therapeutic option in low-flow priapism which is unresponsive to conventional treatments.

4.7 The Preservation of NO-Mediated Nitrergic Relaxations

The initial experiments using a perfusion period of up to 48 hours showed that although the smooth muscle tone gradually diminished with time, the EFS-induced nitrergic relaxations appeared to be preserved. Experiments using 4 hours exposure to conditions of hypoxia, glucopenia and acidosis in combination, showed that the EFS-induced nitrergic relaxations were in deed preserved. Previous studies have reported a similar phenomenon using bladder detrusor muscle suggesting that although the

contractile machinery is impaired following ischaemia, the neural pathways are preserved (198).

The role of NO in ischaemia-reperfusion remains controversial. Although there have been no previous studies investigating the preservation of nitrergic pathways in corpus cavernosum following ischaemia, investigators have utilised cardiac myocytes to investigate whether NO is cytoprotective or detrimental (208). As already stated, the interaction of NO with superoxide results in the formation of peroxynitrite which is considered cytotoxic in high concentrations in ischaemia-reperfusion injury (209;210). However, NO may also offer cytoprotection by scavenging superoxide anions (155) or by inhibiting apoptosis (211).

The cysteine protease family of caspases plays an important role in the signal transduction during apoptosis. The caspase 3 subfamily (caspase-3,-6 and -7) are involved in the effector phase of apoptosis. Previous work has shown that NO may arrest apoptosis through the inhibition of caspase activity by S-nitrosylation (212;213). NO has also been implicated in the control of apoptosis depending on ATP levels (214). A cell study by Leist et al (215) suggested that NO prevents caspase activation by inhibiting the mitochondrial respiration and therefore lowering ATP levels. Cell death is then delayed but eventually cell death by necrosis ensues. However, glucose supplementation to the culture medium reverts the cells back to the apoptotic form. In relation to nitrergic neurones, the protection by NO by stopping the apoptotic cascade would potentially allow them to recover from a transient metabolic insult. This is particularly important in the corpus cavernosum which is subjected to varying degrees of hypoxia depending on the degree of tumescence. Alternatively, the

axons in our tissue strips may be more resistant to ischaemia due to a lower energy requirement to maintain homeostasis compared to the smooth muscle cells.

4.8 Energy Sources and Compartmentalisation During Ischaemia

Smooth muscle can survive during periods of anoxia by utilising substrates in order to generate ATP by glycolysis. The combination of hypoxia and glucopenia reduces both oxidative phosphorylation and glycolysis. A continual supply of ATP is required to ensure that cellular homeostasis is maintained and researchers have proposed that compartmentalisation of the energy sources may occur in smooth muscle such that glycolysis predominantly supplies ATP for cell homeostasis and oxidative phosphorylation supplies ATP for the contractile machinery (198). My experiments showed that in conditions of glucopenia, the reduction in tone was progressive and reduced to the minimum level after a longer time period compared to when hypoxia was used alone. This suggests that impaired oxidative phosphorylation in hypoxic conditions has a greater effect on inhibiting smooth muscle contraction than the inhibition of glycolysis. The ATP utilisation by the contractile machinery is likely to be greater than the ATP required for cellular homeostasis. During glucopenia, oxygen is still available to ensure that oxidative phosphorylation continues by using intracellular stores of glycogen which would explain the longer time period for the tone to reduce in these conditions. Ultimately both conditions have the same effect which is a sustained minimum tone so that energy requirements are minimised.

In order to quantify the changes in intracellular nucleotide levels I attempted to measure the ATP, ADP, AMP and PCr intracellular levels using high pressure

liquid chromatography (HPLC) as described by Levin *et al* (216) . However, this technique proved to be unsuccessful in quantifying the changes. This may have been due to the very small quantity of nucleotide being present in the tissue strips which made it difficult to accurately achieve reproducible results after the tissue had been homogenised. Therefore I measured the changes in the predominant nucleotide, ATP, during conditions of simulated ischaemia associated with smooth muscle dysfunction on reperfusion. Our results showed a fall in tissue ATP levels during perfusion in conditions of simulated ischaemia. This fall in tissue ATP fails to recover when the conditions are reversed. However, the ATP levels did not show complete depletion probably due to the fact that my experimental set up was not completely anoxic and it is likely that additional energy substrates from the degradation of intracellular organelles occurs as the duration of ischaemia increases. The tissue ATP levels are still likely to fall below a critical level which would normally be required for the maintenance of cell viability. It has been proposed that a fall in ATP levels to below 10-15% of normal levels can induce cell death (196).

I proposed that the perfusion of cavernosal smooth muscle in conditions of hypoxia, acidosis and glucopenia combined would result in irreversible smooth muscle dysfunction due to a depletion of ATP leading to cellular death. Interestingly, the histological analysis of the corpus cavernosum following 4 hours of simulated ischaemia did not show histological features consistent with cellular injury. A previous study by Munnariz et al (146) also analysed corpus cavernosum from anaesthetised rabbits which was subjected to ischaemia and reperfusion. Although the ischaemic time was variable and not recorded in the study, 140 minutes of reperfusion were employed. The histological examination of tissue in this study showed adhesion

of polymorphonuclear leucocytes to the corporal endothelium and leucocyte infiltration into the underlying tissue. A study by Spycher and Hauri (143) analysed tissue from patients presenting with priapism of variable duration. In this study the early histological changes (within 12 hours) consisted of minor endothelial defects with occasional lymphocyte infiltration of the trabecular tissue. It was not until the priapism episodes extended beyond 12 hours that intracellular changes in the mitochondria, endoplasmic reticulum and Golgi apparatus became apparent. Therefore there existed a paucity of histological evidence showing disruption in the contractile machinery of myocytes in my *in vitro* model which would account for the failure of the corpus cavernosum smooth muscle to regain its tone once the conditions of simulated ischaemia were reversed.

As I had already shown that intracellular ATP levels were reduced during the conditions of hypoxia, acidosis and glucopenia, I utilised a more sensitive technique in order to verify whether the corpus cavernosum smooth muscle was still viable. TUNEL staining detects DNA fragmentation in the cell nucleus which is generally considered to be specific for cell death, although necrosis has been reported to cause 'non-apoptotic' DNA fragmentation. My experiments using TUNEL staining confirmed the presence of significant cell death in tissue samples exposed to hypoxia, acidosis and glucopenia for 4 hours. This is the first study which has shown significant smooth muscle cell death using TUNEL staining following prolonged simulated ischaemia. Although using hypoxia alone still resulted in the smooth muscle cell death, this was not as significant as that following hypoxia, glucopenia and acidosis combined. However, despite the presence of cell death following hypoxia, the tissue strips still managed to recover the majority of their initial tone.

This suggests that a fixed proportion of viable smooth muscle cells can still generate the same degree of force in the corpus cavernosum. However, after exposure to hypoxia, glucopenia and acidosis, a critical level of ATP depletion leading to cell death is likely to occur which is no longer able to sustain smooth muscle tone. Unlike exposure to hypoxia alone, the loss in smooth muscle tone is partly explained by a reduction in ATP levels around the myofilaments which results in a reduction in myosin ATPase activity and subsequently MLC_{20} phosphorylation is reduced.

Combining these findings, I established that the presence of hypoxia, acidosis and glucopenia in combination results in significant metabolic inhibition of oxidative phosphorylation and glycolysis to such an extent that intracellular ATP levels reduce to a critical level. Once these levels are reached, smooth muscle cell death predominantly by apoptosis occurs which eventually results in a failure of smooth muscle contraction.

4.9 The Investigation of Patients Presenting with Low-flow Priapism

The Doppler studies performed on patients presenting with priapism recruited to this study have confirmed that the priapism was of a low-flow type in three of the seven cases; two patients were considered to have a high-flow picture on Doppler studies although clinically and on the basis of corporal blood aspirations these patients were in keeping with a typical low-flow priapism indicating that false positive results using Doppler studies can occur. The creation of an iatrogenic fistula during needle aspiration may account for the high-flow picture seen on Doppler studies although the initial period of a low-flow priapism would have already resulted in significant smooth muscle dysfunction. Therefore Doppler studies can be misleading in making

an initial diagnosis unless they are performed prior to needle aspiration and care must be taken in the interpretation of these studies.

In all our patients presenting with priapism, blood aspirates from the corpus cavernosum confirmed the presence of hypoxia, acidosis and glucopenia. Although the presence of hypoxia and acidosis is in accordance with a previous study using cavernosal blood aspirations (91), this study is unique in that it is the first study to show that glucopenia is an important parameter in those patients presenting with low-flow priapism which fails to respond to conventional medical and surgical management. Whether the degree of glucopenia is significant enough to completely impair contractile funciton to α-adrenergic agonists has never been investigated *in vivo* as there is a limit to the therapeutic concentration of α-agonists which can be injected into the human corpus cavernosum without unwanted systemic side effects such as uncontrolled hypertension. For that reason I have studied the response of cavernosal smooth muscle to phenylephrine *ex situ*.

The effect of phenylephrine on normal human corpus cavernosum showed a concentration-dependent (0.1-300 μM) increase in tone with an EC_{50} of 2μM. However, corpus cavernosum from patients presenting with priapism showed no contraction to phenylephrine even at the highest concentrations (300μM). In addition to this, EFS did not evoke any response in the priapitic tissue but did show frequency dependent relaxation in control tissue. These results suggest that the presence of hypoxia, acidosis and glucopenia during a priapism episode mediates irreversible smooth muscle dysfunction. This is in accordance with my *in vitro* results showing

irreversible smooth muscle dysfunction in the rabbit corpus cavernosum following exposure to hypoxia, acidosis and glucopenia in combination.

Two patients had already undergone surgical intervention prior to presenting to our unit, one underwent a Winter procedure and another underwent bilateral saphenocorporal shunts which interestingly had already thrombosed by the time of surgery to undertake cavernosal biopsies. The formation of shunts may act solely as an elaborate drainage mechanism for stagnant blood over a prolonged period of time. Although some of the stagnant blood in the corpus cavernosum may drain, smooth muscle dysfunction is likely to have already been established with subsequent infiltration by inflammatory cells and culminating in corporal fibrosis. TUNEL staining of tissue from patients with refractory priapism confirmed the presence of widespread cell death. The long term sequel for patients such as this is that the non-viable smooth muscle undergoes extensive fibrosis leading to eventual impotence (217). The paucity of functional smooth muscle in the corpus cavernosum means that erectogenic therapies such as PDE-5 inhibitors will be unsuccessful. A surgical option in these patients would be to implant a penile prosthesis albeit with technical difficulty due to the extensive corporal fibrosis. Therefore, I propose that in the clinical scenario of prolonged low-flow priapism which is refractory to pharmacological or surgical intervention in the form of shunt surgery, an alternative option involves the immediate implantation of a malleable penile prosthesis. This has the added benefit of easier insertion in the absence of corporal fibrosis and lower morbidity.

4.10 Critique of the study

4.10.1 Study Design

Although this study was initiated by observations in human patients presenting with refractory low-flow priapism who ultimately develop long term erectile dysfunction, the majority of the *in vitro* work was conducted utilising rabbit corpus cavernosum. Rabbit corpus cavernosum has been utilised extensively in the field of erectile dysfunction research and has been found to be a reproducible model for these studies. Cellek and Moncada have shown that rabbit corpus cavernosum is closer to human corpus cavernosum compared to rat and mouse. (218). My initial work demonstrated that human corpus cavenosum has a characteristic concentration response to phenylephrine and that in the presence of hypoxia and acidosis the pattern of smooth muscle relaxation was similar to that in rabbit corpus cavernosum. The intracellular metabolic pathways would be similar in both tissues but the stores of glycogen and alternative substrates is likely to be more abundant in human tissue than rabbit tissue due to the differential size of each species. Therefore we would expect a similar phenomenon with human tissue but probably over a longer ischaemic perfusion period. Therefore I can not directly correlate the time periods for the observed phenomenon in an *in vitro* model using rabbit corpus cavernosum to that which may occur in human tissue.

Although priapism has been reported in horses (219), no such reports of priapism in rabbits exists. In our model we simulated the conditions to mimic those occurring in prolonged low-flow priapism in order to investigate the effects on smooth muscle function. As the study used an *in vitro* model, the question as to whether the phenomenon exists in rabbits would not alter the study.

4.10.2 Experimental protocols

One of the early noticeable features of the experimental set up was the progressive loss in the tone of the control tissue compared to the experimental tissue. This was particularly prevalent during the prolonged 48 hour experiments which showed that by 24 hours, the control tissue had completely lost all of its initial tone. The tissue subjected to hypoxia demonstrated that although the tone was significantly reduced, there was still a degree of recovery of tone following reperfusion. The loss in tone of the control tissue may be a result of oxidative stress. The continuous gassing of the Krebs solution with 95% O_2 would result in the generation of free radicals leading to oxidative smooth muscle cell injury. Normal corpus cavernosum would function at between 25mmHg to 100mmHg whereas 95% O_2 was measured to be equivalent to 100mmHg in both the chamber and organ bath. Tissue strips which were exposed to hypoxic conditions may sense the reduction in pO_2 and undergo a degree of cellular shutdown. This in itself may offer cytoprotection to further oxidative insults.

4.10.3 Metabolic studies

Experiments which used a combination of hypoxia and glucopenia to reproduce conditions of metabolic inhibition showed irreversible smooth muscle dysfunction. I then attempted to reproduce these pharmacologically by using iodoacetic acid to inhibit glycolysis and myxothiazol to inhibit cytochrome c and therefore oxidative phosphorylation. Iodoacetic acid rapidly reduced the tone of the precontracted tissue strips. However, iodoacetic acid is a sulfhydryl reagent causing irreversible damage to glycerol phosphate dehydrogenase and therefore my experiments which investigated the reversal of hypoxia and glucopenia could not be reproduced. The loss in tone was much more rapid which indicates that both glucose

and glycogen metabolism is inhibited as opposed to when glucopenia is used whence glycogen metabolism can still continue intracellularly and the reduction in tone is much slower. Addition of myxothiazol which is a potent cytochrome c inhibitor resulted in a much slower reduction in the tone. Although initially it was difficult to explain this phenomenon, it may be due to myxothiazol having poor tissue penetration. Reviewing the previous work which has been performed using this compound, most studies have been performed on cell cultures and therefore it may be more appropriate for cell based experiments (220). Experiments could have been repeated using cyanide which is also an inhibitor of cytochrome c but these would reproduce the conditions of total anoxia when we know that complete anoxia does not occur in the corporal microenvironment. By using a mixture of 95% N_2 and 5% CO_2, total anoxia does not occur in this set up due to the equilibration through the apparatus tubings, reservoir etc and this is more representative of the hypoxia which occurs in the corpus cavernosum *in vivo*.

Tissue strips were not weighed as per protocols described elsewhere (88). As each experiment analysed the results as a ratio of the initial tone, variability would be based on an alteration in conditions within the same experimental protocol. Inter-strip variation was reduced by using the same experimental set up and gas supply throughout all the experiments. For each experiment two tissues strips would be superfused by solutions from the same organ bath.

Although the TUNEL staining experiments performed on tissue strips exposed to hypoxia showed that there was no significant increase in TUNEL positive nuclei, I used this finding to explain the almost complete recovery of smooth muscle tone

when hypoxia was reversed. However, measurement of the tissue ATP levels in this tissue would also have been useful in order to analyse the change in the ATP levels during these particular experiments and correlate the results to changes in the TUNEL staining of the same tissue.

4.11 Future developments

Organ bath studies have allowed the establishment of a basic model and allowed us to formulate a possible sequence of events which occur during prolonged priapism. During the development of the study, it was noted that Ca^{2+} measurements using FURA 2 loaded tissue have been conducted elsewhere (88). Although previous studies investigating the effects of hypoxia on corporal smooth muscle have shown an elevation in the intracellular Ca^{2+} levels (88) subsequent experiments showed a reduction in the intracellular Ca^{2+} following exposure to acidosis (151). The effects of glucopenia on intracellular Ca^{2+} have yet to be established. In conditions of hypoxia, acidosis and glucopenia, it is uncertain as to whether the overall Ca^{2+} will increase, decrease or remain unaltered. As mentioned in this discussion, the effects of hypoxia on intracellular Ca^{2+} levels using a variety of tissue remains variable. FURA2 loading techniques in tissue are not the optimum technique to ascertain transient Ca^{2+} fluxes. A better model to develop is one of isolated corpus cavernosum cells and utilise the technique to measure the Ca^{2+} transients in conditions of acidosis, hypoxia and glucopenia on the isolated cells. By depleting sarcoplasmic stores of Ca^{2+} using compounds such as caffeine and selectively inhibiting L-Type voltage gated channels using glibenclamide, we can investigate the effects of ischaemia on Ca^{2+} mobilisation from extracellular and intracellular stores. This would aid our understanding as to

whether Ca^{2+} transients in the corpus cavernosum are predominantly due to mobilization of intra or extracellular sources.

My experiments using putative agents failed to prevent irreversible smooth muscle dysfunction. However, a number of potential agents could still have a potential role in preventing smooth muscle dysfunction. Possible additional agents to those already mentioned include adenosine triphosphate –MgCl as we know that ATP depletion occurs during the period of simulated ischaemia. This compound would provide an additional source of energy synthesis.

Finally, the effects of ischaemia and reperfusion on corpus cavernosum could be further quantified biochemically by measuring the amount of lipid peroxidation and myeloperoxidase activity in the corpus cavernosum. This could be performed by homogenising the tissue following ischaemia and reperfusion or by collecting the superfusate at timed intervals in order to establish whether this is a time dependent phenomenon.

4.12 Conclusion

Although the incidence of low-flow priapism is rare in the general population, the paucity of research into this condition has led to both uncertainty and limited therapeutic options in the management of this condition. Currently the surgical management has been based on case series of differing corporal drainage operations. The pharmacological management using α-adrenergic agonists does have a solid scientific foundation but is not always successful. The long term outcome for patients failing these treatment options is the development of corporal fibrosis and ultimately

erectile dysfunction which is devastating for young men of reproductive age groups. The aim of this thesis was to gain a better understanding of the underlying pathophysiology mediating refractory low-flow priapism. I hypothesised that the exposure of the corpus cavernosum to prolonged hypoxia or acidosis alone would result in irreversible smooth muscle dysfunction. However, this was disproved. Using my findings from clinical patients, I found that the predominant factors mediating the irreversible dysfunction were hypoxia and glucopenia in combination. When these two parameters coexist, significant ATP depletion in the corpus cavernosum smooth muscle cells results in cellular death which eventually manifests as irreversible smooth muscle dysfunction.

5.Bibliography

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APPENDICES

Appendix A

Presentations and Lectures

British Society for Sexual and Impotence Research, London 2003

Effects of Acidosis and Hypoxia on Corporal Smooth Muscle Function.

A Muneer, S Cellek, S Minhas, DJ Ralph

Urological Research Society , London 2003

Effects of Prolonged Ischaemia on Corporal Smooth Muscle Function

A Muneer, S Cellek, S Minhas, DJ Ralph

British Association of Urological Surgeons annual meeting, Manchester 2003

The effects of ischaemia on corporal smooth muscle contraction and recovery using an in vitro pharmacological model.

A Muneer, S Cellek, S Minhas, DJ Ralph

European Society Sexual Medicine 2003 meeting Istanbul, Turkey

The investigation of cavernosal smooth muscle dysfunction in low flow priapism using an in vitro model

A Muneer, S Cellek, S Minhas, DJ Ralph.

The investigation of smooth muscle dysfunction in patients presenting with low flow priapism

A Muneer, S Cellek, S Minhas, DJ Ralph.

Treatment Options and Outcomes in Idiopathic Stuttering Priapism

A Muneer, P Kumar, JS Kalsi, S Minhas, DJ Ralph

The Fifth World Congress on Urological Research, London 2003

Invited Lecture

The Molecular Basis of Priapism

A Muneer

Urological Research Society, London 2004

Investigation of novel therapeutic options to prevent irreversible cavernosal smooth muscle dysfunction in low flow priapism

A Muneer, S Cellek, S Minhas, DJ Ralph

British Society for Sexual Medicine, London 2004

Investigation of novel therapeutic options to prevent irreversible cavernosal smooth muscle dysfunction in low flow priapism

A Muneer, S Cellek, S Minhas, DJ Ralph

Therapeutic Options and Outcomes in Idiopathic Stuttering Priapism

A Muneer, P Kumar, JS Kalsi, J Pryor, S Minhas, DJ Ralph

Effect of Hypoxia and substrate depletion on guinea pig corpus cavernosal smooth muscle

P Kumar, S Minhas, A Muneer, DJ Ralph, CH Fry

Invited Lecture

An update on ischaemic priapism

A Muneer

American Urological Association Annual Meeting, San Francisco 2004

The investigation of irreversible corpus cavernosum smooth muscle dysfunction in low flow priapism using an in vitro model

A Muneer, S Minhas, S Cellek, DJ Ralph

European Society of Sexual Medicine, London 2004

Investigation of novel therapeutic options to prevent irreversible cavernosal smooth muscle dysfunction in low flow priapism

A Muneer, S Cellek, DJ Ralph, S Minhas

BAUS Annual Meeting, Manchester, 2006

Investigation of novel pharmacotherapies to prevent irreversible smooth muscle dysfunction in ischaemic priapism

A Muneer, S Cellek, S Minhas, DJ Ralph

Appendix B

Published Abstracts

Muneer A, Cellek S, Minhas S, Ralph DJ. The effects of ischaemia on corporal smooth muscle contraction and recovery using an *in vitro* pharmacological model. BJU Int 2003; 92 (2):78

Muneer A, Kalsi J, Kumar P, Minhas S, Ralph D. Therapeutic options and outcomes in idiopathic stuttering priapism. Int J Imp Res 2003; 15 (6):S47

Cellek S, Muneer A, Kumar P, Minhas S, Ralph DJ. The investigation of smooth muscle dysfunction in patients presenting with low flow priapism
Int J Imp Res 2003; 15(6):S64

Muneer A, Cellek S, Minhas S, Ralph DJ. The effects of prolonged hypoxia and acidosis on corporal smooth muscle contraction.
BJU Int 2003; 92(7):833

Muneer A, Cellek S, Minhas S, Ralph DJ. The investigation of irreversible corpus cavernosum smooth muscle dysfunction in low flow priapism using an *in vitro* model. J Urol 2004; 171 (4):431.

Muneer A, Cellek S, Minhas S, Ralph DJ. Investigation of novel therapeutic options to prevent irreversible cavernosal smooth muscle dysfunction in low flow priapism. J Sex Med 2005; 2(1):43

Muneer A, Cellek S, Minhas S, Ralph DJ. Investigation of novel therapeutic options to prevent irreversible cavernosal smooth muscle dysfunction in low flow priapism. BJU Int 2006; 97(3):57

Appendix C

Publications to date

Muneer A, Cellek S, Dogan A, Kell PD, Ralph DJ, Minhas S. Investigation of cavernosal smooth muscle dysfunction in low flow priapism using an *in vitro* model. Int J Impot Res 2005; 17(1):10-18

International Journal of Impotence Research (2005) 17, 10–18
 0955-9930/05 $30.00
www.nature.com/ijir

Investigation of cavernosal smooth muscle dysfunction in low flow priapism using an *in vitro* model

A Muneer[1,2], S Cellek[2], A Dogan[3], PD Kell[1], DJ Ralph[1] and S Minhas[1]*

[1]*The St Peter's Andrology Centre, University College London, London, UK;* [2]*Wolfson Institute for Biomedical Research, University College London, London, UK; and* [3]*Department of Histopathology, University College London, London, UK*

(13 000 g, 4°C). A measure of 100 μl of the super- positive nuclei.

Appendix D

Patients Information sheet and consent form

Patient Information Sheet - CONFIDENTIAL
Investigators : Mr D J Ralph (Principal), Mr A Muneer, Mr S Minhas
Version: 3
Date: 11/7/03
Project ID:03/0158

Erectile Dysfunction in Priapism

Prospective tissue

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

We are conducting the above study in order to investigate why men presenting with prolonged erections (priapism) fail to respond to treatment.

Why have I been chosen?

As this condition is rare, you have been chosen because you are one of the few patients that we have seen with this condition.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What is involved in the study ?

Your decision to take part in the study will allow our investigators to analyse tissue specimens which were taken as a biopsy before you have a penile prosthesis inserted. The data collected will be anonymous and collected by the Investigators above. We will analyse the tissue to see whether specific features exist in all the similar cases like yours. This will help to define future treatment options in this condition.

What are the possible benefits of taking part?

Although there is no intended clinical benefit to yourself, the study will help to understand this condition more fully.

What information will be held?

All the information collected will be strictly confidential. The tissue samples will be coded so that your details are not passed on or stored in any way. We will use information such as the duration of your erection before your operation and the treatment given before the insertion of the penile implant. All the data will be collected, handled and stored at UCl and UCLH.

What happens to your tissue?

You should be aware that the tissue samples are being donated as a gift and may be used for future research. Any future research will be reviewed by the ethics committee.

What will happen to the results of the research study?

The results of the study will be published in a reputable urology journal.

Who is organising and funding the research?

The costs involved in the study to analyse the tissue will be paid by our own St Peters Andrology Fund.

Who has reviewed the study?

The study has been reviewed by the UCLH ethics committee.

Contact for further information

For further information please contact Mr A Muneer, Research Fellow Tel .

You will be given one copy of this information sheet and a signed consent form.

University College London Hospitals NHS
NHS Trust

CONFIDENTIAL

Centre Number:
Patient Identification Number:

UCLH Project ID number: 03/0158
Form version: 1.5/03

CONSENT FORM

Title of project: **Corporal Smooth Muscle Dysfunction in Ischaemic Priapism**

Principal investigator : Mr D J Ralph.
Additional Investigators : Mr A Muneer, Mr S Minhas
Department of Urology, Institute of Urology and Nephrology, UCLH

1. **I confirm that I have read and understood the information sheet dated 11/7/03 (version 3.) for the above study and have had the opportunity to ask questions.** ☐

2. **I confirm that I have had sufficient time to consider whether or not I would like to have my tissue samples included in the study** ☐

3. **I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.** ☐

4. **I understand that sections of my medical notes will only be looked at by the investigators named above.** ☐

5. **I give permission to allow my tissue samples to be used in the above study and I understand that they are being donated as a gift and may be used for future research in this Department.** ☐

University College London Hospitals NHS

NHS Trust

Centre Number:
Patient Identification Number for this study:

UCLH Project ID number: 03/0158
Form version: 1

CONSENT FORM - CONFIDENTIAL

Title of project: **Corporal Smooth Muscle Dysfunction in Ischaemic Priapism**

Name of Principal investigator : Mr D J Ralph

Name of patient	Date	Signature

Name of Person taking consent	Date	Signature

Mr A Muneer

Researcher (to be contacted if there are any problems)

Comments or concerns during the study

If you have any comments or concerns you may discuss these with the investigator. If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL hospitals. Please quote the UCLH project number at the top this consent form.

1 form for Patient;
1 to be kept as part of the study documentation,
1 to be kept with hospital notes