# Investigating the role of hydrogen sulfide in the myometrium

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By

Hayley Robinson

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Department of Cellular and Molecular Physiology
Institute of Translational Medicine

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## List of Abbreviations

ANOVA – Analysis of Variance

ATP - Adenosine-5'-triphosphate

AOAA - *O*-(carboxymethyl)hydroxylamine hemihydrochloride

AUC – Area under the Curve

BCA - β-cyanoalanine

$BK_{Ca}$ -- large conductance Calcium Activated Potassium Channels

Bp – base pairs

$Ca^{2+}$ - Calcium Ions

cAMP – Cyclic Adenosine Monophosphate

CBS - cystathionine β-synthase

CCE – Capacitative Calcium Entry

$CH_3SH$ - Methanethiol

$CH_3SCH_3$ - dimethylsulfide

CICR – Calcium Induced Calcium Release

$Cl^-$ - Chloride Ions

$Cl_{Ca}$ – Calcium Activated Chloride Channels

CSE - cystathionine γ-lyase

Cys - Cysteine

DAB - 3,3'-Diaminobenzidine

DADS - Diallyl disulfide

DAG – Diacylglycerol

DATS - Diallyl trisulfide

DMSO - Dimethyl Sulfoxide

DTB - Drug & Therapeutics Bulletin

DTPA - Diethylene triamine pentacetic acid

$EC_{50}$ - Half Maximal Effective Concentration

ECL - Enhanced Chemiluminescence

ET-1 - Endothelin-1

Ethe1 - ethylmalonic encephalopathy 1

FSH - Follicle stimulating hormone

HA – Hydroxylamine

HBSS – Hanks Balanced Salt Solution

hCG - Human chorionic gonadotropin

High $K^+$ - potassium chloride

HPLC – High performance Liquid chromatography

HRT - Hormone replacement therapy

$H_2S$ - Hydrogen sulfide

$HS^-$ - Sulfhydryl ion

ICC - interstitial cells of Cajal

IKCa – Intermediate Conductance Calcium Activated Potassium Channels

Indo-1AM – Indo-1 acetoxymethyl ester

IP3 - Inositol-1,4,5, triphosphate

IP3R - Inositol Triphosphate Receptor

$K^+$ - Potassium Ions

kDa – Kilo Daltons

$K_{ATP}$ – ATP-sensitive potassium channel

Kir- Inward Rectifier Potassium Channels

Kv –Voltage Gated Potassium Channels

$LOGEC_{50}$ – LOG Half Maximal Effective Concentration

LH - *luteinizing hormone*

M – Moles

mg – Milligrams

$Mg^{2+}$ - Magnesium Ions

$Mg_2SO_47H_20$ – Magnesium Sulfate

MLC –Myosin Light chain

MLCK – Myosin Light Chain Kinase
MLCP – Myosin Light Chain Phosphatase

mM – Millimolar

mRNA – Messenger Ribonucleic Acid

MRTB – Myometrial Research Tissue Bank

3MST - 3-mercaptopyruvate sulfurtransferase

mV – Millivolts

NaHS – Sodium hydrogen sulfide/ Sodium hydrosulfide

$Na_2S$ – Sodium Sulfide

$Na^+$ - Sodium Ions

NCX - Sodium Calcium Exchanger

NaCl - Sodium Chloride

NHS - National Health Service

$O_2$ - Oxygen

OTR – Oxytocin receptor

PAG - D,L propylargylglycine

$pH_i$ - intracellular pH

$pH_0$ - Extracellular pH

$PIP_2$ - Phosphatidylinositol 4, 5 Biphosphate

pKa - Dissociation Constant

PLC - Phospholipase C

PLP, Vitamin $B_6$ - Pyridoxal 5' Phosphate

PMCA - plasma membrane ATPase

PMT - Photo Multiplier Tube

$^{31}P$ NMR – Phosphorous nuclear magnetic resonance

RC0G – Royal college of Obstetricians and Gynaecologists

ROCC - Receptor Operated Calcium Channels

ROS - Reactive oxygen species

RyR - Ryanodine Receptors

$RS_nH$ – Hydropolysulfide

$R\text{-}S_n\text{-}R'$ – Organic poly sulphides

$S^{2-}$ - Sulfide ion

$S_2O_3^{2-}$ - Thiosulfate ion

$SO_3^{2-}$ - Sulfite

$SO_4^{2-}$ - Sulfate

SDS – Sodium Dodecyl Sulfate

s.e.m – Standard error of the mean

SERCA – Sarcoplasmic Reticulum Ca-ATPase

$SK_{Ca}$ – Small Conductance Calcium Activated Potassium Channels

SOCC – Store Operated Calcium Channels

SOCE – Store Operated Calcium Entry

SR – Sarcoplasmic Reticulum

STOC'S - Spontaneous Transient Outward Currents#

SUR - Sulphonylurea receptor subunit

TBS-T – Tris Buffered Saline Solution with Tween 20

Trp – Transient Recepter Potential Channels

TST – Thiosulfate Sulphur transferase

VOCC – Voltage Operated Calcium Channels

WHO – World Health Organisation

WHSL - Womens Health Specialist Library

# Investigating the role of hydrogen sulfide in the myometrium

Hayley Robinson

## Abstract

Preterm births are increasing worldwide; currently 7 % of UK births are preterm. Prematurity is the principal cause of neonatal mortality and a major cause of paediatric morbidity. Uterine contractility before term leads to pre-term labour. Therefore to reduce pre-term delivery, new pathways and drugs that inhibit uterine contractility are of interest. The gasotransmitter $H_2S$ has been shown to inhibit myometrial contractility without much mechanistic insight and thus is of potential interest. Hydrogen sulfide ($H_2S$) is produced *in vivo* from L-cysteine, by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). At least two enzymes degrade $H_2S$; thiosulfate sulphur transferase (TST) and ethylmalonic encephalopathy 1 (Ethe1). Thus $H_2S$ will be regulated within cells. NaHS, which releases a rapid bolus of $H_2S$, reduces myometrial contractility. However it is not clear if an $H_2S$-generating system is present throughout gestation or if more physiological modes of $H_2S$ production can affect contractility. Previous studies used NaHS as a $H_2S$ producer, which is toxic and releases $H_2S$ as a non-physiological bolus and thus alternative $H_2S$ donors suitable for drug development are sought. A new $H_2S$ generating compound, GYY4137, developed to slowly release $H_2S$ which better reflects physiological conditions, appears to be such a drug.

The aims of this work were to: (1) characterise the non-pregnant human myometrial tissue to determine the inherent spontaneous activity, to monitor whether the contractions were stable enough to assess $H_2S$ effects when compared to the term human myometrium. In addition, to monitoring changes in contractility in response to age, menopausal state and whether the women have endometriosis, (2) examine throughout gestation, the effects of GYY4137 on rat myometrial contractility, (3) investigate the differences in response to GYY4137 in non- pregnant versus pregnant human myometrium (4) compare GYY4137 effects to those of NaHS and L-cysteine in rat and human tissues, (5) elucidate the mechanism of $H_2S$ effects, and (6) determine the myometrial expression of enzymes governing tissue $H_2S$ levels.

Non-pregnant human myometrium gave rise to stable spontaneous contractions. The older women become the lower the amplitude and area under the curve of contractions. High $K^+$ depolarisations were also diminished with advanced age. As women reach post menopause contractions are found to decline when compared to pre-menopausal women. Women with endometriosis showed decreased amplitude with increased frequency of contractions when compared to their fertile, healthy counterparts. This finding suggested a potential involvement of altered myometrial activity in women suffering this condition.

NaHS, L-cysteine and GYY produce uterine relaxation in a dose-dependent manner using rat and human tissues. NaHS and GYY4137 effects increased throughout gestation using rat myometrial tissue, possibly due to changes in $H_2S$ removal rates. TST, a $H_2S$ breakdown enzyme was not detectable in different gestation rat as well as in non- pregnant and term pregnant human myometrial tissue, implying no

involvement.. Labouring rat myometrium however was not affected by either $H_2S$ producer. These data suggest that $H_2S$ contributes to uterine quiescence until labour onset. Term human myometrial contractions, both spontaneous and oxytocin-stimulated produced similar inhibitory responses to $H_2S$ producers NaHS and GYY4137. Non-pregnant spontaneous human contractions were unaffected on application of $H_2S$ producers. GYY4137 and L-cysteine decreased Ca transients, suggesting it affects L-type Ca channels, perhaps via sulfhydration of residues. These data were further supported upon use of BayK 8644 a calcium channel opener showing reduced effects of L-cysteine. $K_{ATP}$ channels were also shown to be involved in the mechanism of $H_2S$ in the myometrium as use of $K_{ATP}$ channel blocker glibenclamide caused reduced effects of the $H_2S$ producer GYY4137. These data suggest that $H_2S$ is an attractive target for therapeutic manipulation of human myometrial contractility and drugs such as GYY4137 will be effective. Both CBS and CSE are present in all the tissues tested in this thesis. It was also demonstrated that these enzymes were down regulated at term perhaps showing a role in preparing the myometrium for the onset of labour as the enzymes have been shown to further decline in labouring tissues.

In conclusion, the work I have undertaken in this thesis strengthens the evidence of a physiologically important role for $H_2S$ in the myometrium and suggests it targets ion channels to affect calcium signalling and thus contractions.

# Chapter 1 - Introduction

## 1.1The Uterus

### 1.1.1 Anatomy of the Uterus

*Human Uterus*

The uterus is a hollow, pear-shaped organ with thick muscular walls. It is situated between the rectum and the bladder. The uterus is separated into several regions and for descriptive purposes; it is divided into the fundus, body and cervix (**Figure 1.1**). The upper area of the uterus is the fundus. The fundus is a broad curved region which leads into the uterine tubes, or fallopian tubes terminating at the ovaries. Below the fundus is the body of the uterus, triangular in shape. Moving downwards to the lower, narrow part of the uterus called the cervix.

The human uterus is a single chambered simplex structure arranged to usually support singleton pregnancies (**Figure 1.1**). The layers of the uterine wall include the myometrium, endometrium, and perimetrium. The myometrium is located between the endometrium (the inner layer of the uterine wall) and the perimetrium (the outer uterine layer).

The myometrium is the layer under investigation within this thesis and is the middle layer of the uterine wall. The myometrium is the spontaneously active smooth muscle layer and consists predominately of poorly defined muscle fibre bundles arranged in circular (inner layer), longitudinal (outer layer) and diagonal orientation, held together by connective tissue. In portions of the human uterus however the arrangement of fibres is much more dispersed as the layers of muscle bundles are spread transversely, obliquely and longitudinally (Ramsey 1994). This layer accounts for the increase in uterine size during pregnancy and is responsible for the powerful contractions resulting in the expulsion of the placenta and the foetus during labour.

The endometrium is an internal mucous membrane lining which is adhered closely to underlying tissue throughout the uterine cavity and is non-excitable. During the menstrual cycle the endometrium grows to a thick, blood vessel-rich, glandular tissue layer to allow for successful implantation of the fertilised ovum. If this occurs the endometrium at site of implantation will then adapt to form part of the placenta.

If no embryo is implanted the endometrium will shed during menstruation. Changes within the endometrium are controlled by ovarian hormones, which lead to these distinctive alterations of the endometrium throughout menstruation.

The outermost layer of the uterus is the perimetrium, also called the serosa. It is part of the peritoneal epithelium and is a thin layer which covers the entire uterus. It is not known currently to influence the pharmacology of uterine contraction.

*Rat Uterus*

Uteri between species have different morphological characteristics which are related to function. In rats and other types of rodents the uterus is duplex and consists of two horns which meet at the uterine body above the cervix. This duplex uterus happily accommodates multiple embryos during pregnancy. However the cross sectional morphology of the rat uterus is similar to that of humans. This is separated into the same three distinct layers, the endometrium, the myometrium and the perimetrium. The rat uterus also consists predominately of poorly defined muscle fibre bundles arranged with an outer layer of longitudinal smooth muscle and an inner layer of circular muscle (**Figure 1.2**).

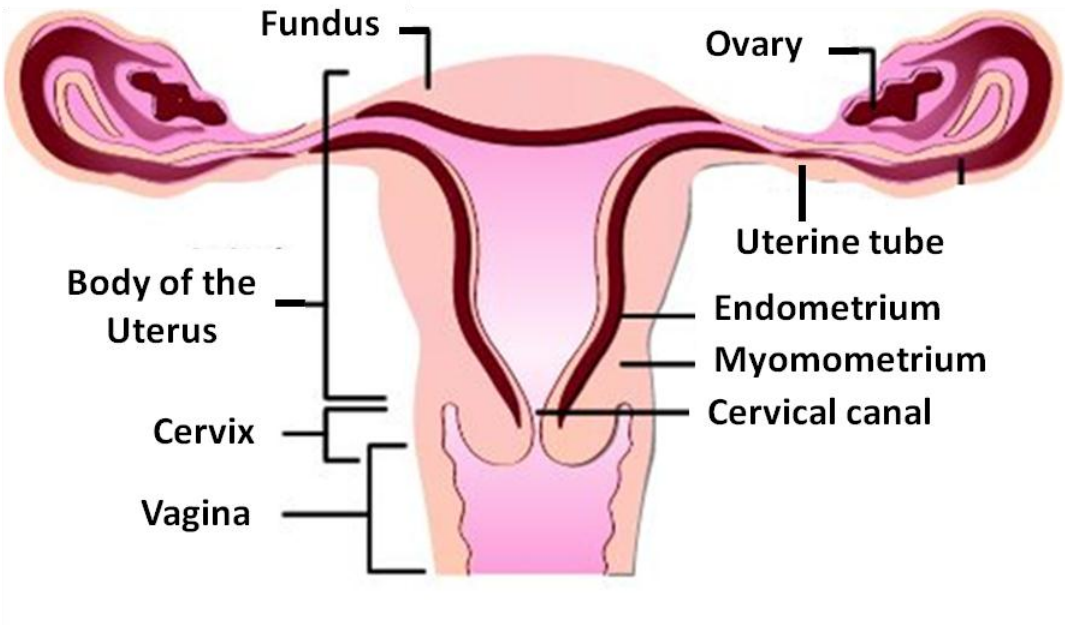



**Figure 1.1 Anatomy of the human uterus.** Adapted from the National Uterine Fibroids Foundation Website.

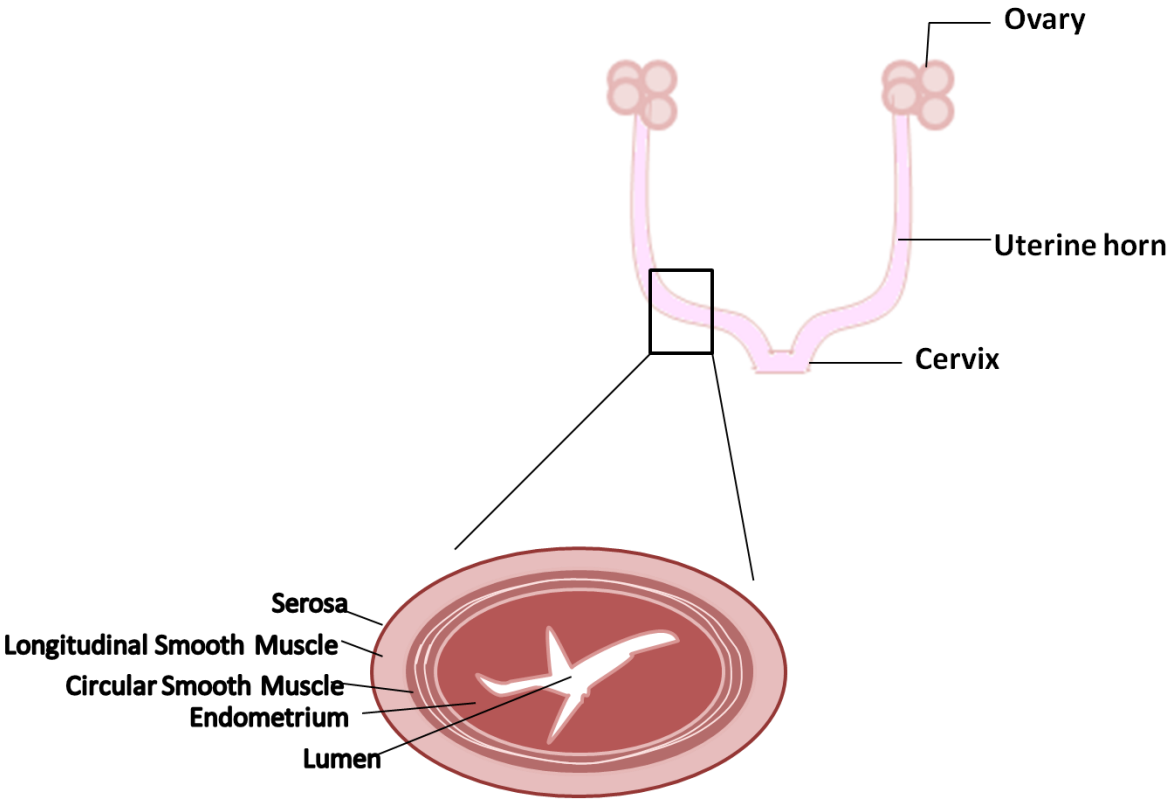



**Figure 1.2 Anatomy of the rat uterus**

### 1.1.2 Uterine Myocytes

The most dominant cell type within the uterus are smooth muscle cells of the myometrium (Young 2007). They are spindle- shaped cells with tapering ends and a single, oval-shaped nucleus located centrally. The smooth muscle cells range from 5-10µm diameter and 30-40 µm long in the non-pregnant uterus. The cells length varies between 300µm to 600µm during pregnancy (Broderick and Broderick 1990).

The generation of contractions is brought about by the interaction of actin and myosin within the myocytes. In relaxed muscle, the myosin cross bridges are detached from actin filaments. During contraction, they attach and provide the contractile force. The contractile machinery (myofilaments, intermediate filaments and dense bodies) occupy 80-90% of the total myometrial cell volume. However the cells also contain nuclei, mitochondria, Golgi bodies and a calcium store called the sarcoplasmic reticulum (Broderick and Broderick 1990).

Myocytes are excitable cells, producing co-ordinated contractions by acting as a syncytium (Wray 1993). This is achieved through cell to cell communication via gap junctions (Wray 1993). Gap junctions are specialised regions of the membrane that permit the rapid spread of electrophysiological activity. In humans and other mammals, gap junctions are scarce in the myometrium of the non-pregnant uterus (Kilarski 1998). Throughout pregnancy the number of gap junctions is low; they have poor coupling abilities and decreased electrical conductance results, favouring quiescence of the myometrium and maintenance of pregnancy. The number of gap junctions increases close to term to enable the rapid spread of electrophysiological activity between myocytes to produce co-ordinated contractions to give birth (Garfield et al 1978, Tabb 1992). The presence of gap junctions is thought to be regulated by the changing of oestrogens and progesterone levels in the uterus (Garfield 1998).

The plasma membrane of the uterine myocyte contains specialised regions called caveolae. Caveolae are omega shaped invaginations of the surface membrane. Within smooth muscle including the myometrium, a high density of caveolae are present, increasing the surface area of the membrane (Noble and Wray 2008).

Uterine caveolae have been indicated in a number of signalling pathways and have been shown in the uterus to have an effect on calcium signalling and contractility (Lee et al, 2001, Kendrick 2004; Smith, Babiychuk et al. 2005).

## 1.2 Myometrial cell excitability

### 1.2.1 Overview

The myometrium is a phasic smooth muscle that exhibits spontaneous and agonist induced contractions. The excitability of a tissue is governed, in part, by the resting potential. Several ions determine the resting membrane potential of myocytes allowing successful action potential generation; these include $Na^+$, $K^+$, $Cl^-$, and most importantly $Ca^{2+}$, the driving force for contractions in the uterus. Influx of calcium is driven by spontaneous changes in membrane potential (Wray 1993). Although many factors are involved in the production of contractions in the myometrium, the primary mechanism underlying phasic activity are those that allow spontaneous depolarisation of the myometrial cell membrane to occur.

### 1.2.2 Resting membrane potential and ion channel activation

Membrane potential is controlled by the distribution of ions across the plasma membrane. This ionic distribution facilitates contraction in myocytes. The ionic gradient within uterine smooth muscle is maintained such that the internal potassium ion content is high, and calcium content is low (Kao 1989). This allows for a quick response when small changes in permeability result in significant movement of ions. The resting membrane potential in the myometrium is estimated to range between -80 and -35mV (Kumar and Barnes 1961, Pressman 1988, Inoue 1990) but is dependent upon the species and the gestational status of the uterus (Parkington 1999). The majority of the work performed on membrane potential in the myometrium comes from Parkington and Coleman (Parkington & Coleman 2001). Contraction and relaxation of the myometrium occurs through the recurring depolarisation and repolarisation of the smooth muscle cell in the form of action potentials (Marshell 1962, Kao 1989). Upon depolarisation an influx of $Ca^{2+}$ occurs as well as $Na^+$. Repolarisation is caused by an outward flow of potassium ions. (**Figure 1.3**)

Depolarisation of the surface membrane is slow and has been associated with pacemaker cell activity; however the presence of any pacemaker cells in the myometrium remains to be elucidated (Shmygol, Noble et al. 2007). Cells known as ICC – interstitial cells of Cajal have been located in other smooth muscles such as urinary bladder and gastrointestinal smooth muscle, and play a pacemaker role (Sanders 2000; Sergeant 2000; Sui, Wu 2004) ICC-like cells have been found in the myometrium (Duquette, Shmygol et al. 2005) but it remains unclear whether they play a pace making role.

Ion channels are discrete membrane-spanning proteins that enable the selective transfer of ions across the impermeable, hydrophobic plasma membrane. The opening and closing of channels allows differences in ionic concentrations to be made and maintained, discriminating between different ions and enabling or excluding their passage on the basis of their charge and size. These ion channels are usually selectively permeable to only one ion or one type of ion and are generally named after the ion to which they allow passage e.g. $Ca^{2+}$ channels, $K^{+}$ channels. Ion channels open by specific changes in their local environment and can involve alterations in membrane potential/ voltage or binding of a specific agonist (Kim et al 2002). The main determinant to the change in transmembrane ion distribution is the expression of ion channels. However, electrochemical gradients also influence the total ion current. A larger electrochemical gradient for a given ion, gives a larger driving force and therefore a greater change in concentration per open channel.

Differences in cell membrane potential and thus changes in the gating of specific ion channels has dramatic consequences for the myometrial cell, in particularly this includes changes in the open probability and conductance of calcium and potassium channels. Upon membrane depolarisation, voltage-gated calcium channels open with a threshold for activation at -60 to -30mV and maximum current flow occurring at -30 to +10mV (Jmari 1986, Amedee 1987, Triggle 1998, Perez-Reyes 2003). Following the opening of these channels, an influx of calcium occurs. Calcium entry into the cell is the major component of the action potential upstroke and further potentiates plasma membrane depolarisation and increases the open probability of these channels (Wray 1993, Shmigol 1998, Wray 2003). Voltage-sensitive $K^{+}$ channels, at a more positive membrane potential, increases its opening probability.

Opening allows the efflux of potassium ions, responsible for the downstroke of the action potential and cell membrane repolarisation (Wray 1993, Wray 2003).

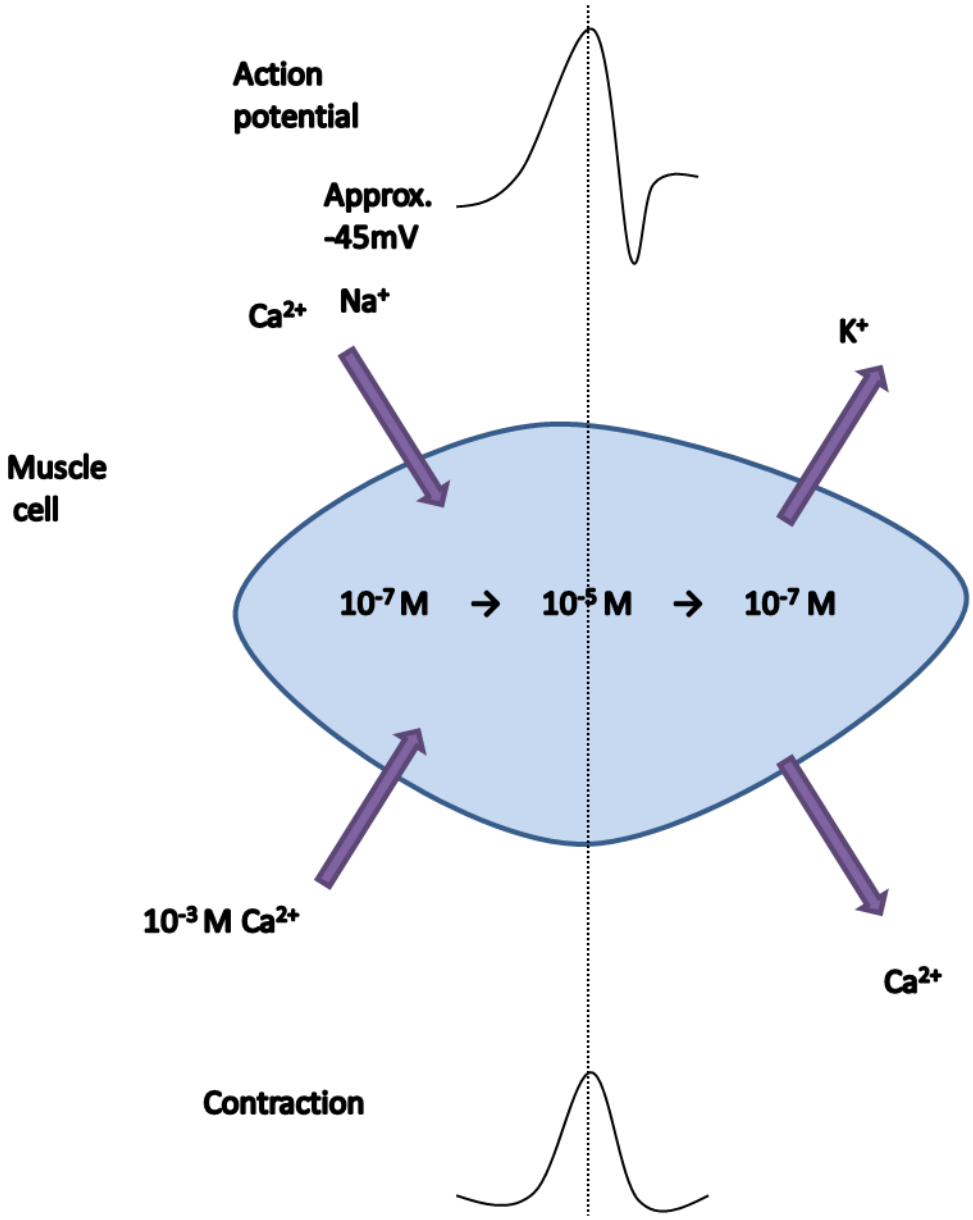



**Figure 1.3 Membrane potential, action potential and contraction** (adapted from (Garfield 1994). A rise in intracellular calcium (from $10^{-7}$ M to $10^{-5}$ M) initiates movements of ions down their electrochemical gradients.

### 1.2.3 Voltage-gated calcium channels

Voltage gated calcium channels (VOCC) are divided into three main groups $Ca_v1$ (L-type), $Ca_v2$, (P-type, N-type and R-type) and $Ca_v3$ (T-type) (Lipscombe, Helton et al. 2004). Two types of VOCC have been identified in the myometrium; L-type and T-type channels (Young 1993, Wray 2003, Lee 2009). They have a complex structure which is composed of four subunits. There is the pore forming subunit α, and three regulatory subunits β, γ, and $\alpha_2/\delta$ (Collins, Moore et al. 2000). The α subunit has been shown to be the subunit that forms the ion conducting pore and to carry the characteristic pharmacological and functional properties of the $Ca^{2+}$ channel for voltage sensing, ion permeability and drug binding whilst the others are classed as auxiliary subunits. Complete receptor function however requires the presence of all subunits and co-expression of the different β subunit isoforms with the same α subunit can result in trafficking of the channel to different membrane compartments within the cell. The extracellular α2 domain provides the structural support required for channel stimulation and is attached to the membrane through the membrane spanning δ subunit. The δ subunit influences voltage-dependent activation and steady-state inactivation and modulates the inactivation kinetics.

T-Type calcium channels comprise of three major α subunits - $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ (Blanks, Zhao et al. 2007). T-type calcium currents have been recorded in human myometrium (Young, Smith et al. 1993; Young and Zhang 2005). These currents are low voltage activated transients and have been implicated in initiation of action potentials in uterine smooth muscle (Young and Zhang 2005) as well as the generation and modulation of the frequency of spontaneous $Ca^{2+}$ transients (Lee 2009).

The main VOCC in the uterus are L-type calcium channels and are crucial for excitation-contraction coupling (Lipscombe, Helton et al. 2004). L-type calcium channels produce high voltage activated long lasting currents that flow through the primary calcium entry route $Ca_v1.2$ calcium channels, and account for the majority of the calcium current measured in the myometrium (Shmygol, Blanks et al. 2007). Calcium entry through L-type VOCC relies on membrane depolarisation, and is a key step for smooth muscle contraction. L-type calcium channels have been found to be

under hormonal control and alter during gestation suggesting a role in parturition (Young, Smith et al. 1993; Collins, Moore et al. 2000).

### 1.2.4 Potassium channels

The main function of potassium channels is to dampen cellular excitability by maintaining the cell membrane potential close to the reversal potential of $K^+$ ions (around -84mV). At this negative potential depolarisation is opposed by an outward $K^+$ current, which causes hyperpolarization or repolarization thereby terminating or rendering action potential generation and ultimately making contraction less probable (Khan 2001).

*Calcium activated $K^+$ channels*

There are three subclasses of calcium-activated $K^+$ channels, the large conductance channel ($BK_{Ca}$), the intermediate ($IK_{Ca}$) and the small ($SK_{Ca}$) conductance calcium-activated $K^+$ channels. All as suggested by their names are activated when intracellular $Ca^{2+}$ levels are elevated (Blatz & Magleby, 1987; Vergara *et al.* 1998).

$BK_{Ca}$ channels are present in the non-pregnant rat (Song 1999), non-pregnant human (Tritthart 1991) and pregnant human myometrium (Khan 1993). Activation of a small number of $BK_{Ca}$ channels has been associated with causing relaxation of the uterus (Brainard, Korovkina et al. 2007).Inhibiting $BK_{ca}$ with tetraethylammonium, barium and 4-aminopyridine thereby reducing the outward potassium current in human myocytes, produces an increase in the frequency of contractions. This supports the role of $BK_{Ca}$ channels in opposing contractility (Khan, Matharoo-Ball et al. 2001). Inhibition of BKca channels however has been shown to have little effect of myometrial contractility in pregnant and non-pregnant rat myometrium (Aaronson, Sarwar et al. 2006).

The $BK_{Ca}$ channels consist of a tetrameric pore forming α subunit, and a regulatory β subunit (Garcia-Calvo 1994, Toro 1998). The β subunit is required as it acts as the calcium sensor. Its ability to enhance $Ca^{2+}$ sensitivity in the human myometrium has been published (Orio et al 2002). Alternative splicing and post translational modifications are thought to result in functionally distinct variants of the receptor which also alter their calcium and voltage sensitivity (Korovkina 2001) hormonal

sensitivity (Holdiman 2002) and phosphorylation of the receptor and its trafficking (Perez and Toro 1994, Bainard 2007). In human myometrium $BK_{ca}$ with decreased sensitivity are up regulated during labour to allow the uterus to be more excitable (Curley, Morrison et al. 2004). In support, Gao et al 2009 also showed down regulation of both the α and the β subunits upon labour onset.

$IK_{Ca}$ channels have a similar structure consisting of a central pore, a calcium sensing region and 6 transmembrane spanning regions (Tharp and Bowles 2009). Unlike $BK_{Ca}$ channels they lack a voltage sensing region so are unaffected by membrane voltage. $IK_{Ca}$ channels play an important role physiologically as they are involved in many systems including the hematopoietic system, and salt and fluid transport. They are also important for maintaining a negative resting membrane potential to help accumulate electrical gradients for ion transport (Jensen, Strobaek et al. 2001; Begenisich, Nakamoto et al. 2004).

$SK_{Ca}$ channels are encoded by four genes – SK1, SK2, SK3 and IK1/SK4. The structure of SK channels is similar to voltage gated ($K_v$) potassium channels consisting of six transmembrane segments (S1-S6), and a selective pore region for potassium conductance (Stocker 2004). SK channels do not contain a calcium binding motif to allow direct interaction with calcium, however the proximal c terminal domain interacts with calmodulin (Maylie, Bond et al. 2004).

Activation of SK channels cause a long lasting hyperpolarisation known as a slow after hyperpolarisation (sAHP) (Vergara, Latorre et al. 1998). There is a role for SK channels in the myometrium as regulators of contractions during gestation and labour (Brainard, Korovkina et al. 2007). Over expression of SK3 in transgenic mice has been shown to delay parturition and reduce the strength of uterine contractions (Bond, Sprengel et al. 2000). Other studies have shown that the expression of SK2 can affect the coordination of uterine contractions by limiting calcium entry though L-type Ca channels (Brown, Cornwell et al. 2007). It has also been shown that SK2 and SK3 are down regulated in human myometrium during parturition (Mazzone and Buxton 2003). In the rat myometrium functional studies show upon inhibition of the this channel was larger than that of $BK_{Ca}$ channel inhibition suggesting a role for SK channels in maintaining uterine quiescence and may in fact contribute more than $BK_{Ca}$ channels.

*Voltage- gated $K^+$ channels*

Voltage dependent potassium channels (KV) are extensively expressed in the uterus and are activated by depolarisation (Brainard, Korovkina et al. 2007). Voltage gated potassium channels have been implicated in maintaining uterine quiescence and the initiation of contractions (Knock, Smirnov et al.1999). They also play a role in maintaining the resting membrane potential of myocytes (Brainard, Korovkina et al. 2007)

There are four major subfamilies of KV channels – KV1 (Shaker) , KV2 (Shab), KV3 (Shaw), KV4 (Shal) (Xu, Yu et al. 1995) The KV4 subfamily seem to play the most important role in the myometrium during pregnancy (Knock, Smirnov et al. 1999). The expression of KV4.2 has been shown to increase before parturition where as KV4.1 and 4.3 appear to decrease throughout gestation (Suzuki and Takimoto 2005). Hormones also affect KV channels. Oestrogen reduces the expression of KV4.3 and its function before parturition allowing increases in myometrial contractility (Song, Helguera et al. 2001). 17β-estradiol and progesterone have also been shown to modulate outward potassium currents in cultured human term myometrial cells (Knock, Tribe et al. 2001).

$K_{ATP}$ channels

The ATP-sensitive potassium ($K_{ATP}$) channel is one of the most abundant potassium channels and most likely contributes to the resting membrane potential in smooth muscle tissues (Teramoto N, 2006). The channel contains heteromultimers of an inwardly rectifying $K^+$ channel (Kir) and a modulatory sulphonylurea receptor subunit (SUR) which is responsible for the ATP sensitivity and pharmacological properties (Inagaki 1995, 1996, Aguilar-Bryan 1998, Isomoto 1996). The mRNA expression of Kir 6.1, Kir 6.2, SUR1 and SUR2B in human pregnant myometrium have been detected and Kir6.1/SUR2B appear to be the predominant isoform of $K_{ATP}$ channel in human myometrium (Curley, Cairns 2002). Another study found the expression of all four $K_{ATP}$ channel subunits in the pregnant myometrium. They found a reduction in the Kir 6.1 and Kir 6.2 subunits with no change in SUB2B within labouring tissue. This reduction could be related to labour onset (Curley, Cairns et al. 2002, Xu 2011)

The functional role of $K_{ATP}$ channels have been assessed through use of channel agonists and antagonists. $K_{ATP}$ channel openers including levcromakalim, diazoxide and pinacidil can inhibit spontaneous and oxytocin-stimulated contractions in isolated human myometrium (Morrison, Ashford et al. 1993). Other openers, also producing an inhibitory affect towards contractions includes diazoxide. Interestingly diazoxide was shown to be less potent in term labouring tissue compared to term non-labouring tissue, suggesting less of a role for KATP channels in labour and overlaps with the reduction in potassium channels expression, allowing for the onset of powerful contractions.

Conversely, $K_{ATP}$ channel blocker glibenclamide induces spontaneous uterine contractions (Bailie, Vedernikov et al. 2002). The effect of $K_{ATP}$ channel manipulation with drugs has variable results between pregnant and non-pregnant myometrium suggesting difference in expression during gestation. (Bailie, Vedernikov et al. 2002; Longo, Jain et al. 2003). Hypoxia has been associated with an increase in $K^+$ efflux from the myometrium which is glibenclamide sensitive suggesting involvement of $K_{ATP}$ channels. An increase in outward potassium current causes hyperpolarisation of the surface membrane and decrease excitability of the smooth muscle cells (Heaton, Wray et al. 1993).

### 1.3 The regulation of calcium [$Ca^{2+}$]i in myometrial contraction

#### 1.3.1 Overview

Contractions arise due to an elevation in intracellular calcium from $10^{-7}$M to $10^{-6}$M. This occurs through $Ca^{2+}$ influx via channels or through release from the internal store. The key mechanism of calcium entry to produce spontaneous contractions is through L-type calcium channels in the uterus. Although calcium can also enter the cell through receptor-operated calcium channels (ROCCs) and store-operated calcium channels (capacitative calcium entry). However inside the cell, $Ca^{2+}$ can be sequestered into the sarcoplasmic reticulum (SR) and/or extruded through the cell membrane by plasma membrane $Ca^{2+}$-ATPase(PMCA) and or $Na^{2+}/Ca^{2+}$ exchanger (NCX) (**Matthew 2004)**.

#### 1.3.2 Calcium influx mechanisms

Calcium influx can occur independent of depolarisation through non-specific cation channels and receptor operated channels (ROCC). Receptor operated currents have been described in a number of smooth muscle tissues with varying degrees of calcium selectivity (Wray 2005, Thorneloe and Nelson, 2005). In the myometrium, G-protein coupled receptors (GPCRs) are activated by a ligand and as a consequence the ligand binds to and activates a G protein leading to activation of second messengers or ion channels. Some receptors when activated stimulate myometrial contraction such as oxytocin, prostaglandin FP and TP receptors where as others such as β2-adrenoceptors and prostaglandin $EP_2$ promote relaxation. The above stimulators activate Gαq and the relaxors activate Gαs.

Oxytocin receptors (OTR) are relatively abundant in pregnant uterine tissues ( Gimpl&Fahrenholz, 2001). In human myometrium, the OTR protein was found to increase at term and after the onset of labour (Kimura 1996). In the rat uterus, the OTR mRNA levels were also increased at term and upon labour onset, the levels fell by 85% in within 24 hours following parturition (Larcher A 1995). Oxytocin binds to OTR's to increase internal $Ca^{2+}$. It does this by inhibiting $Ca^{2+}$ extrusion by suppression of $Ca^{2+}$-ATPase, opening L-type calcium channels and through activation of phospholipase C (PLC) liberating Inositol-1,4,5, triphosphate ($IP_3$) which releases internally stored $Ca^{2+}$ ions (Kao1989, Reimer&Roberts, 1986). Inhibition of calcium

entry highlights the limited role of the SR in the response to oxytocin where as inhibiting the SR reveals no change in the response of oxytocin on myometrial contractions (kupittayanant 2002). This elucidates the role of ROCC via its effect on L-type calcium channels.

Store-operated calcium channels (SOCCs) and chloride channels ($Cl_{Ca}$) have also been implicated in $Ca^{2+}$ entry. SOCC's are activated by a reduction in the sarcoplasmic reticulum (SR) calcium content, which results in calcium entry known as capacitative calcium entry (CCE) or store operated calcium entry (SOCE). This increase in calcium availability may contribute to contractility in the myometrium (Tribe, Moriarty et al. 2000). In the human myometrium CCE has been demonstrated in oxytocin stimulated increases in intracellular free calcium (Monga, Campbell et al. 1999). In the rat myometrium it has been shown that SOCE occurs and that it contributes to oxytocin-induced contraction (Noble 2009). The mechanism behind store operated calcium entry has been linked to transient receptor potential superfamily (Trp). TrpC1, TrpC3, TrpC4 and TrpC6 have been shown to be expressed in human myometrium (Ku, Babich et al. 2006), and are upregulated in term as well as labour suggesting a role in labour (Dalrymple, Slater et al. 2004).

As for chloride channels $ClCa_4$ are the most abundant form found in smooth muscle. Their role in water and salt balance as well as control of excitability has been published in rat uterine myocytes (Jones, Shmygol et al. 2004). $ClCa_2$ channels have been suggested to cause depolarisation via the opening of L-type calcium channels (Arnaudeau, Lepretre et al. 1994, Jones, Shmygol et al. 2004).

### 1.3.3 Calcium efflux mechanism

$Ca^{2+}$ is removed intracellularly in two ways, by the plasma membrane ATPase (PMCA) and the $Na^+/Ca^+$ exchanger. These mechanisms are ATP dependent and remove the bulk of calcium from the cell and if inhibited, the recovery of calcium to resting levels is abolished (shmigol 1999). It is the activity of these proteins that is responsible for the maintenance of the 10,000-fold concentration gradient across the plasmalemma (Matthew 2004). The NCX has a lower affinity for $Ca^{2+}$, but is a higher capacity system (Bradley 2002). It utilises the $Na^+$ gradient provided by Na/K-ATPase to operate the efflux of calcium. During each transport cycle, the energy from $3Na^+$ ions

entering the cells is used to export each $Ca^{2+}$. This reaction can occur in either direction dependent upon the concentration of $Na^{+}$ such that if extracellular $Na^{+}$ is significantly reduced, an increase in intracellular calcium occurs as the exchanger operates in reverse. The PMCA extrudes $Ca^{2+}$ at a lower $[Ca^{2+}]_i$ and is calmodulin dependent (Blaustein 2002). PMCAs affinity to calmodulin is dependent on its isoform. Within uterine cells inhibiting the NCX or PMCA showed 30% of the total calcium extruded was via the NCX and 70%, via the PMCA (Shmigol, 1998). This highlights the predominant mechanism responsible for calcium extrusion in the myometrium is the PMCA.

### 1.3.4 The sarcoplasmic reticulum (SR)

*$Ca^{2+}$ transport into the SR*

There are three mechanisms for removal of $Ca^{2+}$ from the cytosol; the NCX, PMCA, and the sarcoplasmic recticulum $Ca^{2+}$ ATPase (SERCA). The NCX and PMCA are both located on the plasma membrane and extrude $Ca^{2+}$ to the extracellular space and have been discussed earlier. The SERCA is located on the SR membrane, and pumps $Ca^{2+}$ to the SR lumen, which offers a finite capacity for storage (Matthew 2004). Active transport using the energy from ATP hydrolysis is how $Ca^{2+}$ is imported into the lumen of the SR. SERCA takes up $Ca^{2+}$ against the electrochemical gradient at the cost of the hydrolysis of one molecule of ATP (Marin 1999). Myometrial cells have an extensive SR network, which approaches very close to the plasma membrane. There are three isoforms of the SERCA pump, SERCA 1, SERCA 2 and SERCA 3 (Wray and Burdyga 2010). Within animal and human studies SERCA isoforms 2a, 2b and 3 have been identified and change in labour suggesting a potential role of SERCA in pregnancy and contraction (tribe 2000, Khan 1993b). The SR has a large capacity for storing calcium, however experiments have shown that when other calcium extrusion mechanisms are inhibited such as PMCA and NCX, SERCA was unable to act alone, suggesting that SERCA acts in conjunction with plasmalemmal calcium extrusion mechanisms (Matthew, Shmygol et al. 2004). Blocking of SERCA with cyclopiazonic acid CPA and thapsigargin causes an increase in contractility in the uterus (Tribe, Moriarty et al. 2000).

$Ca^{2+}$ within the SR is not uniformly distributed it is located in hot spots (Young&Mathur 1999). Two proteins involved in the distribution of $Ca^{2+}$ within the cell are $Ca^{2+}$ binding proteins; calsequestrin and calreticulin. In the uterine smooth muscle cells calreticulin acts alone as no presence of calsequestrin was found (Milner 1991).

*$Ca^{2+}$ release from the SR*

$Ca^{2+}$ is released from the SR by pores in the SR membrane formed by the tetramerisation of $IP_3R$ or ryanodine receptors (RyR). $Ip_3R$ are activated by agonists leading to the activation of $IP_3$ and diacylglycerol (DAG) from the hydrolysis of phosphoinositide-bis-phosphate ($PIP_2$). $IP_3$ causes release of calcium by binding to the $IP_3R$ on the SR (Bultynck 2003). RyR are activated by an increase in the local [$Ca^{2+}$] and inhibited by phosphorylation by PKC (Bonev 1997). In the uterus all three isoforms have been identified ($IP_3R$ 1- 3) (Morgan, De Smedt et al. 1996). In rat myometrium the expression of $IP_3Rs$ increased with advancing gestation, except for $IP_3R$-2, this increase in expression also coincided with an upregulation of the PLC cascade associated with the release of $IP_3$. In the uterus all three isoforms of RyR have been identified (RyR 1- 3). RyR receptors have not been shown to have any major effect on function (Taggart and Wray 1998, Noble 2009). Further work needs to be done to elucidate its role in the myometrium. Both channels are stimulated to open by the very ion they release, the opening of a single $IP_3R$ or RyR is likely to open adjacent SR channels. This process is called $Ca^{2+}$ induced $Ca^{2+}$ release (CICR). CICR inhibitors have been shown to reduce contractility in the myometrium (Phillippe and Basa 1996). Although there is little evidence that shows CICR has a role in the uterus (Kupittayanant 2002).

Calcium has also been shown to leave the SR in the form of a calcium leak. The SR's calcium load comes from the uptake of calcium by the sarcoplasmic reticulum ATPase (SERCA). The calcium load of the SR is kept at a steady state by release of calcium through vectoral calcium release. Studies of smooth muscle have shown evidence of these releases in the form of calcium sparks from RyRs and Ca puffs from $IP_3Rs$ (Ledoux 2008, Tovey 2001). $Ca^{2+}$ sparks occurring from RyR opening have been shown to target the $Ca^{2+}$-activated $BK_{Ca}$ channels on the plasma membrane. The

sparks increase the local [$Ca^{2+}$] around $BK_{Ca}$ channels and activate them giving rise to spontaneous transient outward currents (STOCs), i.e. small membrane hyperpolarisations. In turn this leads to a decrease in L-type calcium channel opening, a reduction in $Ca^{2+}$ entry and relaxation (Burdyga and Wray 2005). This mechanism has been demonstrated in vascular (Nelson 1995) and utereric (Burdyga and Wray 2005) smooth muscle. Although $BK_{Ca}$ channels are present in the uterus, Calcium sparks and STOCs have yet to be discovered in the uterus (Wray 2007).

### 1.3.5 Excitation-contraction coupling mechanism

Excitation-contraction coupling relies on changes in membrane potential of the smooth muscle cells and intracellular calcium concentrations. Contractions occur upon depolarization of the surface membrane opening L-type $Ca^{2+}$ channels, followed by an influx of $Ca^{2+}$ entry into the cell (**Figure 1.4**). Depolarisation occurs spontaneously (Duquette 2005) or via opening of channels or due to hormones binding to receptors (Miyoshi 2004, Arnaudeau 1994). $Ca^{2+}$ then binds to plasma membrane calmodulin which dissociates forming a calcium-calmodulin complex containing 4 $Ca^{2+}$ ions. This activates myosin light chain kinase (MLCK) causing phosphorylation of serine 19 on myosin light chains (MLC), and triggers the cycling of myosin cross bridges with actin leading to contraction (Wray 2007; Aguilar and Mitchell 2010, Webb 2003). Consequently, a fall in $Ca^{2+}$ concentration through uptake into the intracellular calcium store, and efflux through plasmalemmal calcium extrusion mechanisms, results in the dephosphorylation of myosin by myosin light-chain phosphatase, deactivation of myosin light chain kinase and dissociation of the calcium-calmodulin complex leading to relaxation (Allen 1994, Webb 2003).

Agonists can cause contraction independent of membrane potential changes through release of calcium from the sarcoplasmic reticulum, the entry of calcium through receptor operated channels, or through alterations of the contractile proteins sensitivity to calcium. This is called pharmacomechanical coupling (Wray 1993).

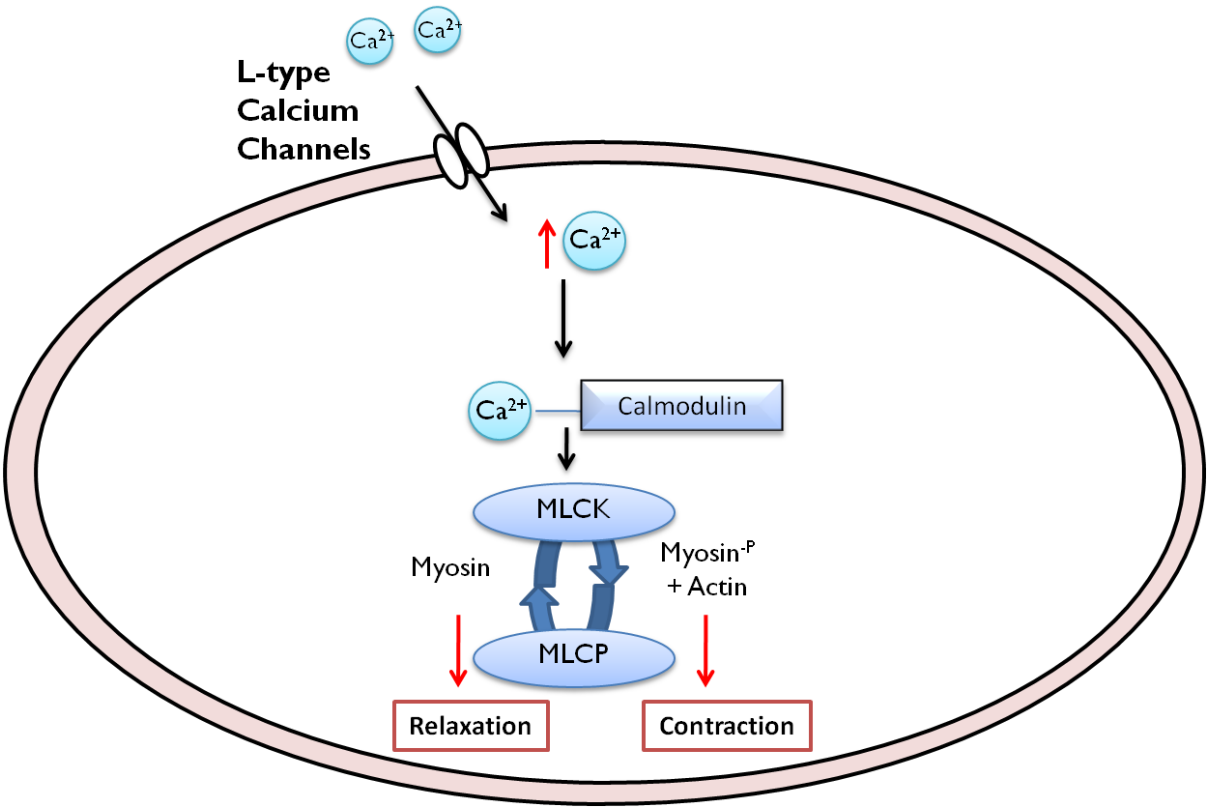



**Figure 1.4 Excitation-contraction coupling pathway in the uterus.**

Action potentials are a result of changes in ionic permeability of the myocyte membrane. Upon depolarisation of the myocyte membrane, L-type calcium channels open and allow calcium influx. Calcium then binds to calmodulin, activating myosin light chain kinase (MLCK), which phosphorylates myosin (Myosin-P) causing cross bridge cycling of myosin and actin and therefore promoting contraction. When dephosphorylation takes place producing myosin light chain phosphatase (MLCP), this leads to relaxation.

### 1.3.6 pH and Contraction

Each contraction causes a small acidification. Uterine pH was first measured by Dawson and Wray in 1985. A value of ~7.1 at 37$^{o}$C was found in pregnant and non-pregnant rat uteri using $^{31}$P NMR spectroscopy. Changes in external pH are transmitted to the cytoplasm where they may affect cellular function (Wray 1993). These changes occur due to physiological processes such as increased activity, hypoxia, respiratory effort and acid base imbalance. The metabolic demands of labour and changes in metabolites, as described previously during contractions, will lead to changes in intracellular pH (pHi). Normal labour is associated with the development of maternal acidemia (Sjosted 1962, Cerri 2000).

A significant alkalinisation has been shown to occur when measuring intracellular pH over the last few weeks of pregnancy, from pH 7.07 (33 weeks gestation), pH 7.12 at 36-37 weeks to pH 7.26 at term (40-43 weeks) (Parratt 1995B). Also the mean value of resting pH, was found to be significantly lower in the non-pregnant women (7.06 ± 0.03; n = 39) when compared to pregnant women (7.14 ± 0.01, n=53)(Parratt 1995A). Direct measurements of intracellular pH in human myometrial tissue has shown that application of a weak acid and base (30mM) produces intracellular pH (pHi) changes of around 0.14pH units (Parratt 1995A) and that changes occur rapidly (<1 minute) as the weak acids and bases cross the cell membrane and dissociate. Intracellular pH is then gradually restored as pH regulatory mechanisms come into play. The intracellular pH change occurring is dependent on concentration of the weak acid or base.

Alteration of pHi in human myometrial strips have been shown to have significant effects on spontaneous contractions (Parratt 1995A and B) such that acidification decreases and alkalinisation increases, spontaneous force and calcium. This is also found in the rat myometrium (Taggart 1993). Intracellular acidification can directly reduce $Ca^{2+}$ current in uterine myocytes and has been shown to directly inhibit force production at the level of cross bridge cycling (Nagesetty&Paul 1994, Smith 1998, Pierce 2003).

Most of the effects of extracellular pH ($pH_0$) are thought to arise from the consequent change in intracellular pH, as $H^+$ diffuse across the plasma membrane and into the cell. The effects however will be slower and probably to less of an extent than direct intracellular pH change. $pH_0$ has been shown to influence L-type $Ca^{2+}$ entry as does pHi (Smirnov 2000, Saadoun 1998, Pierce 2003) and Shmigol 1995 also showed a slow reduction in current when lowering extracellular pH to 6.9. Pierce et al shows upon acidification using changes in $pH_0$ show an increase in frequency and decrease in amplitude were as an alkalinisation produced an increase in force and a decrease in frequency (Pierce 2003).

pH changes during labour, induced via hypoxic events, will have significant effects on the contracting uterus and may affect the progress of the labour. Recently a test based on the work done by Liverpool university and Liverpool women's hospital showing that the uterus produced lactic acid as other muscles do when they work hard, but that when it reaches a certain level the substance starts to inhibit contractions, was established. The test is thought to help doctors monitor lactic acid and determine which women may go on to deliver vaginally and this lactic acid build up could be a reason why some slow labouring women do not respond to induction of labour through use of oxytocin.

## 1.4 Hydrogen sulfide ($H_2S$)

$H_2S$ is well known for its rotten egg smell and toxicological effect, a result of its reaction with metalloenzymes. In the mitochondria, cytochrome oxidase, the final enzyme in the respiratory chain, is inhibited by $H_2S$; this disrupts the electron transport chain, interferes with ATP production and impairs oxidative metabolism (Chou 2003, Reiffenstein 1992). $H_2S$ has a recognition threshold of 0.0047 ppm, at this concentration only 50% of humans can detect the characteristic odour and at ~ 500ppm it can be fatal. However in recent years research has focused on $H_2S$ as a gaseous signalling molecule (Zhao 2001, Mustafa 2009) and its physiological contributions. $H_2S$ has a growing list of functions e.g. long term potentiation (Abe 1996), anti-nociception (Distrutti 2006), exocrine secretion (Schicho 2006) and control of blood pressure(Yang 2008) which has led to the suggestion that $H_2S$ is poised to have a large impact on physiology and medicine. Data from studies on inflammation, atherosclerosis, hypertension and ischaemic injury indicate that it can be cytoprotective especially against oxidative stress and will be useful therapeutically (Szabo 2007). However, it has been associated with diseases such as diabetes and asthma (Yusuf 2005). It can act as an oxygen sensor and is part of the vascular response to hypoxia (Olson 2006) and inhibits production of reactive oxygen species (ROS) leading to increased tissue function (Muzaffar 2008). The reduced cardiovascular risk associated with garlic eating seems to be due to the production of $H_2S$ from polysulfides in the plant (Benavides 2007).

### 1.4.1 $H_2S$ chemistry and compounds used to investigate the effects of $H_2S$

$H_2S$ is a extremely reactive, flammable, colourless gas. It is the sulfur analogue of water, but is somewhat less polar, giving it the ability to dissolve well in both polar and non-polar environments. The gaseous state and lipophilic nature allows $H_2S$ to dissolve into cell membranes and thus no transporters are needed for it to enter or leave the cell (Mathai *et al.*, 2009). $H_2S$ is also a weak acid, and in aqueous solution dissociates according to **equation 1** (**Figure 1.5A**) with pKa 7.05, pH 7.4 at 20°C giving rise to ~30% $H_2S$ and 70% $HS^-$ (Lide, 1998).

Under physiological situations, pH 7.4 and 37°C, give a distribution of about 20% $H_2S$ and 80% $HS^-$. It should be emphasised that $H_2S$ and $SH^-$ may both contribute directly to the biological action of hydrogen sulfide, and that $SH^-$, the predominant sulfide species under biological conditions, is a nucleophile, which readily binds to metal centres in biological molecules (e.g. haemoglobin) or reacts with other compounds. Only the first reaction within equation 1 is relevant for biological samples because the pKa is in the physiological range. The pKa2 for the second reaction is over 12, with some reports suggesting it is as high as 19. $S^{2-}$ is negligible in all physiological experiments (Olson 2012).

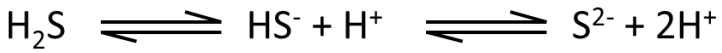
$H_2S$ is a strong reductant, and can spontaneously oxidize to sulfur dioxide or elemental sulfur (among other possibilities) as shown in Equations 2 and 3, respectively (**Figure 1.5B)**. These reactions are not particularly fast in the absence of catalysts, with halftimes on the order of 30 hr (Millero, 1987). In the presence of transition metal ions, such as $Fe^{2+}$, the oxidation rate is greatly increased, and addition of the transition metal chelator diethylene triamine pentacetic acid (DTPA) to an $H_2S$ solution effectively blocks this route of oxidation (Tapley, 1999).

$H_2S$-forming salts are the first compounds used to monitor $H_2S$ effects. However, they require protons from a solvent and their solvation results in an alkaline solution, such as sodium sulfide ($Na_2S$) (**Figure 1.5C)** which requires 2 protons (Olson 2012). Sodium hydrosulfide (NaHS, **Figure 1.5C**) only requires one proton and is commonly used as an $H_2S$-forming compound, since it dissociates instantaneously to $Na^+$ and $HS^-$; the latter then partially binds $H^+$ to form undissociated $H_2S$ (Lowicka 2007). However, as compounds that can release more physiological amounts of $H_2S$ are sought to better control $H_2S$ quantities and monitor its effects, $H_2S$ releasing compounds have been and are still being produced. One drug that slowly releases $H_2S$ following addition to aqueous solution due to its steric hindrance, is GYY4137 (**Figure 1.5C**). The likely mechanism of producing $H_2S$ is through protonation of the sulfide group to form a sulfhydryl moiety followed by hydrolysis to release $H_2S$ (Li 2009). Lee et al confirmed $H_2S$ production, illustrating upon incubation of NaHS and GYY4137 in culture medium using a methyl blue formation assay resulted in the release of measureable amounts of $H_2S$. Liberation of $H_2S$ from NaHS was rapid with

plateau from 30-50 mins and declining to undetectable levels by 90 min. $H_2S$ release from GYY4137 was 10% of that observed with NaHS but was sustained, remaining higher than baseline for up to 7 days. Both $H_2S$ production enzymes Cystathionine beta synthase (CBS) and cystathionine gamma lyase (CSE) use L-cysteine (**Figure 1.5C**) as a substrate to produce $H_2S$ (described below), hence this has been used to investigate $H_2S$ effects also. However, L-cysteine is an important amino acid that protects stomach lining, the intestines and helps in the absorption of essential nutrients from foods also. It is naturally occurring and can be found in most protein rich foods ranging from dairy to poultry products. Naturally occurring garlic derived compounds maybe also a good choice of compound to monitor $H_2S$ effects as human red blood cells convert garlic-derived organic polysulfides into $H_2S$ (Benavides 2007). Allyl-substituted polysulfides undergo nucleophilic substitution at the α carbon of the allyl substituent, thereby forming a hydropolysulfide (RSnH), a key intermediate during the formation of $H_2S$. Organic polysulfides (R-Sn-R') also undergo nucleophilic substitution at a sulfur atom, yielding RSnH and $H_2S$. Diallyl disulfide (DADS), and Diallyl trisulfide (DATS) are the highest producing $H_2S$, garlic-dervied polysulfides producing approximately 35 and 100 µM $H_2S$, respectively on the addition of 100 µM of each polysulfide (Benavides 2007).

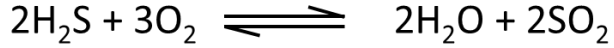
NaHS has been reported to relax different vascular tissues with $EC_{50}$ of 1-300 µM (Olson 2010). Another report described an $EC_{50}$ value of 0.7mM term non labouring myometrium and 2mM for term labouring tissue using L-cysteine (You 2011). GYY4137, a slow releasing $H_2S$ compound has been shown to relax contraction in aortic rings with an $EC_{50}$ of 115 µM (Li 2008). This shows that there is considerable inter-tissue differences in $EC_{50}$ values, although experimental differences may account for much of the variation.

A

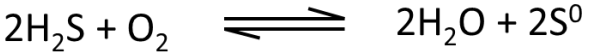



Equation 1

B



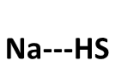

Equation 2



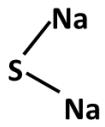

Equation 3

C

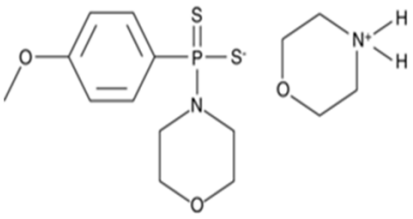
**Sodium Hydrogen Sulfide**



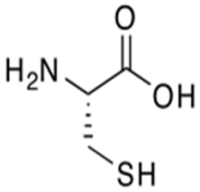

**Sodium sulfide**



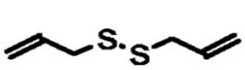

**GYY4137**




**L-cysteine**




**DADS**




**DATS**

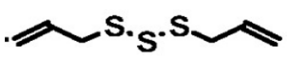



**Figure 1.5 Dissociation of $H_2S$ (A), reduction of $H_2S$ (B) and $H_2S$-forming compounds (C).**

### 1.4.2 Endogenous $H_2S$ production

Hydrogen sulfide is produced in mammalian tissues from L-cysteine by two key enzymes CBS and CSE. Another enzyme that produces $H_2S$ is 3-mercaptopyruvate sulfurtransferase (3MST), although this enzyme seems to be the more important source of $H_2S$ in the brain (See **Figure 1.6**, Ishigami 2009). Alterations in CBS and CSE activity have been associated with functional effects in a variety of tissues (Hosoki 1997). The expression of CSE has been shown to be higher than that of CBS in several smooth muscles (Zhao 2001, Hosoki 1997). Both the CSE and CBS enzymes are expressed in rat and human myometrium (Patel 2009, You 2011). There is also the presence of a minor non-enzymatic route via reduction of elemental sulphur to $H_2S$ using reducing equivalents obtained from the oxidation of glucose. An environment where oxidative stress and hyperglycaemia is high promotes $H_2S$ generation through this route (Searcy 1998).

CBS depends on pyridoxal 5' phosphate (PLP, vitamin $B_6$). This enzyme catalyses a β-replacement reaction with amino acid substrates, including L-cysteine, 3-chloroalanine and serine. Homocysteine, 2-mercaptoethanol and $H_2S$ are nucleophile substrates, allowing for other possible reactions to take place (Miles &Kraus 2004). CBS can catalyse the β-replacement of the sulfhydryl group on cysteine with water, releasing serine and $H_2S$ but traditionally on the sulfhydryl group of homocysteine with the hydroxyl of serine forming cystathionine (Julian 2002). CBS is a homotetramer consisting of 551-amino-acid subunits with a subunit molecular weight of ~63KDa which bind two co-factors (haem and PLP) and two substrates (homocysteine and serine). Several isoforms have been identified although their functional effect of them remains unknown (Singh 2009). The haem component of CBS is reported to function as a cellular redox sensor (Maclean 2002) which could increase $H_2S$ generation in response to oxidative stress (Whiteman 2011). Two inhibitors are available in the form of *O*-(carboxymethyl)hydroxylamine hemihydrochloride (AOAA) and hydroxylamine (HA).

CSE also depends on PLP and is a 405-amino-acid protein consisting of a tetramer formed by two homodimers, with active and stable dimer of ~45KDa (Sun 2009, Whiteman 2011). CSE catalyses the α,γ-carbon elimination of cystathionine to

produce cysteine, α-oxobutyrate and ammonia. Additional cysteine-dependent β- and homocysteine-dependent γ-reactions have been suggested (Chiku 2009) to generate $H_2S$. Additionally CSE may catalyse the β-elimination of cystine (cysteine disulfide) via the formation of thiocysteine, which then decomposes non-enzymatically to $H_2S$ (Chiku 2009). The CSE-catalysed α,β-elimination of cysteine was the predominant source of $H_2S$ accounting for ~70% of the $H_2S$ produced (Chiku 2009). At least two CSE mRNA splice variants have been demonstrated producing long and truncated CSE proteins, although the precise role of these variants in regulating CSE activity has yet to be disclosed (Lu 1992, Levonen 2000). A condition called hyperhomocysteinemia is when there is excess $H_2S$ production (Carson 2010). Two CSE enzyme inhibitors are available, reversible inhibitor, β-cyanoalanine (BCA) and irreversible inhibitor, D,L propylargylglycine (PAG).

Little information is available on 3MST and $H_2S$ synthesis. 3MST is a ~33 kDa monomeric or disulfide-linked dimeric protein containing two rhodanese domains. At least two splice variants of human 3MST are present, but as with CSE and CBS, their regulation and role in $H_2S$ synthesis are not understood and no inhibitors exist (Whiteman 2011).

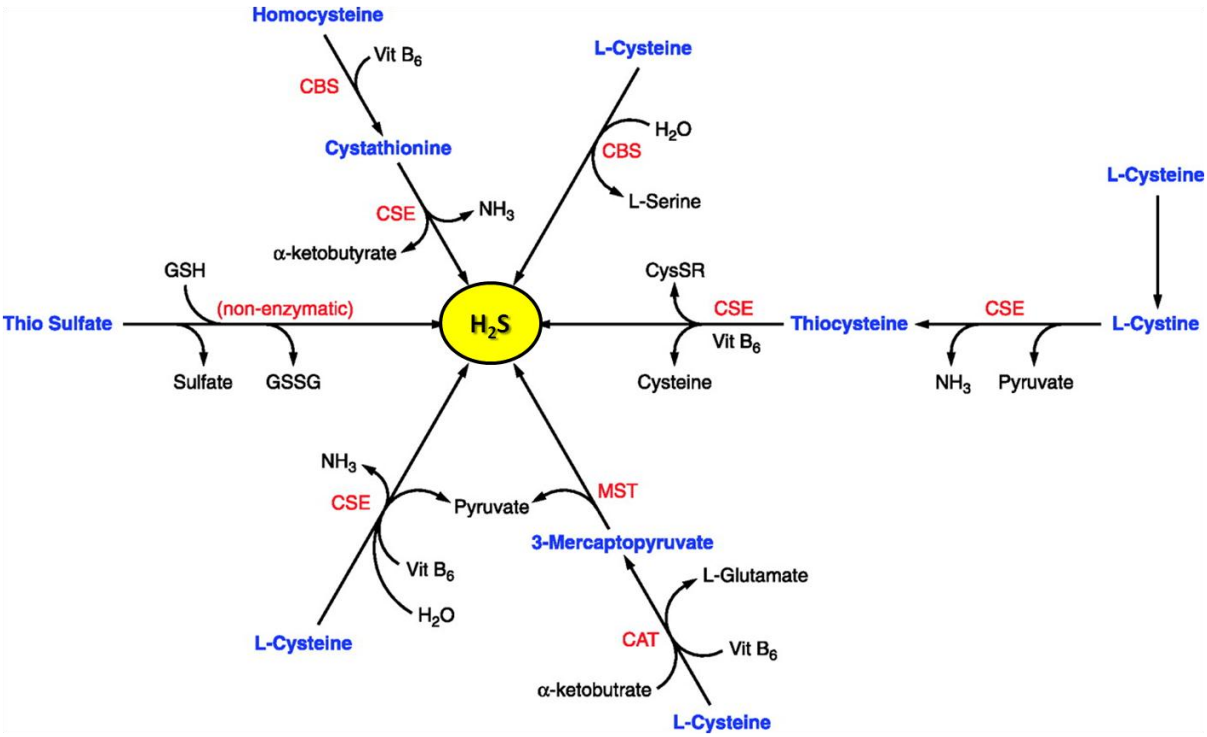



**Figure 1.6 Production of $H_2S$ in mammalian cells. Adapted from Wang 2012.**

CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CAT, cysteine aminotransferase; 3MST, 30mercaptopyruvate sulfurtransferase.

### 1.4.3 $H_2S$ catabolism

*Oxidation*

Oxidation is the loss of electrons or an increase in oxidation state by a molecule, atom, or ion and is the main way to catabolise $H_2S$. It was first reported in 1986 that sulfide oxidation occurred in the mitochondria (Powell 1986). $H_2S$ is rapidly oxidized to thiosulfate ($S_2O_3^{2-}$) by mitochondria and is subsequently converted to sulfite ($SO_3^{2-}$) and sulfate ($SO_4^{2-}$) (Caliendo 2010). The oxidation of $H_2S$ to thiosulfate in the rat liver is influenced by heme compounds (Sorbo 1958, Sorbo 1957), metal protein compounds and ferritin (Wang 2012). The overall oxidation process take place using membrane-bound sulfide-quinone oxidoreductase (SQR) to oxidize $H_2S$ to a persulfide ($S^0$), the electrons are used to reduce ubiquinone, which then enters the electron transport chain. Subsequently, sulphur dioxgenase further oxidizes a persulfide to sulfite, consuming $O_2$ and water. The final steps are converting sulfite to thiosulfate by the sulfur transferase-catalysed transfer of a second persulfide from SQR and excretion of thiosulfate. Thiosulfate is converted to sulfite by thiosulfate sulfurtransferase and sulfite is then further metabolized to sulfate by sulfite oxidase (stipanuk 2010, Olson 2011, Linden 2012). The production of sulfite and sulfate is catalysed by the sulfide-detoxifying enzymes, one of which is Rhodanese (Picton 2002). Rhodanese consists of two isoforms thiosulfate sulfur transferase (TST), the isoform that detoxifies $H_2S$ to sulfite and 3MST which cannot (Ramasamy 2006). Mice lacking ethylmalonic encephalopathy 1 (Ethe1) exhibit elevated sulphide levels, suggesting that Ethe1 is the sulfur dioxygenase involved in $H_2S$ metabolism (Linden 2012). Both Ethe1 and Rhodanese are highly expressed in the liver (Hildebrandt and Grieshaber 2008). $H_2S$ is a powerful reducing agent and is likely to be consumed by endogenous oxidant species in the vasculature also, such as peroxynitrite (Whiteman 2004), superoxide (Chang 2008), and hydrogen peroxide (Geng 2004).

*Methylation*

Primarily methylation takes place in the cytosol. Using thiol S methyltransferase, methylation of $H_2S$ generates smelly gas methanethiol ($CH_3SH$) and then this can slowly be converted to dimethylsulfide ($CH_3SCH_3$) through a second methylation. It

has been shown that oxidation of $H_2S$ happens ~10,000 faster than sulfide methylation (Levitt 1999).

*Scavenging*

$H_2S$ can be scavenged by methemoglobin to form sulfhaemoglobin and is thought to have a short half life (Wang 2012). There is evidence that shows in the presence of methemogloblin, $H_2S$ production is decreased (Yang 2004). Metallo- and disulfide-containing molecules such as horseradish peroxidise and oxidized glutathione can also scavenge $H_2S$ (Smith 1966, Beauchamp 1984).

### 1.4.4 Tissue and blood levels of $H_2S$

Much controversy has been associated with monitoring $H_2S$ production and blood levels. Tissue production and levels of '$H_2S$' in blood has been the subject of much controversy (reviewed in (Whiteman 2009, Olson 2012). $H_2S$ levels in blood serum and plasma of healthy adults have been measured using the methyl blue method and through use of sulfide sensitive electrodes of 20-60µM (Whiteman 2009, Lawrence 2000, Yang 2008, Branceleone 2008). More recently fluorimetric-based methods have been developed employing monobrombimane to trap 'free' $H_2S$ and the resulting dibimane determined by reverse-phase HPLC to show baseline levels of free '$H_2S$' to be in the region of 0.4–0.9 µM (Wintner, 2010). It is therefore likely that other methods measure the total sum of $H_2S$-derived species such as $HS^-$ and $S^{2-}$ and possibly other physiological $H_2S$ 'carrier' molecules that exist at physiological pH and which release $H_2S$ under acidic conditions employed in the analytical processes, rather than 'free' $H_2S$ itself and care should be taken to describe the results as such (Whiteman 2009).

### 1.4.5 $H_2S$ in vascular smooth muscle

Both the CBS and CSE enzyme have been detected in many smooth muscles tissues including airway (Ryu 2009), aorta and mesenteric arteries (Cheng 2004). CSE is the predominant enzyme responsible for $H_2S$ generation in vasculature smooth muscle (Zhao 2001), whereas both CBS and CSE contribute to $H_2S$ generation in gastrointestinal and penile smooth muscle (Fiorucci 2005, d'Emmanuele di Villa Bianca 2009). These data confirm that $H_2S$ generation could play a significant role in smooth muscle.

Modulation of smooth muscle activity has been described as one of the main functions of $H_2S$ in peripheral tissues (Moore 2003). The effects of $H_2S$ on smooth muscle contractile activity have been examined in several tissues and in general it is associated with a reduction in contractile activity. Relaxation has been reported for bronchial (Kubo 2007), GI tract (Teague 2002, Gallego 2008, Dhaese 2009), corpus cavernosum (Shukla 2009) and bladder (Dombkowsi 2006). *In vivo* intraperitioneal administration of NaHS causes relaxation of the rat colon (Distrutti 2006) and iv injection of $H_2S$ in rats induced a transient dose-dependent decrease in mean arterial pressure (Zhao 2001). In vitro, $H_2S$ and NaHS relaxed rat thoracic aorta and portal vein pre-constricted with noradrenaline. $H_2S$ also relaxed mesenteric arteries (Cheng 2004); an effect mimicked by L-cysteine. In addition the relaxing effect of L-cysteine was abolished by the CSE inhibitor, Propargylglycine (PAG) suggesting that cysteine acted through its conversion to $H_2S$. However, some reports in vascular smooth muscle have found increased contraction or different effects dependent upon $H_2S$ concentration (Zhao 2009,Webb 2008). It has been suggested that these differences may be due to the lower conversion efficacy of NaHS to $H_2S$ at high concentrations (Tian 2012). The finding that free $H_2S$ values are up to 100-fold higher in smooth muscle (aorta) compared to liver, blood, heart and kidney, (Levitt 2011) shows its importance in smooth muscle.

These data suggest $H_2S$ is functionally important in smooth muscle as a vasodilator (widening of blood vessels leading to a decrease in blood pressure) in diseases were high blood pressure is a problem, it highlights a role in new drug synthesis as well as help in other diseases such as cystic fibrosis and atherosclerosis (Lowicka 2007).

### 1.4.6 $H_2S$ in the Uterus

Early studies showed that exposure of pregnant rats to 28-100mg $H_2S$/m3 for 7 hours a day on gestation day 6- day 21 postpartum might increase the mean parturition time and prolong labour (Hayden 1990). This was evidence and created interest in the role of $H_2S$ in the uterus. It has been shown that $H_2S$ is produced in rat and human myometrium using a methylene blue technique, both the CSE and CBS were expressed and that hypoxia increases $H_2S$ production (Patel 2009). Further work by You *et al* looked at the expression of CBS and CSE, as well as quantifying the amount of protein and mRNA levels in human non labouring and labouring myometrium, showing a decrease in the CBS (~63kDa, 205bp) and CSE (~45kDa, 149bp) mRNA levels and a decrease in CBS at the protein level in labouring tissue. This demonstrates the enzymes are regulated over gestation and their down regulation in labour suggests the decrease in $H_2S$ production could be involved in labour onset.

$CBS^{-/-}$ knockout mice are infertile and $CBS^{+/-}$ knockout mice have reduced fertility wereas $CSE^{-/-}$ knockout mice have normal fertility which highlight a possible role of $H_2S$ in fertility and that CBS may have an important role in reproduction. It could also give insight and warrants further work in understanding hyperhomocysteinemia (CBS deficiency), which affects the female reproductive function in many ways. Early pregnancy loss, congenital birth defects and maternal obstetric complications such as pre-eclampsia are some of the related abnormalities (Wang 2012). This also suggests the importance of producing new more specific inhibitors of the enzymes, to be used therapeutically.

Myometrial contractility experiments using L-cysteine and NaHS to monitor the effects of $H_2S$ have been performed. In the pre-term (19 day) rat myometrium, L-cysteine and NaHS, $H_2S$ producers decreased contractility at 1µM-1mM L-cysteine concentrations and 1mM NaHS concentration (Sidhu 2001). Hu *et al* studied the effects of NaHS on spontaneous and oxytocin-stimulated term labouring myometrial contractions and showed significant decreases in frequency and AUC at 1µM to 1mM concentrations. Another study showed a dose dependent decrease from 0.1µM to

10mM L-cysteine with an increase in frequency found at and above 1mM L-cysteine in term labouring and non-labouring tissue. They also found that the effects found on labouring tissue were less potent as well as there being a decrease in $H_2S$ production (You 2011). Within this thesis we also found the effects of $H_2S$ were less in the labouring rat myometrium compared to term pregnant myometrium, further suggesting that endogenous $H_2S$ could be involved in the transition to labour. Inhibitors of CBS and CSE, PAG and AOAA blocked or partially blocked effects of L-cysteine on contractility (You 2011). The effect of $H_2S$ and expression of CBS and CSE throughout gestation and in particular late pregnancy in the pregnant rat myometrium as well as in the non-pregnant human versus term pregnant human myometrium has not been researched.

As new ways to relax the uterus are required to help prevent the global increase in pre-term deliveries and as $H_2S$ effects look very promising more work needs to be performed to help understand the mechanism. As well as to investigate new more controlled and physiological $H_2S$ releasing drugs with the potential to be used clinically such as GYY4137 and even naturally occurring compounds such as garlic derived polysulfides. $H_2S$ could also shed some light on the mechanism by which uterine quiescence is maintained during pregnancy and the initiation of co-ordinated contractions in human parturition.

### 1.4.7 Mechanisms responsible for the effects produced by $H_2S$

The main mechanism linked to $H_2S$ effects is via hyperpolarisation through opening of $K_{ATP}$ channels. Data indicates that $H_2S$ relaxes blood vessels mostly, by opening ATP-regulated potassium channels in the vascular smooth muscle cells. First, glibenclamide, a $K_{ATP}$ channel antagonist, attenuated the hypotensive effect of $H_2S$ *in vivo* and vasodilatory effect *in vitro* (Zhao 2001). Second, the vasodilatory effect of $H_2S$ was attenuated when vessels were incubated in a high-$K^+$ medium. Third, patch-clamp studies have demonstrated that $H_2S$ increases $K_{ATP}$-dependent current and induces hyperpolarization in isolated vascular smooth muscle cells (Cheng 2004, Zhao 2001). In smooth muscle cells isolated from the rat mesenteric artery, $H_2S$ increased the open-probability of $K_{ATP}$ channels without altering their conductance (Tang 2005). Interestingly, CSE inhibitors reduced $K_{ATP}$ channel current indicating that endogenous $H_2S$ continuously stimulated the channel under baseline conditions. Unlike the direct effect on smooth muscle cells, the endothelium-dependent component of $H_2S$-induced vasorelaxation is independent of $K_{ATP}$ channels (Cheng 2004). Other studies however have found no role for $K_{ATP}$ channels (Dhaese 2009, Boyarsky 1978, Kubo 2007). In the myometrium although $K_{ATP}$ channels are expressed (Curley 2002) they so far appear to have only a limited functional importance compared to voltage dependent K channels (Heaton 1993, Aaronson 2006, Longo 2003) , thus other targets for $H_2S$ may be important in the myometrium. The only mechanistic data in the myometrium to date is using the $K_{ATP}$ blocker glibenclamide showing it abolishes the effects of $H_2S$ producers NaHS and L-cysteine (Hu 2011, You 2011).

Other reports suggest a potential inhibitory mechanism of $H_2S$ involving an effect on L-Type calcium channels. Recently, a study in cardiomyocytes suggested, $H_2S$ inhibits L-type [Ca] channels through sulfhydration, as NaHS decreased the functional free sulfhydryl groups in the channels (Zhang 2012). In non-contracting (butanedione monoxime treated) cerebral artery, Tian et al, used fluo-4 and showed decreases in Ca levels as NaHS was increased from 0.1 to 1 mM, and suggested that NaHS relaxes these vessels by reducing L-type Ca current. There have however been no simultaneous

measurements of the changes of intracellular Ca with contraction and no research has looked at $H_2S$ effect on L-type calcium channels in the myometrium.

BkCa, SKCa and IKCa have a role in myometrial membrane excitability and $H_2S$ has been associated with stimulating SKCa and IKCa channels in isolated rat mesenteric arteries (Mustafa 2011). BKCa channels have been research more and both a decrease in open probability in HEK 293 cells transfected stably with human BKCa channel alpha subunits (Li 2010) and recently, the opposite has been documented in rat pituitary tumor cells (Sitdikova 2010). This difference could be associated with differences in BKCa channel isoforms. Other studies using blockers iberiotoxin and paxillin in rat mesenteric and cerebral arteries showed $H_2S$ caused hyperpolarisation through the BKCa channel opening (Jackson-weaver 2011).

Other mechanisms implicated in the effects produced by $H_2S$ include myosin light chain phosphotase activation investigated in cardiomyocytes (Dhaese 2009) and activation of Cl-/HCO-3 in smooth muscle cells, another mechanism concerned with mediating excitability (Tang 2010). Lee et al (Lee 2007) explained $H_2S$ vasorelaxant effects via activation of pH regulating mechanisms. Intracellular signalling pathways connected with $H_2S$ effects include PKA, cAMP and PKG (Shukla 2009, Srilatha 2009). A review of the actions of $H_2S$ in neuronal and smooth muscle tissues concluded that it was likely to be targeting different pathways in different tissues (Kimura 2005). It is more likely that $H_2S$ produces effects on ion channels and other targets by sulfhydration i.e cysteine's covalent modification by which -SH groups on cysteine residues of a protein are converted to –S-SH, via addition of sulphur from $H_2S$ (Mustafa 2009). This molecular mechanism is similar to the S-nitrosylation effect of NO, however, unlike S-nitrosylation, S-sulfhydration activates rather represses, its target proteins (Gallyas, 2012). The most widely researched effect of $H_2S$ is on $K_{ATP}$ channels within smooth muscle as discussed, in vascular smooth muscle cells $H_2S$ stimulated single-channel activity of $K_{ATP}$ channels by directly increasing their opening probability (Wang 2012). Recent work has made progress in identifying which residues in the channel are affected by $H_2S$, with Cys 6 and 26 on the extracellular N terminal of the SUR1 subunit of the channel being identified (Jiang

2010). Sulfhydration has also been associated with stabilising cys residues to prevent oxidative stress, damage and preserving protein function (Paul 2012).

Interestingly, recent work identified a channel permeable to HS− anions in the bacterium *Clostridium difficile*, suggesting that the signalling function of HS− anions may not be confined to HS− anion-generating cells and that although there is a long way to go and much research to be performed to elucidate the mechanism of $H_2S$ maybe such channels/ receptors are present in mammalian tissues (Czyzewski 2012).

Concluding that many mechanisms have been related to the effects $H_2S$ generates, suggesting different mechanisms are targeted in different tissues. In the myometrium specifically, only the use of Glibenclamide a $K_{ATP}$ blocker has been performed to elucidate the mechanism of $H_2S$. Confirming further work is needed to unveil its role especially on calcium entry, L-type calcium channels as well as any other potential mechanism that could cause the responses found to $H_2S$ producers.

## 1.5 Clinical aspects

Here I will explain the relevant clinical aspects of my thesis. I characterise non-pregnant myometrial contractility hence I will discuss the menstrual cycle of the non-pregnant uterus, the menopause and endometriosis. I am looking at the effect of $H_2S$ on myometrial contractility of the pregnant myometrium and its potential to help in pre-term birth therefore I will also describe pregnancy and parturition as well as the complications of a pre-term birth.

### 1.5.1 Non -pregnant uterus

The endometrium is the traditionally accepted end target organ for cyclical ovarian hormonal activity. Endometrial cycle of proliferation, differentiation and shedding is the result (the menstrual cycle). Myometrium also express oestrogen receptors (ER) and progesterone receptors (PR) (Geimonen 1998); some myometrial activities are regulated by oestrogen and progesterone.

The main research investigating myometrial function in non-pregnant women over the last two decades have been obtained from the use of open-tipped pressure catheter recordings, 3D ultrasound, and magnetic resonance imaging (Brosens 1998, van gestel 2003, Bulletti 2004, Bulletti and de Ziegler 2006). The contractions observed during the menstrual cycle have been termed 'endometrial waves' (ljland 1996). These contractions appear to involve only the sub-endometrial layer of the myometrium (Aguilar 2010). After menstruation, in the early follicular phase, contractile waves occur once or twice per minute and last 10–15s with low-amplitude (usually, 30 mmHg). As ovulation approaches, the frequency increases to 3–4 per minute. During the luteal phase, the frequency and amplitude decrease possibly to facilitate implantation. When a blastocyst does not implant, the contraction frequency remains low but the amplitude increases considerably (50–200 mmHg) producing labour-like contractions at the time of menstruation (Aguilar 2010). The difference in contractility through the menstrual cycle suggests some cyclical action of oestrogen and progesterone.

### 1.5.2 The menopause

The menopause is defined as the permanent cessation of menstruation brought on by ovarian failure (Rees *et al* 2009). In the UK, the median age for onset of menopausal symptoms is 45.5 to 47.5 years (Burbos 2011). Follicles in the ovaries become less responsive to follicle stimulating hormone (FSH) – which stimulates the follicles before ovulation – until ovulation ceases. The menopause is a retrospective definition made following one year of amenorrhoea (Rees *et al* 2009). It includes different phases, peri menopause, menopause and post menopause (Rees *et al* 2009). The peri-menopause (or pre-menopause) is the period from the onset of menopausal symptoms, which is when ovarian function begins to decline, to the menopause, when oestrogen production ceases as no follicles. Post menopause are the years following menopause (when periods have stopped for at least twelve months). The age at which the menopause occurs is determined by genetics, environmental factors such as smoking, and surgery (oophorectomy), chemotherapy or radiotherapy (Rees *et al* 2009). Premature ovarian failure (early menopause) is where the ovaries stop functioning in women under the age of 45 years (Holloway 2011).

Common vasomotor symptoms include hot flushes, night sweats, sweating, palpitations, insomnia, sleep disturbances, shivering, increased pulse, feeling faint, and nausea. Vaginal and urinary symptoms include vaginal infections, post-coital bleeding, painful sex, itching or irritation, decreased libido (hormonal causes: atrophic changes (loss of elasticity in vaginal tissues), decreased lubrication, change in sensory perception), dysuria, atrophic vaginitis – dryness, urinary frequency/urgency, stress and urge incontinence. Other symptoms include changes in bleeding pattern, irregular periods, heavier periods, long gaps of amenorrhoea, skin itching or crawling sensations, and joint pain. Symptoms of the menopause can be managed with lifestyle changes (such as regular exercise and weight reduction), complementary treatments (homeopathy) prescribed alternatives (such as progestogens to control hot flushes) and hormone replacement therapy (HRT). HRT is a combination of oestrogen and progesterone which help to relieve the symptoms of menopause.

There is little research looking at the differences in contractility with onset of the menopause. Domali *et al* showed *invitro* the effects of endothelin-1 (ET1) on the myometrial contractility of pre and post menopausal women showing observations from segments of equilibration periods from both groups, concluding pre-menopausal myometrium contracted quicker, and were more frequent than post menopausal myometrium, however no analysis was performed. Although the main finding of this research was the long-lasting effectiveness of ET1 in strips collected from post menopausal women compared with pre menopausal women, perhaps the effect of ET1 is enhanced by the oestrogen deficiency after menopause. They also found that there was no change in the response to High $K^+$ in either group and suggested that High $K^+$ and ET1 affect uterine contractility through different mechanisms and that ovarian steroids may play a regulatory role in human uterine responsiveness to ET1 (Domali 2001).

### 1.5.3 Endometriosis

Endometriosis is one of the most common gynaecological disorders. It affects 10–15% of all women in the reproductive years. The incidence is 40–60% in women with dysmenorrhoea and 20–30% in those with subfertility (WSHL 2010). Endometriosis is a chronic, inflammatory condition characterised by growth of endometrial tissue in sites outside the uterus, most commonly in the pelvic cavity, but also in other parts of the body (RCOG 2006). The condition is predominantly found in women of reproductive age, from all ethnic and social groups (RCOG 2006). This ectopic tissue responds to the hormonal changes of the menstrual cycle, with subsequent bleeding, inflammation, and pain. If the ovaries are affected, endometriotic ovarian cysts may develop (Bulun 2009). Symptoms associated with endometriosis may include infertility defined as the inability to conceive and is one of the problems associated with endometriosis. As this inability is often not absolute, the term 'subfertility' is preferred. Dysmenorrhoea is severe uterine pain during menstruation.

Although the condition may be asymptomatic, common symptoms include dysmenorrhoea, dyspareunia, non-cyclical pelvic and abdominal pain, and subfertility (RCOG 2006). The cause of endometriosis is not known, but several factors are thought to be involved in its development. These include retrograde

menstruation (backward movement of menstrual fluids); embryonic cells giving rise to deposits in distant sites around the body; an abnormal quantity or quality of endometrial cells; failure of immunological mechanisms; angiogenesis; and the production of antibodies against endometrial cells (Gazvani 2002, Rock 1992, Seli 2003, Kyama 2003, Oral 1996).

Pain due to endometriosis can be functional, neuropathic, due to inflammation, or result from a combination of these. It may be evoked by a low intensity, normally innocuous stimulus (allodynia), it may be an exaggerated and prolonged response to a noxious stimulus (hyperalgesia), or it may be spontaneous in the absence of any apparent peripheral stimulus (Lundeberg 2008). In addition, oestrogens and prostaglandins probably play key modulatory roles in endometriosis and the pain it causes (Lundeberg 2008). Consequently, current medical treatments for the condition include drugs such as non steroidal anti-inflammatories (NSAIDs), combined oral contraceptives, progestogens (Provera, Mirena coil), antiprogestogens (Danazol and *Gestrinone)* and gonadotrophin releasing hormone analogues, as well as surgical excision of endometriotic lesions. However, management of pain in women with endometriosis is often inadequate.

Retrograde contractions are contractions propagating from the cervical end of the uterus towards the fundus aiding in sperm transport or possibly in retention of iron for example, following blood losses at menstruation (Kunz and Leyendecker 2002). In the pregnant uterus, these retrograde contractions may also have roles in the maintenance of early pregnancies within the uterine cavity (de Vries et al. 1990), but possibly also in causing endometriosis, as menstrual debris enters the peritoneal cavity. Despite the common occurrence and the huge economic burden of endometriosis, the many biological studies using a range of models have not yet identified the causative mechanisms. Changes in myometrial contractions could be involved in increasing the back flow of menstrual debris in to the pelvic cavity, giving rise to endometriotic deposits. Only *in vivo* techniques have been investigated to look for changes in myometrial contractility in response to endometriosis. In 1995, Salamanca and Beltran in a study of inner myometrial contractility using transvaginal

sonography in women with endometriosis found a predominantly retrograde pattern of subendometrial contractions during menstruation (Salamanca 1995). In 2002, intrauterine pressures were recorded showing increased frequency, amplitude and basal pressure tone within infertile endometriosis patients when compared to infertile patients without endometriosis (control). Retrograde bleeding was found in 73% of these patients with endometriosis compared to 9% of the control group. This study also shows 45% endometrial debris within the cul-de-sac of endometriosis patients compared to 0% in controls which could implant (Bulletti 2002). Both studies indicate abnormal alterations of uterine contractility at the time of menses are involved in the development of endometriosis. No *in vitro* studies to look at myometrial contractions of women with endometriosis have been performed.

### 1.5.4 Pregnancy

The uterus has a number of important functions that are fundamental for successful pregnancy to occur. It provides an appropriate environment for implantation of the fertilised ovum, after which time it undergoes alterations in size and structure to accommodate to the needs of the growing embryo. During pregnancy, the uterus provides nourishment for the fetus and also serves as a mechanical barrier throughout the entire developmental stage. As gestation progresses and term approaches however, the uterus undergoes a number of preparatory changes in readiness for the onset of labour and for the activity required for successful delivery of the fetus and placenta.

### 1.5.5 Labour

Human labour is the process whereby the products of conception (fetus, placenta and membranes) at a gestation where the fetus is viable (term) are expelled from the genital tract. In humans the process begins naturally at term (37- 42 weeks of gestation). Labours that occur between 24-37 weeks of gestation are referred to as pre-term labours.

The safe passage of the fetus through the birth canal is dependent upon five unknown factors of labour (Beazley 1995):

i) The efficiency of uterine contractions

ii) the 'give' of the pelvis

iii) the moulding potential of the fetal head

iv) the fortitude of the mother

v) the adaptability of the child's physiology.

The process of parturition is continuous but traditionally it is divided into three stages:

***The first stage***

This is from the onset of uterine contractions to full dilation of the cervix. It is the subdivided into the latent phase and the active phase.

The time onset of spontaneous labour is difficult to determine and indeed the trigger for the commencement of this physiological process is still unknown. Clinically, the extrusion of the mucus 'plug' from the cervix, which appears as a jelly-like substance streaked with blood, commonly indicates that the labour process is about to commence. This occurs during the latent phase as the cervix undergoes extensive changes from a sphincter, designed to retain the products of conception to a dilator. The ground substance of the cervix hydrates, collagen is denatured and there is an increase in hyaluronidase. As a result, the ground substance changes from a gel to a fluid medium permitting deformation of the cervix (Gee &Olah 1993).

During the active stage of labour the cervix dilates rapidly. The rate of dilation was described by Freidman in 1955 as 1cm per hour. This is adhered to today as the rate of 'normal' progress in labour; however this changes amongst different populations. Throughout the first stage of labour, contractions increase in their frequency and duration becoming more powerful and painful to the mother.

***The second stage***

This is from full dilatation of the cervix to delivery of the infant. It can be subdivided into two phases; the descent phase, when the presenting part of the infant descends through the maternal pelvis, triggering a 'need to push' response from the mother and a second expulsive phase which results in active maternal pushing and delivery of the infant.

***The third stage***

From delivery of the infant to separation and expulsion of the placenta and membranes.

### 1.5.6 Premature Labour

Preterm birth is defined as any delivery, regardless of birth weight, that occurs before 37 completed weeks of gestation but after the gestation of viability (WHO 1969). Prematurity is the principal cause of neonatal mortality (Mathews 2004) and a major cause of paediatric morbidity and long-term disability (Hack 2002, Mccormick 2002); it is associated with at least 50% of all paediatric neurodevelopmental disorders (Goldenberg 1998). The incidence is gradually increasing and currently complicates 7% of all pregnancies. These rates however, vary considerably between races and populations being about 8.8% in Caucasian and 18.9% in black communities (Lyon 1994).

The aetiology of spontaneous premature labour is often unknown. Risk factors include: low socioeconomic status, low maternal weight at time of conception, maternal smoking, uterine abnormalities, cervical incompetence, antepartum haemorrhage, pre-eclampsia, multiple gestations, previous preterm labour and infection.

In some cases prevention of contractility can be a useful way to prevent pre-term labour and improve neonatal outcome but in cases of antepartum haemorrhage, pre-eclampsia and infection this may not be the case. As of yet there is no clinical evidence for the use of tocolytic drugs in prolonging gestation.

The choice of tocolytic drug remains controversial. Magnesium sulphate, calcium channel blockers, prostaglandin synthase inhibitors, nitric oxide donors, β-adrenergic receptor agonists, oxytocin antagonists as well as progesterone (P4) have all been suggested to be effective (Arrowsmith 2010). All help to relax the uterus to promote quiescence. There is no consensus on the best tocolytic of choice although the rate of pre-term delivery has not declined. This suggests the invention used is not effective and new ones are needed and that a better understanding of the mechanisms of force production by the uterus may therefore lead to the development of improved tocolytic agents.

Overall, there is a need to find better ways to treat uterine dysfunctions. Recent evidence suggests a physiological role for $H_2S$ for example in the control of blood pressure and in changing contractility. Enzymes that produce $H_2S$ have been found in the myometrium and $H_2S$ causes a decrease in contractility suggesting $H_2S$ could give insight into the mechanism of quiescence and potentially help in pre-term birth. However, there are many unknowns in the understanding of $H_2S$, its mechanism and its physiological relevance in the myometrium.

## 1.6 Thesis Aims

The aims of my thesis was to investigate my hypothesis which is –
$H_2S$ relaxes the myometrium and could potentially help therapeutically to reduce the incidence of preterm birth. Reduced contractility in the presence of $H_2S$ has been indicated in other smooth muscles as described in my introduction as well as few studies in the uterus. I examined $H_2S$ through the use of three $H_2S$ producing compounds NaHS, GYY4137 and L-cysteine. NaHS is the most widely used $H_2S$ producing compound although its toxicity and release of $H_2S$ in a non-physiological large bolus reveals the need for alternative $H_2S$ donors suitable for drug development. A new $H_2S$ generating compound, GYY4137, developed to slowly release $H_2S$ and thus better reflects physiological conditions and therefore is a more promising drug for clinical use. L-cysteine is a substrate for $H_2S$ production through the presence of enzymes CBS and CSE within tissues, an alternative and more physiological way to produce $H_2S$ compared to NaHS. Dose response curves were performed for novel compound GYY4137 upon myometrial contractility.

I investigated whether the response to $H_2S$ producers changes throughout gestation and its potential involvement in quiescence through use of different gestational state rat myometrium as well as non-pregnant and term pregnant human myometrium. Firstly I characterised the non-pregnant human tissue samples received to see how contractility changed with age and pre or post menopausal hormonal status. I also looked at whether $H_2S$ has an affect on oxytocin stimulated term pregnant myometrial contractions. I explored the mechanism of $H_2S$ action in the myometrium. As suggested by other studies on smooth muscle, $K_{ATP}$ channels are the most widely researched for its involvement in the relaxant affect produced by $H_2S$. In the myometrium, L-type calcium channels are a key target to initiate changes in contractility. Therefore I examined the affect of $H_2S$ on both L-type calcium channels and $K_{ATP}$ channels through use of calcium sensitive indicator indo-1, high K, BayK as well as $K_{ATP}$ channel inhibitor glibenclamide.
As myometrial contractility is reduced throughout gestation I carried out immunohistological analysis on sections of myometrium from non-pregnant and term pregnant women to look for the distribution of $H_2S$ production enzymes CBS

and CSE and performed Westerns to indicate any differences in expression of both enzymes present within these tissues. Rhodhanese is involved in detoxifying $H_2S$. Rhodanese consists of two sulfurtransferase components, thiosulfate sulfurtransferase (TST) and mercaptopyruvate sulfur-transferase (MST), with close structural and size similarities. There is evidence that rhodanese, particularly TST, might be involved in detoxification of $H_2S$

Therefore I examined the presence and expression of TST within the myometrium to see if there is any difference between tissues. Overall, looking at both production and breakdown of $H_2S$ to assess whether or not the alterations in the responses found in contractility in response to $H_2S$ were due to their upregulation or down regulation.

# Chapter 2 - General Materials and Methods

## 2.1 Tissue

**Both human and animal tissues were used for the work performed in this thesis.**

### 2.1.1 Animal Tissue

In this study female non-pregnant and time mated Wistar rats (Charles River, KENT, UK) were used. For the time mated Wistar rats the male was placed in the cage on Day 0 and left overnight to mate. The pregnant rat was allowed to reach 14, 18 and term, (day 22) and killed prior to labour. Labouring animals were also used and were taken on delivery of the first pup. All animals were humanely killed by cervical dislocation under $CO_2$ anaesthesia in accordance with UK Home Office legislation. Pups were delivered by caesarean section and killed by decapitation in accordance with UK Home Office legislation. Once all pups had been removed the uterus was dissected and immediately placed into physiological saline solution (PSS): (154mM NaCl; 5.6mM KCl; 1.2mM $Mg_20_4.7H_2O$; 10.9mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES); 8mM glucose; 2mM $CaCl_2$, pH 7.4). The uterine tissue was then rinsed and cleaned, removing any foetal membranes, placenta, fat and any excess blood prior to myometrial dissection.

### 2.1.2 Human Tissue

The human tissues used for these studies were non-pregnant and pregnant myometrial biopsies. Non-pregnant tissues were collected from women undergoing hysterectomy and pregnant tissues from elective caesarean section. Ethical approval was sought separately for the use of pregnant and non-pregnant hysterectomy myometrium within this project and granted (Appendix 2). For myometrial tissue from the Liverpool Women's NHS Foundation Trust Hospital Myometrial Research Tissue Bank (MRTB) a further application was submitted to the hospital (Appendix 3). Access was granted from the MRTB.

Patients were consented by research midwives and clinicians at the preoperative clinic. Written informed consent was obtained and proformas detailing any relevant clinical history and medical conditions was also filled in (Appendix 4).

Biopsies from non-pregnant women undergoing hysterectomy were taken immediately after the removal of the uterus from the lower half of anterior uterine wall, so as to approximate the area where the biopsy was removed from the pregnant uterus. For pregnant tissues, full thickness biopsies measuring 1cm x 1cm were taken after delivery from the middle of the upper edge, lower segment uterine incision at time of caesarean section. All biopsies were then placed immediately into chilled Hanks Balanced Salt Solution(HBSS): (137mM NaCl; 5.1mM KCl; 0.44mM $KH_2PO_4$; 0.26 $Na_2HPO_4$ ; 5mM glucose, 10mM HEPES, pH 7.2) and then transferred to the physiology department. All biopsies were collected and handled using a protocol to prevent tissue degradation and to ensure all condition for experiments were the same. Biopsies were used either on the day of collection (within 3 hours of removal from organ) or the next day after storage at 4 $^{o}$C. Biopsies were never used more than 18 hours after delivery. Observations from Crankshaw et al (Hillcock and Crankshaw (1999) Senchyna and Crankshaw (1999) and Popat and Crankshaw (2001)) have indicated that human myometrium stored in PSS for up to 18 hours and at room temperature do not behave differently upon exposure to a number of different agents compared to fresh samples. Unpublished results obtained within our group and my own findings would also confirm this.

## 2.2 Tissue Preparation and Dissection

Each tissue type was placed in a dissection dish containing PSS solution at room temperature.

### 2.2.1 Animal Tissue Dissection

A small section approximately 1cm in length of the uterine horn was cut from the ovarian end. An incision to the uterine tube longitudinally was cut and opened out, then pinned out with the myometrium facing upwards. The surface of the tissue was cleaned using a cotton cue tip to remove any serosal cells. Strips of longitudinal myometrium were dissected avoiding any underlying circular smooth muscle and endometrium and were placed into physiological saline solution.

### 2.2.2 Human Biopsy Dissection

Samples were transferred from HBSS into PSS where they were cleaned and trimmed of any blood, endometrium, and peritoneum. Using blunt dissection an opening was made between muscle bundles to expose the inner tissue not subjected to any external trauma. Small longitudinal strips of muscle were dissected from the biopsy, each approximately 1x5mm and placed into physiological saline solution.

In specimens from pregnant women, hypertrophy of the smooth muscle bundles enabled dissection of strips where the majority of the muscle fibres were running in the same direction. Dissection of tissue from non-pregnant women was more difficult and it was composed of more interwoven fibres.

## 2.3. Chemical and Solutions used for Contractility Experiments

All chemicals used were purchased from Sigma (Dorset, UK) unless otherwise stated. In stock solutions of certain substances that did not dissolve within PSS, solvents such as ethanol and DMSO were used. These substances do not alter the myometrial strip contractile activity as reported previously. (Taggart &Wray, 1997, Taggart & Wray 1998, Noble & Wray 2002).

**High Potassium Solution**

119.6mM NaCl; 40mM KCL; 1.2mM $Mg20_47H_2O$; 10.9mM HEPES; 8mM glucose; 2mM $CaCl_2$,

pH 7.4

**GYY4137**

A 1mM GYY4137 (Santa Cruz biotechnology, USA) stock solution was prepared in PSS. The solution pH was adjusted (pH7.4) before use and made fresh before each experiment. Then serial dilutions were prepared from the stock to produce 1nM, 1μM, 0.1mM. GYY4137 is a $H_2S$ donor.

**Sodium hydrogen sulphide (NaHS)**

A 1mM NaHS solution (Alfa Aesar, uk) was prepared in PSS. The solution pH was adjusted (pH7.4) before use and made fresh before each experiment. NaHS is a $H_2S$ producer.

**Dithiothreitol (DTT)**

A 1mM DTT solution (Sigma Aldrich, uk) was prepared in PSS. The solution pH was adjusted (pH7.4) before use and made fresh before each experiment. DTT is a reducing sulphydryl modifier. DTT transforms disulphide bonds to thiol groups.

**Diamide (DM)**

A 1mM DM solution (Sigma Aldrich, uk) was prepared in PSS. The solution pH was adjusted (pH7.4) before use and made fresh before each experiment. DM is a oxidant sulphydryl modifier. Diamide converts thiol groups to disulphide bonds.

**L-Cystiene**

A 1mM L-cysteine stock solution was prepared in PSS. The solution pH was adjusted (pH7.4) before use and made fresh before each experiment. Then serial dilutions were prepared from the stock to produce 1nM, 1µM, 0.1mM. L-cysteine is the substrate $H_2S$ production enzymes use in tissue to form $H_2S$.

**D-cysteine,**

A 1mM D-cysteine stock solution was prepared in PSS. The solution pH was adjusted before use and made fresh before each experiment. D-cysteine is the opposite stereoisomer of L-cysteine. Isomers are molecules that have the same molecular formula but differ in the way the atoms are arranged around the central atom.

**Oxytocin**

A 1mM Oxytocin was prepared by dissolving the lyophilized powder in double distilled water. From the 1mM stock a further diluted stock of 10µM solution was prepared, aliquoted and stored at -20°C. For use in contractility experiments a concentration was used that would not cause a tonic response but would augment

contraction frequency and force. A concentration of 0.5nM was used for human myometrial strips.

**BayK 8644** (BayK)

BayK was dissolved in ethanol (63mg/ml) to produce a 10 mM stock solution. A 0.1 µM solution was used in rat tissue and was diluted using PSS. Above 0.5 µM concentrations were not used as tonic rather than phasic contractions were found. BayK is a calcium channel agonist and increases the open probability to L-type calcium channels (Lauven et al 1999).

**L-serine**

A 1mM L-serine stock solution was prepared in PSS. The solution pH was adjusted before use to 7.4 and made fresh before each experiment. L-serine is an analogue of L-cysteine in which the sulphur atom is replaced with an oxygen atom.

**Sodium pyruvate**

A 1mM sodium pyruvate stock solution was prepared in PSS. The solution pH was adjusted before use to7.4 and made fresh before each experiment.

**Propylargylglycine (PAG)**

A 1mM PAG stock solution (Santa Cruz biotechnology, USA) was prepared in PSS. The solution pH was adjusted to 7.4 before use and made fresh before each experiment. $H_2S$ is produced by two enzymes Cystathionine gamma lyase (CSE) and Cystathionine beta synthase (CBS). PAG is a CSE enzyme inhibitor.

**Aminooxyacetic acid (AOAA)**

A 1mM AOAA stock solution was prepared in PSS. The solution pH was adjusted before use to 7.4 and made fresh before each experiment. AOAA is a CBS inhibitor.

**Hydroxylamine (HA)**

A 1mM HA stock solution was prepared in PSS. The solution pH was adjusted before use to 7.4 and made fresh before each experiment. HA is a CBS inhibitor.

**Garlic**

Solutions from 0.0025- 1 mg/ml Garlic powder(100% pure garlic powder, RAJAH) were prepared in PSS. The solution pH was adjusted before use to 7.4 and made fresh before each experiment.

**Glibenclamide**

A 10mM stock solution was prepared every week in DMSO, then aliquoted and stored at -20°C. For use in contractility experiments a 1mM solution using this stock was prepared. Glibenclamide is a $K_{ATP}$ channel inhibitor.

## 2.4 Measurement of Intracellular calcium

The concentration of intracellular ions such as $Ca^{2+}$, $Na^{+}$ and $H^{+}$, are important in the regulation of cell function. Calcium is the main ion responsible for smooth muscle contraction, although pH differences can also be responsible for some of the changes we see in contractility especially in response to agonists. The work in this thesis involves establishing not only changes in smooth muscle contractility under differing conditions but also the associated changes in intracellular calcium ($[Ca^{2+}]_i$), and intracellular pH ($pH_i$).

Fluorescent probes are the most popular method for establishing intracellular concentrations of ions. This is because (Tsien 1989):

- In most cell types the probes can be incorporated into the intact functioning cell without any breaching of the plasma membrane.
- Indicators allow ion activities or free concentrations to be measured.
- Fluorescent probes can be used at all levels of organisation from whole organs to isolated tissue fragments to populations of disaggregated cells to single cells to subcellular domains of single cells.
- Simple application of fluorescent indicators only need a fluorometer but also sophisticated instruction is available leading to dual-wavelength ratio fluorometry, digital ratio imaging microscopy and scanning confocal microscopy.

For a fluorescent compound to be a useful indicator, the fluorescence intensity must change as a function of the concentration of the measured ion. In addition a valuable property is that either the excitation or emission spectrum should change in shape, thus allowing dual wavelength measurements (Valdeolmillos & Eisner, 1991).

### 2.4.1 Fluorescence process

Fluorescence is a process that begins with excitation. A photon of energy is applied via an external source such as a lamp or laser and the energy is absorbed by the fluorophore. This forms an elevated 'excited' energy state of the fluorophore. This excited state however, is not infinite and some of the energy becomes dissipated revealing a more relaxed state but does not completely reach its original (ground

energy state). Fluorescence then concludes with the emission of a photon of energy from the fluorophore returning back to the ground state. Some energy is dissipated during the excited state therefore the energy of the photon emitted is lower and is therefore of a longer wavelength than the initial excitation photon. This difference is called the Stokes shift and is fundamental to fluorescence techniques as it allows for emission photons to be detected against a background of excitation photons.

This process of fluorescence can reoccur as long as the fluorophore is not irreversibly destroyed (i.e photobleaching) therefore the same fluorophore can be repeatedly excited and detected. Each fluorophore is also capable of generating thousands of photons. For fluorescence to be detected an excitation source, a fluorophore, wavelength filters to isolate emission photons from excitation photons and a detector that registers emission photons and produces an output that can be recorded are required. For detection of cellular ions such as calcium, as used in this thesis, often the fluorescent indicator used has different excitation or emission spectra for its free and ion-bound form. Fluorescence systems such as these are called ratiometric systems since the ratio of the optical signals (ie. excitation or emission spectra) can be used to measure the change in the free and ion bound indicator forms which in turn can be used to quantify ion concentrations.

### 2.4.2 Fluorescent indicators and measurement of intracellular calcium and intracellular pH (cytosolic free)

The fluorescent indicators Indo-1 and Carboxy SNARF-1 have been used in this dissertation.

Indo-1 AM (**Figure 2.1**) have binding sites which are modelled on the $Ca^{2+}$ selective chelator ethylene glycol bis(β-aminoethyl ether) N, N'-tetracetic acid (EGTA). EGTA at pH 7 is normally occupied by two protons, but the incorporation of the aromatic rings in the fluorescent indicators lowers the PKa of the amine nitrogens to 6.5 or below thus eliminating all the proton interference for pH>6.8. $Ca^{2+}$ binding diverts the nitrogen lone pair electrons away from the aromatic system, causing large spectral changes. The more electron donating or withdrawing the aromatic nucleus, the higher or lower the $Ca^{2+}$ affinity (Tsien 1980). Indo-1 is a dual emission calcium

sensitive indicator. Two emission wavelengths are collected, calcium free (approx 500nm) and calcium bound (400nm), once excited at about 340-350nm (**Figure 2.3**).

5- (and 6)-Carboxy SNARF-1 AM (**Figure 2.2**) is used by exciting the dye at between 488 nm -530 nm, while monitoring the fluorescence emission at two wavelengths, the proton-bound and unbound forms of SNARF emit at 590 and 640 nm (**Figure 2.3**), respectively. These are pH-dependent emission shifts going from yellow-orange to deep-red fluorescence as conditions become more basic. The absorption spectrum of the carboxy SNARF-1 pH indicator undergoes a shift to longer wavelengths upon deprotonation of its phenolic substituent (Invitrogen). The use of dual emission spectroscopy increases the precision of the measurement, reduces the effects of instrumentation shift, movement artefacts or loading dye content, and permits the quantification of the signal without knowing the concentration of intracellular indicator. The fluorescent signal is also independent of the cell thickness (valdeomillos & Eisner, 1991, Grynkiewicz, Poenie et al. 1985).

These indicators were incorporated into cells as ester AM (acetoxymethyl ester) derivatives. This form of the indicator is readily cell permeable as it is uncharged and hydrophobic and thus can cross lipid membranes, gaining entry to the interior of cells (Kao, 1994). The carboxyl groups in the indicator are essential to the ability of the indicator molecule to sense $Ca^{2+}$; therefore the AM groups must be removed once the AM ester has entered the cell. The AM esters are cleaved by cytosolic esterases once inside the cell which restricts the indicator to the cytosolic compartments. This is because the polycarboxylate form of both indicators is multiply charged and thus becomes trapped within the cell.

Tissue loading with indicators can be improved by adding the detergent Pluronic F-127 as it is a mild non ionic surfactant and dispersing agent for AM esters. Pluronic is presumed to sequester the AM ester in micellar from, thus preventing precipitation and the micelles are presumed to serve as a steady source to replenish AM esters taken up by cells (calcium in living cells, Whitaker, 2010). Both Pluronic acid and AM ester stock solutions in Dimethylsulfoxide (DMSO) are mixed intimately before

dispersal into an aqueous medium, facilitating the solubilisation of the water-insoluble dyes in physiological media and improving the loading of indicators into cells, this methodology was incorporated into the loading technique.

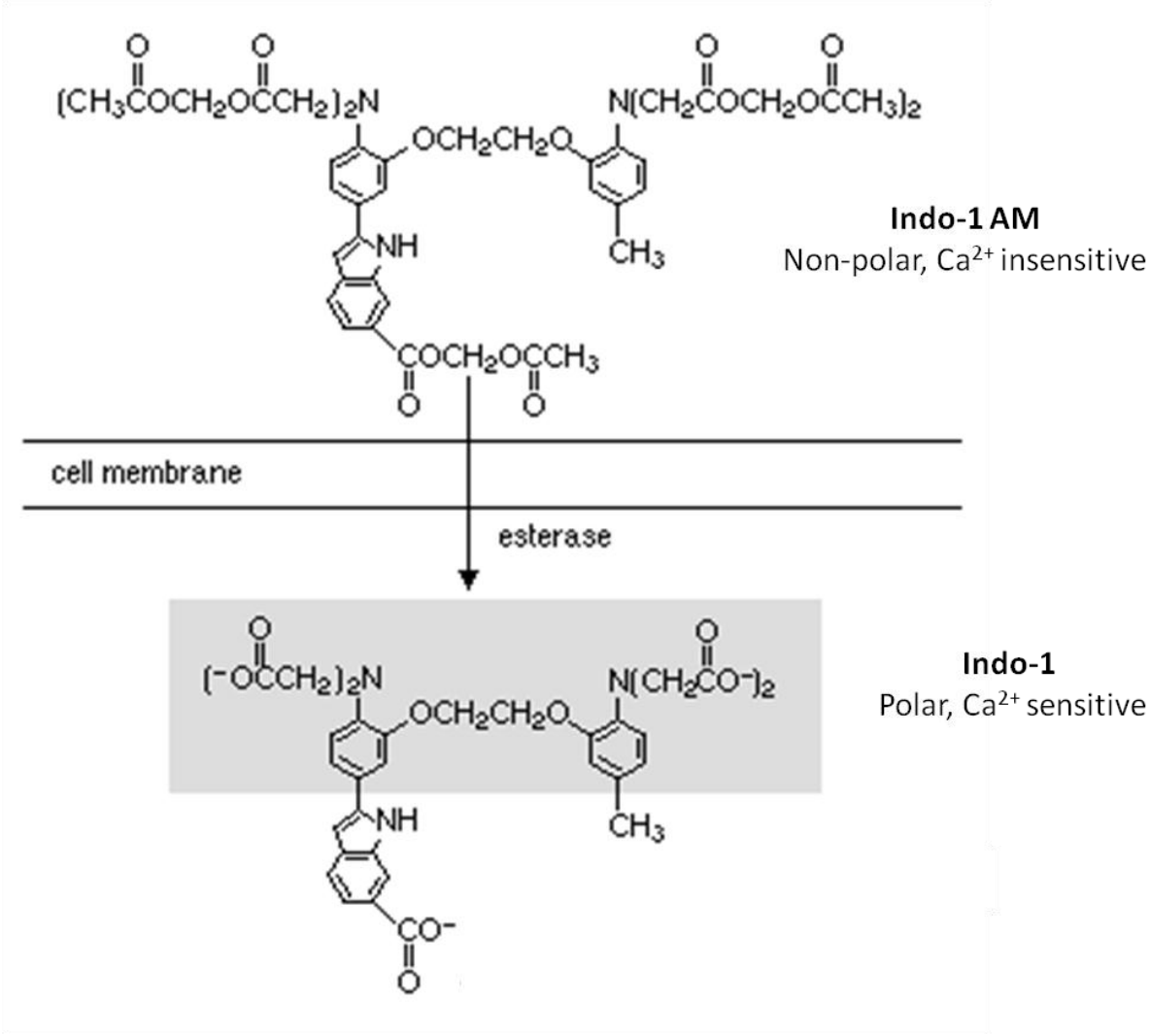



**Figure 2.1 Diagram of the structural modifications involved in loading cells using Indo-1 AM.** The membrane-permeable acetoxymethyl (AM) ester derivative of Indo-1 (Indo-1 AM) is insensitive to $Ca^{2+}$ions and therefore does not bind to $Ca^{2+}$. However, once inside the cells it is readily hydrolysed to Indo-1 by ubiquitous endogenous esterases, releasing the ion sensitive indicator. Cleavage if the ester produces a polar form of the indicator which is cell impermeable and is therefore retained within the cells interior. (Figure adapted from Johnson I, current protocols).

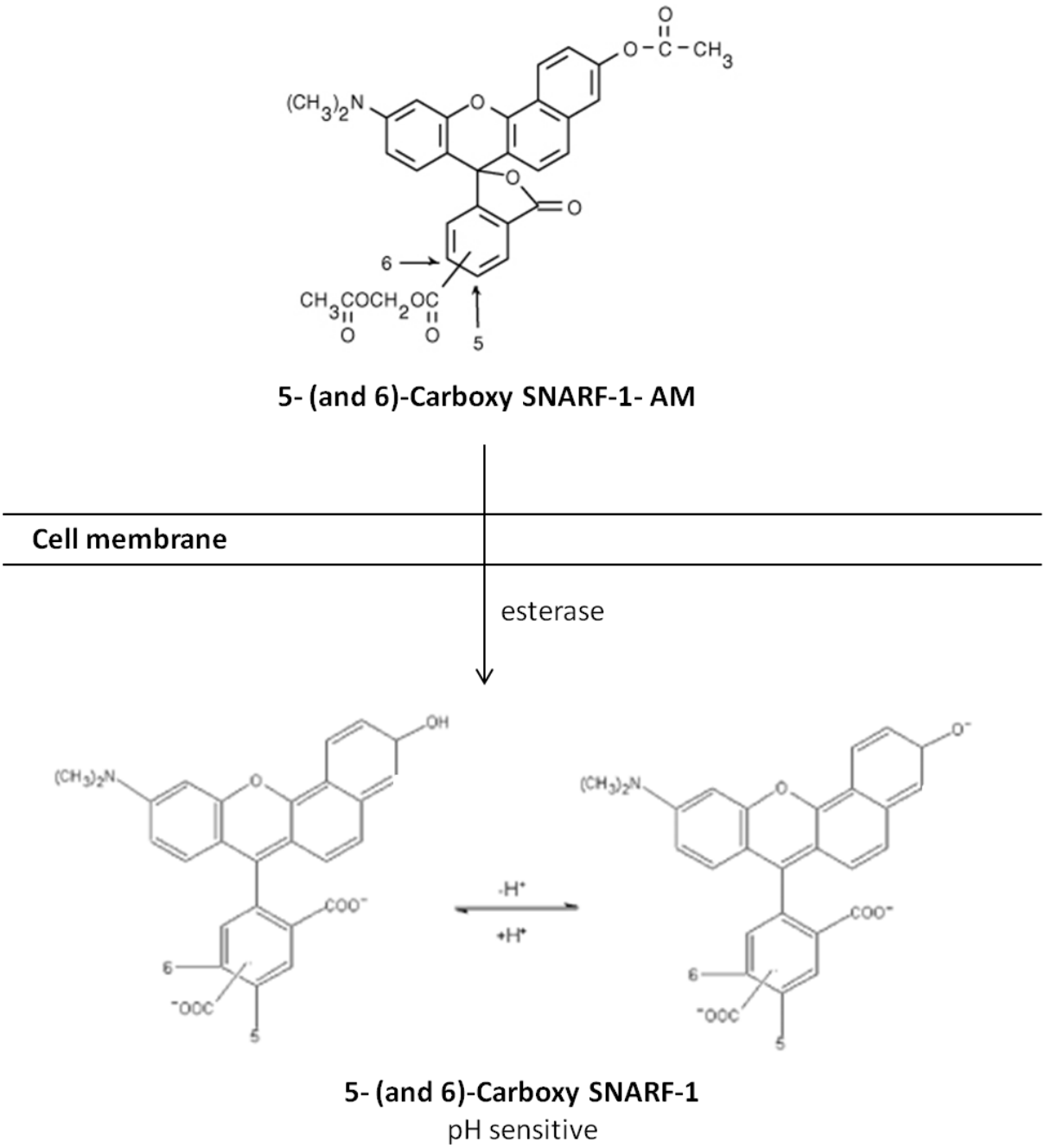
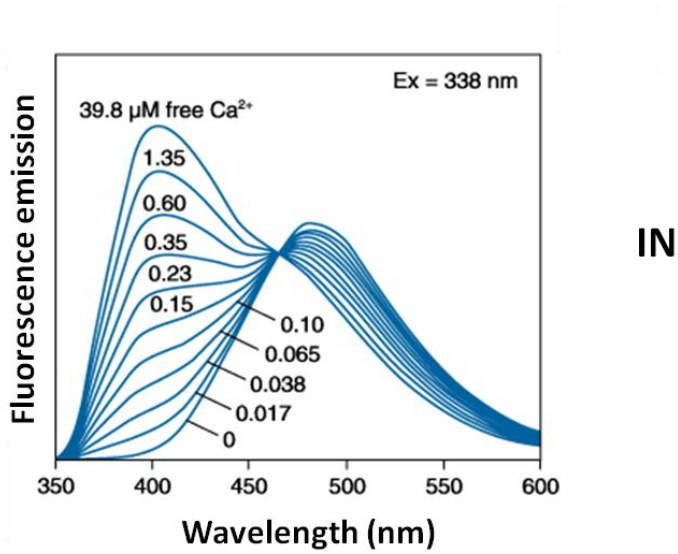
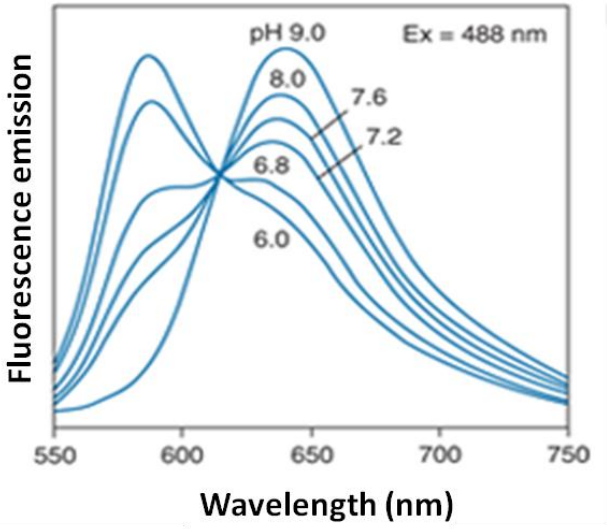



**Figure 2.2 Diagram of the structural modifications involved in loading cells using 6-(and 5) carboxy SNARF-1 AM.** The membrane-permeable acetoxymethyl (AM) ester derivative of SNARF-1 (SNARF-1 AM) is not affected by pH. However, once inside the cells it is readily hydrolysed SNARF-1 by ubiquitous endogenous esterases, releasing the pH sensitive indicator. Cleavage if the ester produces a polar form of the indicator which is cell impermeable and is therefore retained within the cells interior. (Johnson I, current protocols).




**INDO-1**




**5- (and 6)-Carboxy SNARF-1**

**Figure 2.3 Fluorescence emission spectra of Indo-1 and 5-(and 6)- Carboxy SNARF-1.**

$Ca^{2+}$- dependent emission spectra of Indo-1 excited at 338nm, shifts from~475nm in $Ca^{2+}$-free medium to ~400nm when the dye is saturated with $Ca^{2+}$. pH-dependent emission spectra of carboxy SNARF-1 when excited at 488nm shifts from ~640 nm when proton bound form ~590nm when in its proton unbound form. Figures taken from Invitrogen Handbook.

### 2.4.3 Advantages and disadvantages of indicators

*Photobleaching*

Photobleaching is the irreversible destruction of the excited fluorophore. Another factor contributing to loss of signal is leakage of fluorophore out of the cell. For mammalian cells the loss rate is maximal at 37$^{o}$C and drops sharply as temperature is lowered (Kao, 1994). To help reduce photobleaching, detection sensitivity can be increased to maximum so allowing a reduction in the intensity of excitation. This was undertaken in all experiments. Also neutral density filters were inserted in front of the excitation light to reduce light intensity in the tissues (Tsien 1989, Haugland 1995).

*Autofluorescence*

Tissue autofluorescence results from naturally occurring cellular fluorophores, such as NADH, and riboflavin. If the contribution from autofluorescence is significant this will affect the total fluorescence readings and thus an estimate of autofluorescence is required in order to calculate intracellular calcium. It has been shown that myometrial strips have autofluorescence at 340nm (luckas et al 2000), which will increase the 500nm signal in response to an increase [Ca]i. However the same author showed that this autofluorescence was very small (<5%) and the signals changed in opposite directions, thus the autofluorescence of myometrial strips is not significant.

*Subcellular compartmentalisation of fluorophores*

Indicators can be compartmentalised into other organelles such as sarcoplasmic reticulum and mitochondria. In order to correct for the contribution of the above fluorescence signal, an estimate of their contribution to the signal can be made via quenching with manganese (Mn) (Hesketh et al, 1983). The measured emission following quenching is subtracted from the total signal, the difference equalling the signal derived from cytosolic free indicator.

Despite these disadvantages, preventative steps have been put in place to avoid cell toxicity and compartmentalisation of the dye. These steps include minimising the indicator loading time (<4 hours), loading in a dark place to avoid photobleaching, and therefore loss of fluorescence as well as loading at lower temperatures (room temperature).

## 2.5 Force, Calcium and $pH_i$ Measurements

Indo 1AM and Carboxy SNARF-1 AM were prepared by dissolving in DMSO. There is no significant effect of DMSO on myometrial contractility at the concentration used for Indo-1 AM and Carboxy SNARF-1 AM loading (Taggart, Menice et al. 1997).

### 2.5.1 Calibration of force

Force was calibrated to Newtons (N). The electrical signal from the transducer was amplified and converted to a digital signal and record on a computer using Axoscope software (**Figure 2.4**). Force was calibrated by comparing force traces to traces obtained from a known amount of force. This was done by suspending weights from the transducer and converting to force using the equation: N=kg.ms-2 where 1kg is 9.8N.

### 2.5.2 Loading Tissue with Membrane Permeable Calcium Indicator Indo -1

To monitor changes in intracellular calcium concentrations $[Ca^{2+}]_i$ the ratiometric calcium indicator Indo-1 acetoxymethyl ester (Indo-1AM, Molecular Probes, Oregon, USA) was used. To load myometrial strips 50µg of Indo-1/AM was dissolved in 50µl of a solution of 200µl dimethyl sulphoxide (DMSO) containing 0.05g pluronic acid. This solution was added to 4ml of PSS and vortexed creating a 12.5µM solution. This solution was separated into two 5ml vials to which the dissected myometrial strips (no more than 6 strips per 2mls) were added. These were then incubated for 3 hours at room temperature on a rotating platform, once loaded tissue was transferred to PSS.

### 2.5.3 Loading Tissue with $pH_i$ Indicator SNARF

To monitor changes in $pH_i$ the ratiometric pH indicator caroboxy SNARF-1 (carboxy SNARF-1 AM, Molecular Probes, Oregon, USA) was used. To load myometrial strips 50µg of carboxy SNARF-1/AM was dissolved in 50µl of a solution of 200µl dimethyl sulphoxide (DMSO) containing 0.05g pluronic. This solution was added to 8ml of PSS and vortexed creating a 11µM solution. 4mls of this solution was separated into two 5ml vials to which the dissected myometrial strips (no more than 6 strips per 2mls) were added. These were then incubated for 2 hours at room temperature on a rotating platform, once loaded tissue was transferred to PSS.

### 2.5.4 Simultaneous Measurements of Calcium and Force or $pH_i$ and Force

All strips were stretched to a resting tension of 2mN to ensure the degree of stretch exerted to all samples was standardised across experiments. Loaded myometrial strips were transferred to a dissection dish to attach aluminium foil clips either end of the strip. These strips were then placed into a 1ml perfusion bath above an inverted microscope. The strips were held in place by attaching one clip to a fixed hook inside the perfusion chamber and the other to a hook attached to a force transducer connected to a digi data acquisition system. This system includes the software Axoscope which records the contractility data and output from the photo multiplier tubes (PMT's) for ratio metric measurements of indo-1AM and carboxy SNARF-1AM. Once the tissue was attached a stretch of 2mN was given as a standard resting tension for all experiments. In the bath the strips were super-perfused with physiological saline solution pH7.4 at 37°C and spontaneous contractions were left to equilibrate for 60-90miniutes. The inverted microscope objective was focused onto the tissue and the fluorescent dye now loaded in the tissue was excited by ultraviolet illumination from a xenon lamp at a wavelength of 340nm (indo-1AM), 530nm (Carboxy SNARF-1AM). Light emitted at both wavelengths for each dual emission indicator was detected by the photomultiplier tubes and recorded using Axoscope software (**Figure 2.4**). The rise and fall of intracellular calcium which is responsible for myometrial contractions is measured by the changes in the Indo-1AM ratio calculated from the shifts in the emission signals from the 400nm and 500nm photomultiplier tubes, as described above. The rise in intracellular pH is

measured by the changes in the SNARF-1 ratio calculated from the shifts in the emission signals from the 590nm and 640 photomultiplier tubes.

When incubation experiments were performed (**Figure 2.5**) for monitoring the effects of GYY4137, NaHS, and PSS (control) strips were placed on the rig for a control period and then incubated in test solution for 45 minutes, within a hot plate in a fume hood at 37$^{o}$C and then strips were re-attached between the fixed hook and force transducer and contractions monitored after 5 minutes of re-attachment.

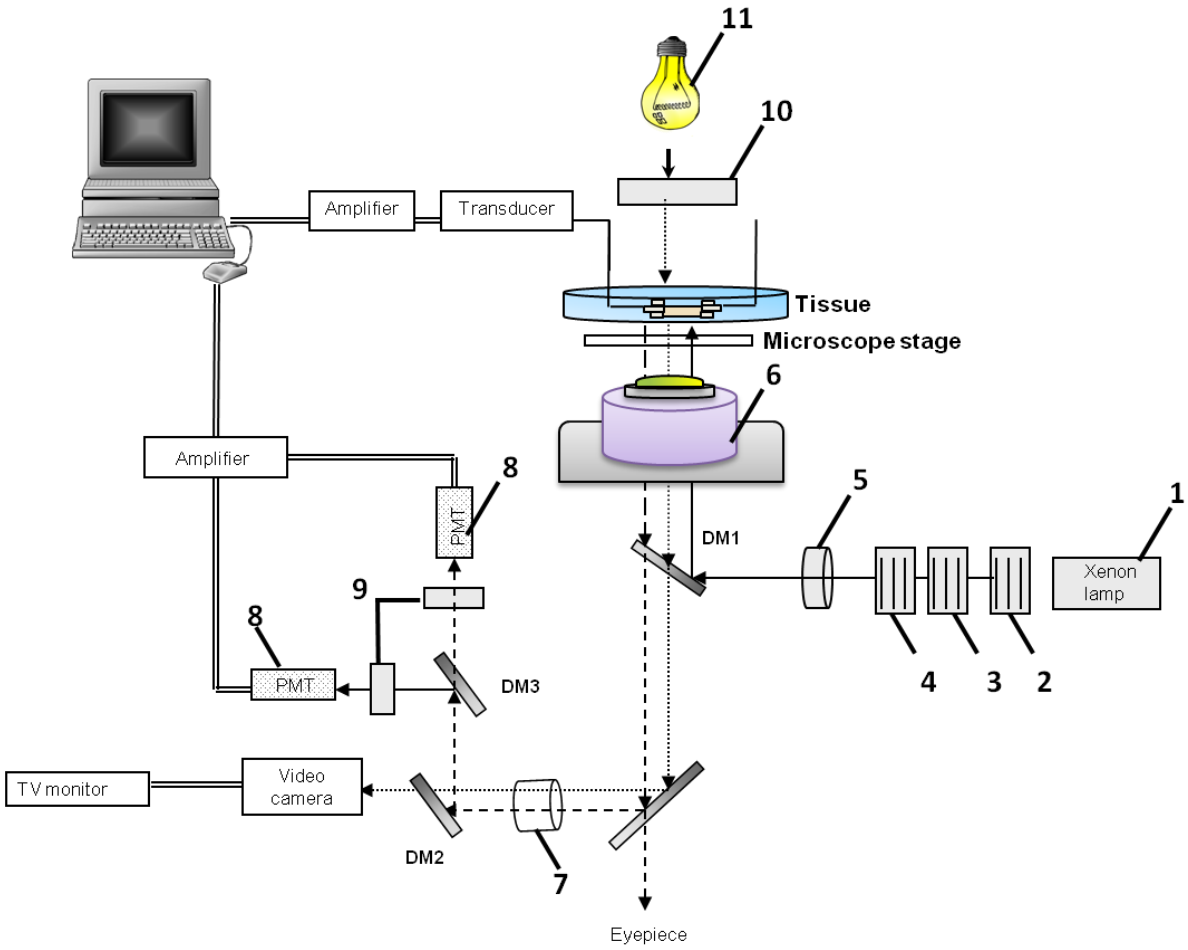



**Figure 2.4 – Diagram of Equipment designed to record Simultaneous Measurements of Force and Calcium or Force and intracellular pH ($pH_i$).**
To excite the calcium fluorescent dye Ultraviolet illumination is provided by a xenon lamp **(1)**. A heat filter **(2)** and neutral density filter **(3)** are placed in the path of light to reduce the light intensity and heat on the excitation filter **(4).** A mechanical shutter **(5)** in the excitation pathway keeps the tissue light exposure time to a minimum to prevent photobleaching. The excited light hits the dichroic mirror 1 (DM1) which is angled to reflect onto the indicator loaded tissue strip through a focused microscope objective **(6)**. The light emitted by the fluorescence indicator is then passed back through the microscope objective, through dichroic mirror1, reflected by a sliding mirror and directed through and adjustable diaphragm **(7)**. The light then hits a DM2 mounted at 45°. The longer wavelength emitted light is diverted to the video camera and the shorter wavelength light is passed through a DM3 directed to the photomultipliers (**8**, PMTs). The DM3 splits the emitted light and passes it through emission filters **(9)** of specific wavelengths for both indo-1 and 6-(and 5) Carboxy SNARF-1 emissions. To avoid interference from the microscope light with the fluorescence measurements, a long pass filter **(10)** is positioned in front of the microscope lamp **(11).**

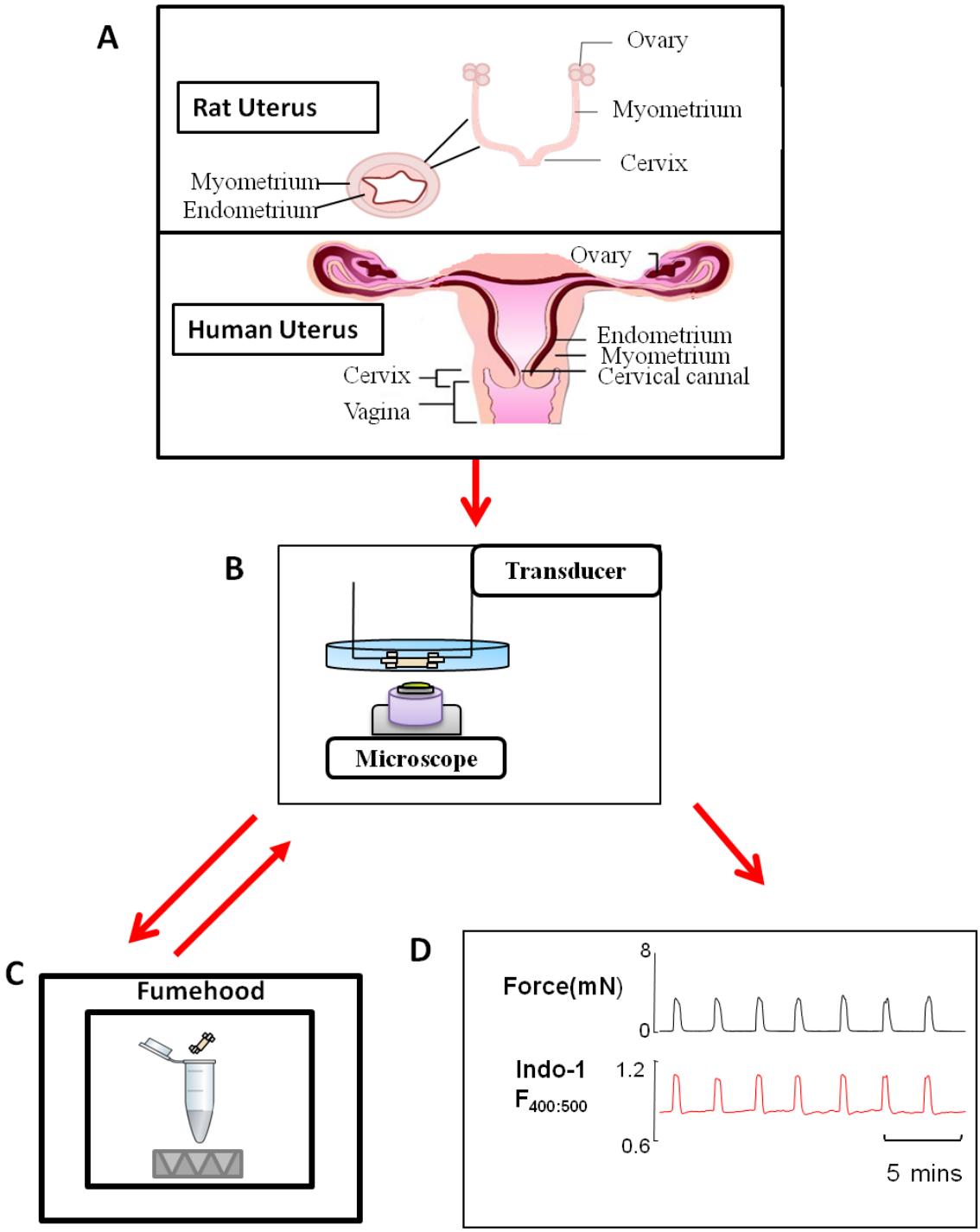



**Figure 2.5- Diagram of the incubation technique to monitor the contractility changes using GYY4137 and NaHS.** This was performed as $H_2S$ is a very potent gas and is poisonous at ppm levels. Firstly 1x5mm myometrial strips from rat of varying gestation and human tissue were dissected (A). Then once the strips were clipped up attached to a fixed hook and force transducer (same set up as Figure 2.4) and monitored for a control period (B). After establishing a control period strips were incubated in a fumehood at 37$^{o}$C for 45 minutes (C) and then placed back on the rig to monitor changes in contractility (B).All ultimately to produce a force trace, underlying calcium transients were also monitored using the same technique via the use of Indo-1 (D).

### 2.5.5 Measurements of Force alone

The same experimental setup as described in section 2.5.4 was used for force alone measurements except the tissue was not loaded with a fluorescent dye. For control periods, once spontaneous contractions were established the strips were left for approximately 30minutes – 60minutes to allow for a sufficient control period of contractions of similar force, duration and frequency to develop. Any treatments to be used were then either super-perfused through the tissue or incubated at 37$^{o}$C, pH 7.4 (**Figure 2.5**) and the effect on contractility was measured.

A time control showing a stable period of contractions is depicted in Figure **2.6**. These are 5 hour traces showing that my dissected myometrial strips can contract for over 5 hours before any decline in force is seen due to tissue fatigue within both animal and human tissue. An incubation control for both animal and human tissue is also shown showing no change in contractility in control PSS solution before and after incubation re-attachment (**Figure 2.7**).

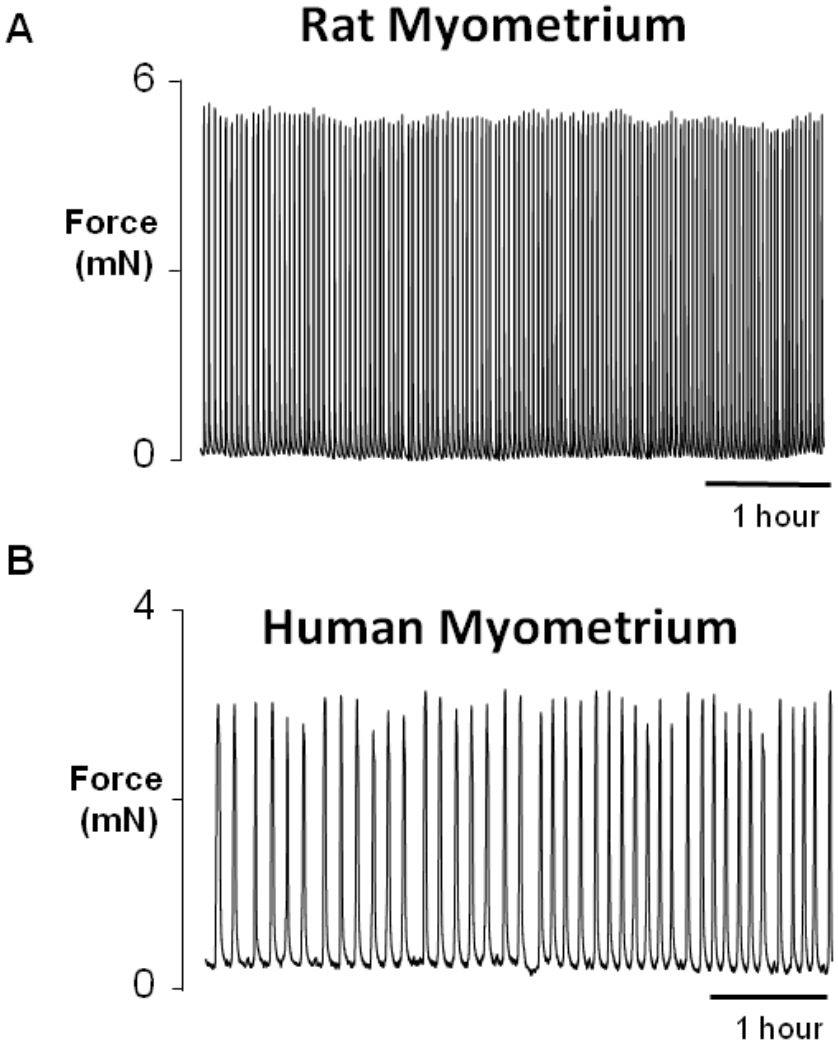



**Figure 2.6 Control traces** - showing a 5 hour period of myometrial contractions generated by a small longitudinal strip of myometrium dissected from A) a term rat myometrium and B) a term human biopsy.

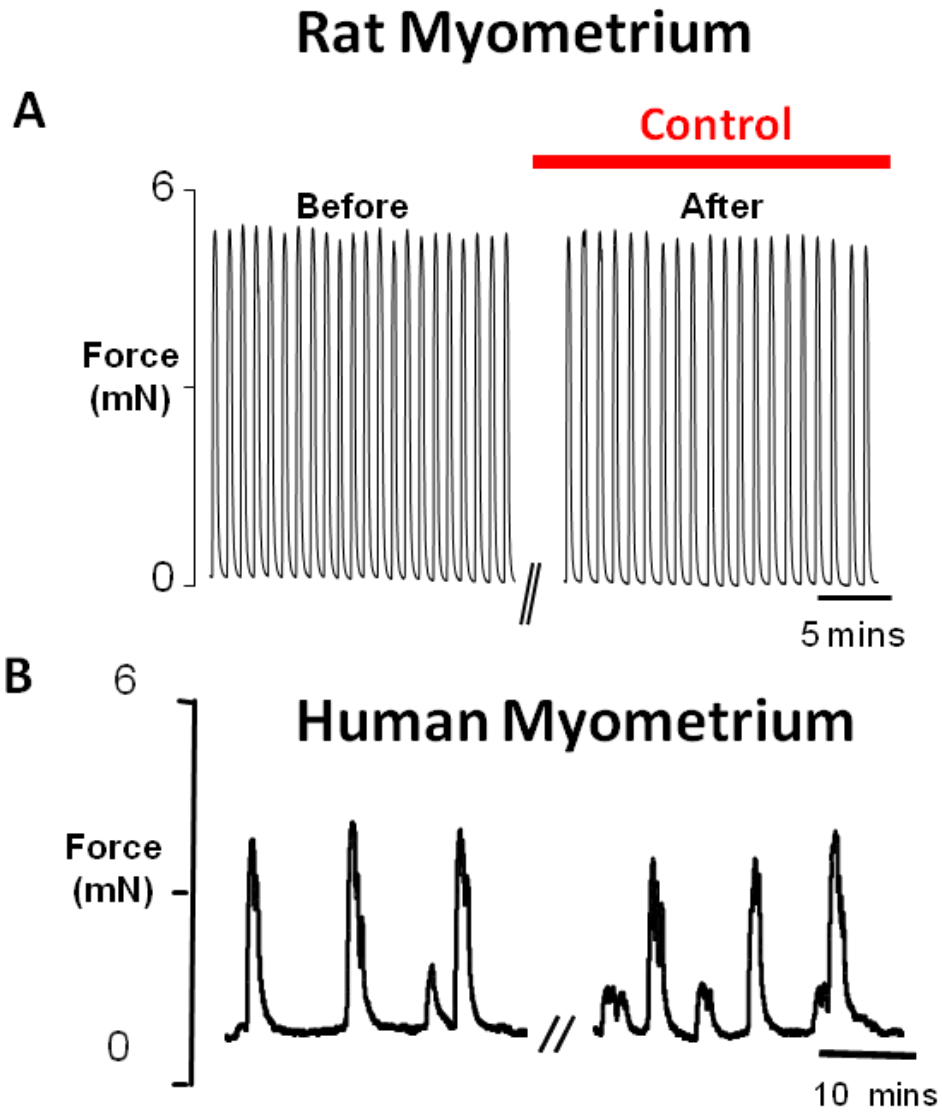



**Figure 2.7 Control incubation technique traces**. Control contractility before and after incubation in physiological saline solution (PSS) of a term rat (A)and human (B) myometrial strip . (Strips were placed under a resting tension of 2mN and superfused continually with physiological saline solution (pH 7.4) at 37°C before and after the 45 minute incubation periods in PSS or NaHS (pH 7.4) at 37°C.

### 2.5.6 Analysis of Contractility Data

There are four main parameters that were analysed for contractility data. All analysis was carried out in Origin Pro (Version 8.5).

**Force amplitude**

Force of contractions (mN) was measured by subtracting the value of the baseline from the peak of the contraction to be measured. An average of 10-30 minutes worth of contractions was taken for the control period which was then compared to the average obtained from any treatment periods. 10 minutes worth of contractions was assessed in the rat myometrium were as 30 minutes was monitored in the human myometrium to accommodate the slower rate of contractions.

**Duration**

Duration was measured by calculating the time (minutes) at the half maximal amplitude ($t_{50}$) point of the contraction. The duration of the same contractions used for the average force control period were measured and the same for the treatment period.

**Frequency**

A period of control contractions was selected and the time period between the beginning of one contraction to the beginning of the next was measured. This was done for 3-6 contractions and the average time was calculated. From this average the number of contractions in a ten minute period for rat contractility or 30 minute period for human contractility was calculated for both control and treatment periods.

**Area under the Curve (AUC)/Mean Integral of Force**

A period of 10-30 minutes control and treatment contractions were selected and the area under each contraction in the time period was measured. This gives an indication of the overall effects of frequency, duration and force.

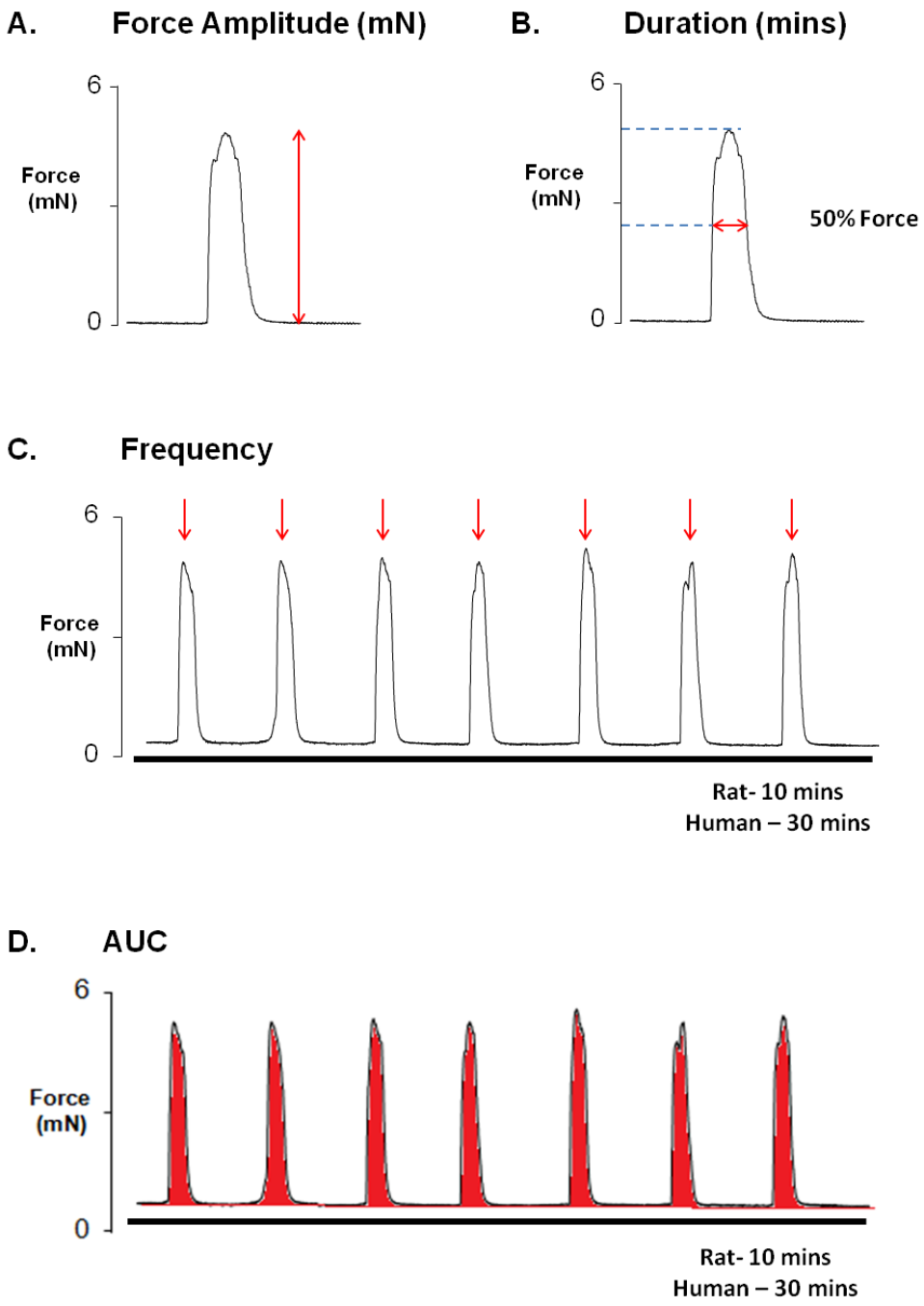



**Figure 2.8 Parameters measured for contractility.**

A) Force amplitude of contraction represented in force (mN) and corresponds to the height of the contraction (represented by the red arrow), B) Duration of contraction is represented as how long a single contraction lasts (minutes) and was measured at half the maximal peak of contraction (dotted blue lines show contraction height and half the contraction height, red arrow indicates duration), C) Frequency of contraction, D) AUC of contraction, a measure of the overall contractile activity. This data was obtained over 10 minutes for rat contractility and 30 minutes for human contractility.

## 2.6 Immunohistochemistry

### 2.6.1 Fixation of tissue for immunohistochemistry

Small pieces of myometrium approx 0.5mm x 0.5mm x 0.5mm were dissected and placed into neutral buffered formalin containing 10 % formalin. The fixed samples were left for 24 hours in the physiology fridge before transferring back to Liverpool Women's Hospital. The fixed samples were processed and embedded in paraffin wax by Kelly Harper at Liverpool Women's Hospital for human sections, and animal sections in the pathology department in the University of Liverpool Veterinary School and 4µm sections were cut, then mounted onto glass slides. Sections were cut from 6 pregnant and non-pregnant women as well as different stage gestation rats. For control tissues and antibody optimisation animal tissue was also used.

Aorta and Kidney were dissected from 22 day pregnant Wistar rats, also non-pregnant, 10, 14, 18, 22 day as well as labouring rat uterus were dissected. They were then placed in Formalin solution, neutral buffered, 10%. Embedding and sectioning was carried out in the pathology department in the University of Liverpool Veterinary School.

### 2.6.2 Staining for CBS and CSE

CBS enzyme – CBS monoclonal antibody, Abnova, clone 3E1 : 1:50

CSE enzyme- CTH monoclonal antibody, Abnova, clone 4E1-1B7 : 1:150

Both CBS and CSE antibodies have been used multiple times in recent literature (Rashid 2012, Kasparek 2011, Fu 2012).

Beta Actin - beta Actin antibody, Abcam : 1:500

Sections from human myometrium were labelled appropriately before combining in a metal rack. The rack was placed into a glass bath containing 100% Xylene for 30minutes to remove the paraffin wax from the section. The sections were then dipped in a series of ethanol baths from 100%. 95%, 85%, 70% and 50% in order to rehydrate the sample before being placed into a bath of distilled water for 5 minutes. The slides were then placed into a bath of boiling 14mM Sodium Citrate buffer pH6.0 in a microwave for 20mins to allow optimum antigen retrieval. The slides were allowed to cool for 20minutes on ice before washing with distilled water. Each slide was removed of excess water before circling the tissue section with

ImmEdge Hydrophobic barrier pen (Vector Laboratories) which allows reagents to remain localized. To block endogenous peroxidise activity 3% hydrogen peroxide (30% Hydrogen peroxide in tris buffered saline (TBS) solution) was dropped onto each tissue section and incubated for 30 minutes in a humid chamber. Slides were then rinsed with TBS-T (0.05% Tween20) and incubated for 1hour with non specific block 5% Bovine Albumin Serum (BSA) dissolved in 0.05% TBS-T. Slides were then rinsed again in TBS-T before overnight incubation with the appropriate antibody in blocking solution at 4°C. As a negative control one section from both non-pregnant hysterectomy and term pregnant patients, as well as a multiple stage rat myometrium slide, were incubated overnight in blocking solution alone. As a positive control an animal section that is known to express the protein of interest was used and for an antibody control beta actin was used.

The following day the primary antibody was removed by 3 X 15 minute washes with TBS-T

0.05% before incubating with secondary antibody at room temperature for 1hour (impress

Universal Antibody anti-mouse Ig /anti-rabbit Ig, peroxidise polymer detection kit, Vector Labs). The secondary antibody was then removed by 3 X 15minute washes with TBS-T

0.05%. As a HRP labelled secondary was used 3,3'-Diaminobenzidine (DAB) was used for developing (3,3′-Diaminobenzidine (DAB) Enhanced Liquid Substrate System tetrahydrochloride, Sigma). DAB was dropped onto the positive control slide first and placed under a microscope until a brown colour developed. The reaction was stopped by placing slide into distilled water. All slides from pregnant and non-pregnant human were developed for the same time to reduce any bias in the experiments; the same was done for the different stage gestation animal tissue. Once all slides were developed the slides were counterstained with Haematoxylin by incubating with the stain for 1 minute followed by washing with tap water for 5 minutes. The slides were then dehydrated by dipping in a series of ethanol baths from 50%, 70%, 85%, 95% and 100% before being placed into xylene. This removed any excess paraffin pen that was encircling the section. Cover slips were then mounted using DPX mountant and left to dry overnight before image capture.

### 2.6.3 Image Capture of Myometrial Biopsy Sections – DAB Stained.

The setup used was provided by Liverpool Women's Hospital university department and was composed of the following -

**1.** Nikon Eclipse 50i Microscope, Nikon Corporation, Tokyo 100-8331, Japan

**2.** Nikon DS-Fi1 digital camera Head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan

**3.** Nikon Digital control unit DS-U2 USB, Nikon Corporation, Tokyo 100-8331, Japan

**4.** Nikon C-Mount TV adaptor, 0.63x, Nikon Corporation, Tokyo 100-8331, Japan

**5.** NIS-Elements-F software, developed for Nikon Instruments

**6.** Personal computer (minimum specification 1GB RAM, 2.8GHz processor)

After launching the NIS- Elements-F software a slide was placed on the microscope stage and moved to an area that contained a section of myometrial biopsy. The settings were adjusted to normal mode, 640x480 resolution, 1280x960 quality capture, and high colour contrast and sharpness. The microscope was calibrated using a scale slide for each objective

– 4x, 10x and 40x. The whole tissue section was scanned on 4x objective and 10 x objective to examine which areas would be most representative of the entire tissue for photographing. The negative control was examined first to make sure there was no staining

– if any staining was present then the experiment would have to be repeated. All slides were blinded to the observer to reduce any bias. After locating the areas with a high amount of staining the 40x objective was used to photograph 10 different areas of the section. This was repeated for all sections in each immunohistochemistry.

## 2.7 Western Blotting

### 2.7.1 Protein Extraction

A small section of myometrium was dissected, blotted onto filter paper and weighed to obtain a maximum of 1 gram of tissue. This was done for compiled non-pregnant, 14, 18, and 22 day rat myometrial strips as well as pregnant and non-pregnant hysterectomy human. Once weighed the samples were snap frozen with liquid nitrogen and stored at -20°C. Once a sufficient number of samples were reached the tissue was removed from the freezer, diced with a scalpel and ground to a fine powder keeping it cold with liquid nitrogen before placing into protein extraction buffer – RIPA (Radio-Immunoprecipitation Assay) Buffer (1xPBS, 1.0% IGEPAL,0.5% Sodium Deoxycholate, 0.1%Sodium dodecyl sulphate). Just before use 30µl of100mM Sodium orthovanadate, 90µl Aprotinin, and 30µl of 10mg/ml of PMSF (phenylmethanesulfonylfluoride) was added to each 3ml of RIPA. For each 1g of tissue 3ml of prepared RIPA buffer was added, the tissue was then homogenised in 5ml Bijou tubes using IKA Ultra Turrax T258N on level 5. The homogeniser was cleaned between each protein extraction and the samples all placed on ice. The homogenate was transferred to Eppendorfs and centrifuged at 12,000 RPM at 4°C for 10minutes. The supernatant was transferred to new Eppendorfs tubes and recentrifuged at 12,000 RPM at 4 C for 10minutes. Any samples that were in separate Eppendorfs were pooled and mixed, aliquoted and stored at -20°C.

### 2.7.2 Protein Assay of Extract from Human Myometrial Biopsies

A detergent compatible (Dc) technique was used to quantify the level of protein within samples. Bio-Rad *DC* Protein Assay is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay, but with the following improvements: The reaction reaches 90% of its maximum colour development within 15 minutes thereby saving valuable time, and the colour changes not more than 5% in 1 hour or 10% in 2 hours after the addition of reagents.

The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead

to colour development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm.

The protocol I used was firstly to make a 2ml/mg protein standard stock (0.01g BSA in 5ml RIPA) using nominal protease free bovine serum albumin (BSA). Then from this, solutions of 1, 0.5, 0.3, 0.25, 0.1 and zero mg/ml standards were produced within testubes. Extracted protein samples were defrosted and diluted in RIPA (usually 1:50) within test tubes. All solutions were then vortexed. The Dc reagent was then prepared, 20 µl of reagent S to each ml of reagent A (an alkaline copper tartrate solution). 500 µl of this reagent was then added to each of the standards and proteins of interest (a solution of 100 µl ). Then addition of 4mls of reagent B(a dilute Folin Reagent) was added to each test tube and vortexed immediately. After 15 minutes absorbances could be read using a wavelength of 750nm. A standard curve was produced each time the assay was performed and used to acquire protein concentrations of test solutions.

### 2.7.3 Preparation of Protein Samples for Western Blotting

Once the protein content for the samples had been calculated the samples were then prepared to run western blots. 25µg of protein was prepared in 3x loading buffer/Laemmili buffer (1M Tris-HCl pH 6.8 2.4ml , 20% Sodium dodecyl sulphate 3ml, Glycerol 3ml, Bromophenol blue 6mg, beta mercaptoethanol 1.6ml). The samples were then boiled at 100°C for 5minutes, placed into ice ready for loading onto a gel.

### 2.7.4 Western Blotting

**Antibodies**

CBS enzyme – CBS monoclonal antibody produced in mouse, Abnova, clone 3E1, – Dilution 1:250

CSE enzyme- CTH monoclonal antibody produced in mouse, Abnova, clone 4E1-1B7, – Dilution 1:200

TST enzyme – Anti-TST antibody produced in rabbit, Abcam, ab96543– Dilution 1:1000

Beta Actin - beta Actin antibody produced in mouse, Abcam, Ab8227, – Dilution 1: 5000

Secondary Antibody - Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated, A0545 1:100,000

Secondary Antibody – Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated, A2554, – Dilution 1:10000

1mm spacer plates and glass plates were cleaned using 70% Ethanol and distilled water before being assembled into a casting kit (Omni PAGE kit). 12% acrylamide tris-HCL resolving gels (30% Acrylamide:Bis-Acrylamide ,distilled water, 1.5M Tris-HCL pH8.8, 10% SDS, 10%APS, TEMED) were cast, overlaid with distilled water and left to polymerise for 1hour at room temperature. The distilled water was poured off and a 2.5% acrylamide stacking gel was cast (30% Acrylamide:Bis-Acrylamide, distilled water, 0.5M Tris HCl pH 6.8,10% SDS, 10% APS, TEMED) and 1mm 10 well combs inserted. This was then left to polymerise for 30minutes. Once the gels were fully polymerised they were inserted into the gel tank containing fresh running buffer (25mM Tris-base, 192mM glycine, 0.1% SDS, distilled water, pH8.3) and the combs were removed. 8µl of protein standard was added to wells on both ends of the gel (SeeBlue® Plus2 Pre-Stained Standard) and 20µl of the prepared proteins were added to the remaining wells. The gel was then run at 150 volts for 90 mins or until the loading buffer ran off the end of the gel. The gels were then removed from the tank and moved to blotting cassettes for protein transfer to nitrocellulose membrane (Whatman Protran nitrocellulose membrane).

The assembled cassettes were placed into the gel tank on top of a magnetic stirrer surrounded by ice. Transfer buffer was poured into the tank (Tris-Base, glycine,

distilled water, 10% methanol) and the tank was run at 40V for 1hour at room temperature. Once transferred the nitrocellulose membranes were placed into blocking solution (5% BSA dissolved in TBS-T 0.1%) for 2 hours. The antibodies to be used were diluted to the appropriate concentrations in prepared antibody solution (TBS, 0.1%BSA, 0.1% Sodium Azide) and the membranes were incubated in containers overnight at 4°C on a rocker. The following day the primary antibody was removed and the membranes were washed 3x10minutes with TBS-T 0.1% then incubated with the appropriate secondary antibody diluted in 5% BSA for 1hour at room temperature. The membranes were then washed for 1x 15mins and 4 x 5minutes before incubation for 5minutes with chemiluminescent (ECL) substrate for horseradish peroxidase (HRP) enzyme (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). The membranes were then transferred to a film cassette where they were exposed to film for the required amount of time (CL-XPosure Film, ThermoFisher scientific). The films were then developed using Kodak processing chemicals developer and fixer.

### 2.7.5 Analysis of Western Blots

Analysis of Western blots was carried out in Image J
(http://rsb.info.nih.gov/ij/index.html).
The original blot was scanned and saved as a TIFF file. The image was then opened in ImageJ. Using the rectangular selection tool a rectangle was drawn around the first band in the image. This same rectangle is then used to select every band on the image. Once all have been selected a profile plot is generated showing peaks which represent the density of each band. Using the wand tool each peak was highlighted which gave the area and percentage of each peak. This process was repeated for the loading control used (actin) so that there were two set of percentage values – one for Actin and one for the samples. The percentage value for the sample was then divided by the loading control to get an adjusted density for each sample.

## 2.8 Statistics

For statistical analysis and construction of graphs and tables Microsoft excel, origin 8 and Graphpad Prism 5 were used.

All data was normally distributed. The standard deviation, standard error of the mean were calculated. As the data followed the Gaussian distribution Student's t-test,
ANOVA, and Spearman's correlation were used, as detailed in each results chapter.

For all tests significance was taken as a p value less than 0.05 ($p<0.05^*$).

# Chapter 3 -

## Characterisation of non- pregnant human myometrium: Examining the differences in myometrial contractility from pre-and post menopausal women and from women with endometriosis

### 3.1 Abstract

This chapter characterises the contractile activity of non-pregnant human myometrium particularly looking at age of women, menopausal state and the effect of endometriosis. Characteristics of spontaneous and high $K^+$ depolarisation-induced contractile activities of each tissue type are discussed.

Myometrial tissue was obtained from 40 women aged between 27-74 years and contractility was assessed. The contractile functions of the uterus and the changes it undergoes from pre-menopausal to post-menopausal state will be discussed and any changes in myometrial contractility and responses as women age. Here its indicated that in the non-pregnant state there is a significant decrease in contractility for both spontaneous and depolarised-induced contractions, with age. Hence, concluding that there is a decrease in the contractile activity of the myometrium in the post-menopausal state. Muscle atrophy and down regulation of Ca channels may account for this.

Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity. Altered contractions of the uterine muscles (myometrium) may be involved in the pathogenesis, as they may increase the back flow of menstrual debris in to the pelvic cavity, where it can be implanted and give rise to endometriotic deposits. I found that the forces of myometrial contractions from women with endometriosis are decreased when compared to fertile, healthy, pre-menopausal women, having a hysterectomy for menorrhagia, irregular bleeding, or pelvic pain, but the frequency of these contractions increased. These data suggest a potential involvement of altered myometrial activity in women suffering with this condition.

In summary this data provides the most extensive *in vitro* characterisation of non-pregnant human myometrium to date and shows significant effects of aging and endometriosis on its activity.

## 3.2 Introduction

The smooth muscle of the uterus, the myometrium, is active throughout a woman's life, not just during labour and delivery. The mechanism generating the contractile activity and its physiological regulation are reasonably well understood and have been described within **Chapter 1**.

The purpose of this chapter is to characterise and consider how aging, menopausal state and endometriosis may affect uterine function in non-pregnant women. Characterising this tissue is necessary background information needed to further my work on the effects of $H_2S$ on the non-pregnant compared to the term pregnant human myometrium. There has however been little previous *in vitro* work investigating non-pregnant myometrium, discussed below.

The first recording of contractile activity in the non-pregnant uterus was performed by Heinricius using a balloon technique in 1889 (Heinricius 1889). The main research investigating myometrial function in non-pregnant women over the last two decades have been obtained from the use of open-tipped pressure catheter recordings, 3D ultrasound, and magnetic resonance imaging (Brosens 1998, van gestel 2003, Bulletti 2004, Bulletti and de Ziegler 2006). The contractions observed during the menstrual cycle have been termed 'endometrial waves' (Ijland 1996). These contractions appear to involve only the sub-endometrial layer of the myometrium (Aguilar 2010). After menstruation, in the early follicular phase, contractile waves occur once or twice per minute and last 10–15s with low-amplitude (usually, 30 mmHg). As ovulation approaches, the frequency increases to 3–4 per minute. During the luteal phase, the frequency and amplitude decrease possibly to facilitate implantation. When a blastocyst does not implant, the contraction frequency remains low but the amplitude increases considerably (50–200 mmHg) producing labour-like contractions at the time of menstruation (Aguilar 2010). However there are few *in vitro* studies performed on non-pregnant human myometrial strips. Domali *et al* showed the effects of endothelin-1 (ET1) on the myometrial contractility of pre and postmenopausal women showing observations from segments of equilibration periods from both groups, concluding pre-menopausal myometrium contracted

quicker, and were more frequent than post menopausal myometrium, however no analysis was performed. Although the main finding of this research was the long-lasting effectiveness of ET1 in strips collected from postmenopausal women compared with premenopausal women, perhaps the effect of ET1 is enhanced by the oestrogen deficiency after menopause. They also found that there was no change in the response to KCl in either group and suggested that KCl and ET1 affect uterine contractility through different mechanisms and that ovarian steroids may play a regulatory role in human uterine responsiveness to ET1 (Domali 2000). The only other study using strips mounted in organ baths demonstrated smaller contractions produced by the non-pregnant myometrium when compared to non labouring term pregnant myometrium, whilst time between contractions was lower in the non-pregnant myometrium. This study also suggested that SK/IK channels are present and functional in myometrium from pregnant and non-pregnant women (Rosenbaum 2012). This chapter will explore more extensively, the statistical differences in contractility of the non-pregnant myometrium, specifically in pre and post menopausal women.

In non-primate species, the myometrium consists of two distinct layers—an outer longitudinal layer and an inner circular layer. However, in the human, the myometrial substructure is not so well defined (Huszar and Naftolin, 1984). Looking at the oestrus cycle in the non-pregnant rat, four distinct periods are found: pro-oestrus, oestrus, metoestrus and dioestrus. Pro-oestrus typically lasts 12–14 h, oestrus 25–27 h, metoestrus is shortest lasting 6–8 h and dioestrus lasts 55–57 h (38, 39). Mating occurs during the oestrus period (‘heat’) and as rats are nocturnal breeders, under normal lighting conditions, oestrus occurs overnight, typically commencing between 16.00–22.00 h. Ovulation is timed to coincide with copulation, and usually occurs 8–11 h after the onset of oestrus (Hafez 1970). During pro-oestrus electrical and mechanical activity of the rat myometrium has shown a relative quiescence with little propagation of any electrical events. Noble et al showed using longitudinal myometrial tissue strips that $Ca^{2+}$ signalling and mechanical activity are greatest in metoestrus and dioestrus compared to pro-oestrus and oestrus (Wray 2008). $Ca^{2+}$ signalling still needs to be addressed in the non- pregnant human myometrium.

The first contractions felt by a woman are those occurring once menstruation has started, possibly those giving rise to uterine cramping (dysmenorrhoea) often associated with menstruation. Non-pregnant myometrium has been shown to undergo different patterns of contractility during the menstrual cycle; one being 'focal and sporadic bulging of the myometrium', (Togashi 2007; Togashi et al. 1993) giving rise to sustained contractions and the other, rhythmic, 'wave-like' contractions, sometimes called uterine peristalsis, which are thought to aid in the sloughing of the endometrium (Bulletti et al. 2000; de Vries et al. 1990;
Lyons et al. 1991). The female steroid hormones change during the menstrual cycle and influence the pattern of myometrial activity in women and other animals (Wray and Noble 2008). These hormones will also control levels of ATP and other metabolites needed for contraction (Crichton et al. 1993; Wray and Tofts 1986) and can affect excitability (Parkington et al. 1999). Recordings of uterine pressures in the non-pregnant uterus as well as MR imaging have shown that the pattern of myometrial activity, such as the direction of contraction propagation throughout the uterus varies with the different phases of the menstrual cycle, already discussed (Bulletti et al. 2000; Kunz and Leyendecker 2002; Nakai et al. 2003; Togashi 2007), although diurnal variations, have not been observed (Kido et al. 2006). The pattern of contractile activity in the non-pregnant uterus is closely related to uterine function. Thus antegrade contractions, that is contractions propagating from the fundus towards the cervical end of the uterus, favours forward emptying or discharge of uterine content i.e. menstrual blood, (Lyons et al. 1991) whilst cervico-fundal contractions aid in sperm transport or possibly in retention of iron for example, following blood losses at menstruation (Kunz and Leyendecker 2002). In the pregnant uterus, these retrograde contractions may also have roles in the maintenance of early pregnancies within the uterine cavity (de Vries et al. 1990), but possibly also in causing endometriosis, as menstrual debris enters the peritoneal cavity, discussed below.

The menopause is another major feature in a woman's life. With the gradual decline in hormonal secretion from the ovaries, up to the point when the ovaries cease to function, the menopause is characterised by a loss of regular menstruation and

eventually permanent cessation of menses (Burger et al. 2002). One might assume that following the menopause, uterine activity ceases. However, rhythmical myometrial contractions have been seen by ultrasound examination in postmenopausal women (de Vries et al. 1990) and one previous study has reported spontaneous activity in strips from human post-menopausal myometrium *in vitro* (Domali et al 2001). However to date no study has looked at the function of myometrium from women much over 40, and certainly not for women in their 50s or even older, nor at the activity of the non-pregnant uterus with age, menopausal state described within this chapter.

There is growing awareness of the potential importance of abnormal myometrial function in common disorders of reproduction. Endometriosis is one of the commonest benign gynaecological conditions; it causes severe pelvic pain, painful periods, painful sexual intercourse and infertility. Endometriosis has a prevalence of 38.5% and 5.2% in infertile and fertile women, respectively (Wheeler 1989, Verkauf 1987). Despite the common occurrence and the huge economic burden of endometriosis, the many biological studies using a range of models have not yet identified the causative mechanisms. Changes in myometrial contractions could be involved in increasing the back flow of menstrual debris in to the pelvic cavity, giving rise to endometriotic deposits. Only *in vivo* techniques have been investigated to look for changes in myometrial contractility in response to endometriosis. In 1995, Salamanca and Beltran in a study of inner myometrial contractility using transvaginal sonography in women with endometriosis found a predominantly retrograde pattern of subendometrial contractions during menstruation (Salamanca 1995). In 2002, intrauterine pressures were recorded showing increased frequency, amplitude and basal pressure tone within infertile endometriosis patients when compared to infertile patients without endometriosis (control). Retrograde bleeding was found in 73% of these patients with endometriosis compared to 9% of the control group. This study also shows 45% endometrial debris within the cul-de-sac of endometriosis patients compared to 0% in controls which could implant (Bulletti 2002). Both studies indicate abnormal alterations of uterine contractility at the time of menses are involved in the development of endometriosis. An interesting histological study on human non-pregnant myometrial sections, staining for nerve fibres illustrated

increased nerve fibre density in both the endometrium and myometrium of endometriosis women implying a role in the mechanism of pain generation in this disease (Tokushige 2007). This chapter is the first *in vitro* study to look at myometrial contractions of women with endometriosis.

The aims of this chapter were:

1) to characterise the *in vitro* contractile activity of the non-pregnant human myometrium

2) to compare activity between pre- and post-menopausal women,

3) to examine the influence of age on contractility

4) to examine the effects of women suffering from endometriosis on contractility

## 3.3 Methods

### 3.3.1 Tissue

Non-pregnant human myometrial tissue was obtained from women undergoing a hysterectomy. I was blinded to the details of the sample until experiments had been performed and analysed. Once performed details including age, menopausal state, medication, reason for hysterectomy and whether they had endometriosis was obtained. Further details of how the tissue was obtained are described in the general methods section, **Chapter 2.**

Non-pregnant samples were obtained from hysterectomy specimens. The indications for surgery were as follows: menorrhagia (25), prolapse (8), irregular bleeding (1), pelvic pain (2) and not stated (4). Therefore the total number of non-pregnant samples in this study group was 40, with 22 women in the pre-menopausal group, 8 in the post menopausal group and 10 in the endometriosis group (all pre-menopausal). The endometriosis group was then separated into no medication including women with mirena coil (n=6) and those on medication (n=4). Medication included gonadotropin-releasing hormone therapy (GnRHa,2), hormone therapy (1) and Provera (1). All surgery was performed under general anaesthesia and the biopsy was removed immediately following removal of the uterus at surgery. The median age of all 40 women studied was 52.2 years.

Endometrium was scrapped off all biopsies. Looking down the microscope all strips dissected were clearly muscular. The tissue size used for all experiments was standardized at 5x2x1mm. Within hysterectomy samples, the tightly bound muscle fibres, made dissection of individual muscle fibres more challenging compared to the pregnant tissue in which identification of the longitudinal muscle fibres was easier. Strips could be attained just as easily in the non-pregnant human myometrium from pre, post-menopausal and endometriosis samples.

### 3.3.2 Measurement of tension

Tissue preparation and measurements of tension are the same as those described in **Chapter 2.**

### 3.3.3 Solutions

PSS and High $K^+$ solutions were produced as described in **Chapter 2**.

### 3.3.4 Statistics

Contractility measurements were made and analysed blinded to the woman's age, menopausal state, and condition. Significance was tested by Student's t-test with $P<0.05$ taken as the significance value. When more than two groups were tested ANOVA with Bonferroni post hoc test were performed. All data was normally distributed. All values represent the mean ± s.e.m where 'n' is the number of samples with each representing a different woman. In some cases results are expressed as percentage of control contractions were control is 100%.

## 3.4 Results

Data were obtained from 40 non-pregnant hysterectomy samples. These samples varied in age, (From 27 to 74 years) and menopausal status, 32 pre-menopausal, including 10 who had endometriosis and 8 post menopausal women. These details and other relevant information is given in **Tables 3.1** and averages in **Table 3.2**.

For analysis of contractile parameters, including time to establishment of activity, data are first analysed and presented from the entire 40 patients and then divided into the following sub-groups (1) menopausal status, (2) the effects of endometriosis and (3) the effects of medication. There was no significant difference between BMI, and time to spontaneously contract in any of the sub groups. However age was significantly increased in the post–menopausal group as expected. These differences are explored later in this chapter.

**Table 3.1 – Demographics for non-pregnant women -** showing Age, reason for hysterectomy, BMI, medication and if they had endometriosis or not for each patient (n=40)

| Age (years) | Reason for Hysterectomy | BMI | Medication | Endometriosis? | Pre or Post menopausal? |
|---|---|---|---|---|---|
| 45 | Menorragia | 25.3 | NONE | NO | Pre |
| 50 | Not stated | 25.2 | NONE | NO | Pre |
| 48 | Menorragia | 22 | Mirena coil | NO | Pre |
| 34 | Irregular bleeding | 26.1 | Implanon | NO | Pre |
| 44 | Menorragia | 28 | NONE | NO | Pre |
| 37 | Menorragia | 23.7 | Mirena coil | NO | Pre |
| 43 | Pelvic pain | 28.2 | NONE | NO | Pre |
| 27 | Menorragia | 22.7 | Tranexamic and mefenamic acid | NO | Pre |
| 46 | Menorragia | 21 | NONE | NO | Pre |
| 32 | Not stated | 27.2 | Mirena coil | NO | Pre |

| | | | | | |
|---|---|---|---|---|---|
| 44 | Not stated | 23.7 | Norethisterone until previous day | NO | Pre |
| 27 | Not stated | 23 | NONE | NO | Pre |
| 44 | Menorragia | 30.4 | Mirena coil | NO | Pre |
| 45 | Menorragia | 29.4 | Mirena coil | NO | Pre |
| 48 | Menorragia | 29.9 | NONE | NO | Pre |
| 37 | Pelvic pain | 29 | NONE | NO | Pre |
| 46 | Menorragia | 35.4 | NONE | NO | Pre |
| 46 | Menorragia | 23.2 | NONE | NO | Pre |
| 46 | Menorragia | 27.4 | NONE | NO | Pre |
| 31 | Menorragia | 24.9 | NONE | NO | Pre |
| 27 | Menorragia | 23.8 | NONE | NO | Pre |
| 46 | Menorragia | 29 | NONE | NO | Pre |
| 44 | Menorragia | 34.9 | NONE | Endometriosis (stage1) | Pre |
| 48 | Menorragia | 22.9 | NONE | Endometriosis (stage1) | Pre |
| 39 | Menorragia | 29.4 | Mirena coil | Endometriosis (stage1) | Pre |
| 47 | Menorragia | 25.5 | NONE | Endometriosis (stage 3) | Pre |
| 44 | Menorragia | 33.3 | Mirena coil | Endometriosis (stage 3) | Pre |
| 31 | Menorragia | 21.0 | Mirena coil | Endometriosis (stage 4) | Pre |
| 39 | Menorragia | 27.2 | GnRHa | Endometriosis (stage 4) | Pre |
| 47 | Menorragia/Fibroids | 31.2 | GnRHa | Endometriosis (stage 2) | Pre |
| 39 | Menorragia | 25.9 | Hormone therapy | Endometriosis (stage 2) | Pre |

| | | | | | |
|---|---|---|---|---|---|
| 45 | Menorragia | 29.2 | Provera | Endometriosis | Pre |
| 71 | Prolapse | 24.1 | HRT 17 years | NO | Post |
| 62 | Prolapse | 24.4 | HRT 6 years | NO | Post |
| 47 | Prolapse | 28.0 | NONE | NO | Post |
| 63 | Prolapse | 28.1 | NONE | NO | Post |
| 58 | Prolapse | 20.0 | NONE | NO | Post |
| 69 | Prolapse | 24.7 | NONE | NO | Post |
| 62 | Reduce risk of ovarian cancer | 32.2 | NONE | NO | Post |
| 74 | Prolapse | 32.0 | NONE | NO | Post |

Rows highlighted in pink are pre-menopausal women, in purple are pre-menopausal women with endometriosis and in blue are post-menopausal women.

**Table 3.2 – Summary of demographics of all 40 non-pregnant women as well as separated into subgroups of menopausal status (pre and post menopausal women) and endometriosis-** showing average age, BMI and times to contract for each group.

**Averages**

| Age (years) | BMI | Subgroups | Time to spontaneous activity (mins) |
|---|---|---|---|
| **45.6** | **26.8** | **All women (n=40)** | **43.7** |
| **41.1** | **26.8** | **Pre-menopausal (n=32)** | **48.5** |
| **63.2*#** | **26.7** | **Post menopausal (n=8)** | **24.7** |
| **42.3** | **28.1** | **Endometriosis (n=10)** | **54.7** |

* Represents a significant difference between pre-menopausal and post menopausal, and # between pre-menopausal and endometriosis patients using ANOVA with Bonferroni pos hoc test

### 3.4.1 Establishment of spontaneous contractions *in vitro.*

All tissues were superfused with physiological salt solution (PSS) at 37 $^{o}$C, pH 7.4 until spontaneous contractile activity was established. In general from my observations the commencement of contractions occurred within 1 hour of continuous perfusion, average time to contract for entire 40 patients was 43.7±4.5 minutes. Once contractions reached equilibrium, contractions were monitored for 1-2 hours and then high $K^+$ solution applied to enable maximal force to be obtained.

All samples contracted apart from those dissected from a 71 year old woman, the second oldest in this cohort.

**Table 3.3 Contractile parameter averages for the entire 40 non-pregnant patients studied within this chapter.**

| | **Mean (± S.E.M)** |
|---|---|
| **n- number** | 40 |
| **Amplitude (mN)** | 2.4mN±1.6 |
| **Frequency (no. Per 30 minutes)** | 6.4± 0.6 |
| **Duration ( minutes)** | 0.9 ± 0.3 |
| **AUC (30 minutes)** | 9.1± 1.0 |
| **High $K^+$ response (mN)** | 2.4± 0.4 |

Amplitude of contraction is peak force (mN); Frequency of contraction is the number of contractions occurring in 30 minutes; Duration of contraction is duration of contraction (min) measured at the half maximal peak of contraction; AUC is a measure of the overall contractile activity occurring in 30 minutes and High $K^+$ response is the maximal force of contraction measured in mN.

### 3.4.2 Comparing the contractility of pre-menopausal women with post menopausal women.

Firstly, the contractility of all 40 women in **Table 3.1** was assessed in response to menopausal status. The average age of this group was 45.6 and average BMI was 26.8 as found in **Table 3.2**.

**(a) Contraction amplitude**

The mean force amplitude of spontaneous contractions recorded for post-menopausal women (n=8) was significantly reduced, 0.3±0.1 mN, compared to pre-menopausal women (n=32), 2.9 ±0.4 mN. Representative recordings of spontaneously contracting myometrium from pre-menopausal and post menopausal women are shown in **Figure 3.1**.

**(b) Frequency**

Frequency of contractions was greater (**Figure 3.1**) for post-menopausal women, 8 ± 1.8 contractions per 30 minutes; pre-menopausal women: 6 ± 1.3 contractions per 30 minutes. No significant difference was found.

**(c) Duration**

The mean duration of contraction was shorter (**Figure 3.1**) in post-menopausal women; 0.5 ± 0.2 minutes, pre-menopausal women; 1.0 ± 0.1 minutes. The difference however did not reach significance.

**(d) Area under the curve (AUC, 30 minutes)**

AUC of contractions, which is an index of the total work done by the tissue over a given time period, was calculated by measuring the area under the contraction curve in 30 minutes. For post-menopausal women mean AUC calculated was 1.4± 0.6 a.u compared to 11.1± 1.6 a.u recorded for pre-menopausal women. Contractile activity of post-menopausal women was significantly reduced compared to pre-menopausal women.

**(e) High $K^+$ depolarisation-induced contractions**

Depolarisation was produced by external elevation of [$K^+$] in the PSS to 40mM for 2 minutes. Under application of a $K^+$ depolarisation stimulus, the pattern of contractile activity is noticeably increased compared to spontaneous phasic contractions, high $K^+$ producing a maintained 'tonic like' force of contraction. As the response to high $K^+$ is thought to measure the maximal contractile activity that the tissue can yield via membrane depolarisation and consequently opening of L-type calcium channels, I examined and compared this response in both the pre and post-menopausal groups. The high $K^+$ response achieved by post-menopausal myometrium was significantly lower, 0.5±0.2mN compared to the pre-menopausal myometrium 2.7± 0.4mN.

**(f) Time to commencement of spontaneous activity**

Spontaneous contractions occurred *in vitro***,** in pre-menopausal myometrium (n=32) after 48.5 ± 5.0 minutes compared with post menopausal (n=8) myometrium after 24.7± 7.4 minutes. This was proven to be a significantly lower time to spontaneously start contract in the post menopausal women using a Student's t-test.

**Table 3.4** summarises the mean values of contraction amplitude, frequency, duration, AUC and High $K^+$ responses of spontaneous contractions as well as the mean time to spontaneously contract in pre and post-menopausal myometrium (n=40).

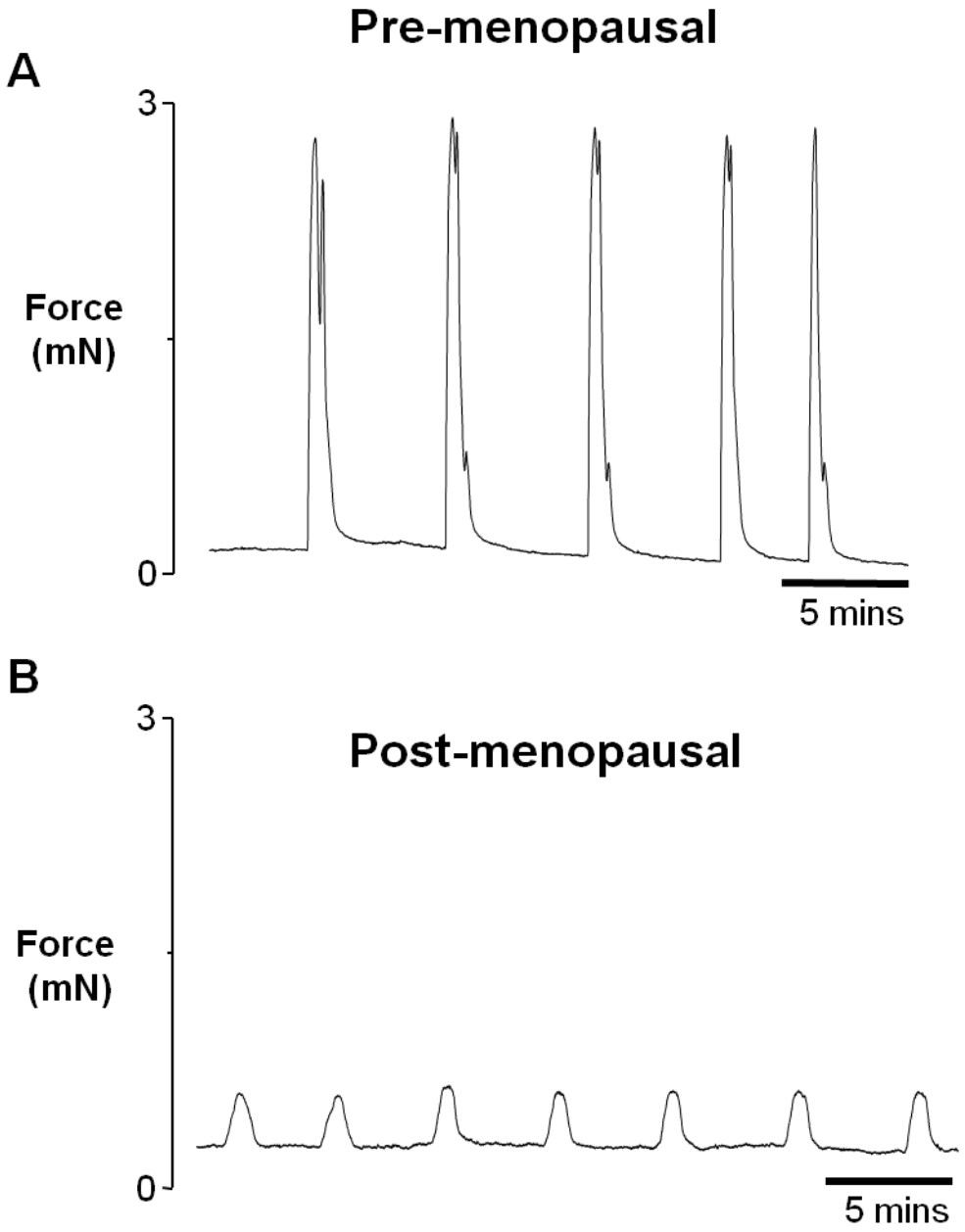



**Figure 3.1 Contractility of pre and post-menopausal women.**

Representative recordings of spontaneous contractions of human non-pregnant myometrium from women that have undergone hysterectomy's; and are in (A) pre-menopausal and (B) post-menopausal. In this and subsequent figures, the tissues were superfused with PSS (pH 7.4) at a flow rate of 1.5ml/min and maintained at 36-37 $^{o}$C.

**Table 3.4 Summary of spontaneous contractile activity of pre and post-menopausal non-pregnant myometrium.**

| | Pre-menopausal | Post menopausal | *P*-value |
|---|---|---|---|
| **n- number** | 32 | 8 | --- |
| **Amplitude (mN)** | 2.9mN±0.4 | 0.3mN±0.1 | **0.001*** |
| **Frequency (no. Per 30 minutes)** | 6.0± 1.3 | 8 ± 1.8 | **0.266** |
| **Duration (minutes)** | 1.0 ± 0.1 | 0.5 ± 0.2 | **0.076** |
| **AUC (30 minutes)** | 11.1± 1.6 | 1.4± 0.6 | **0.007*** |
| **High $K^+$ response (mN)** | 2.7± 0.4 | 0.5± 0.2 | **0.044*** |
| **Time to spontaneous activity (minutes)** | 48.5 ± 5.0 | 24.7± 7.4 | **0.032*** |
| **Average age** | 41.1 | 63.2 | **$2.5x10^{-9}$*** |
| **Average BMI** | 26.8 | 26.7 | **0.921** |

*denotes significance recorded at p<0.05 level

Table 3.4: Student T-test identified that mean force amplitude of contraction, AUC of contraction, mean High $K^+$ response and time to spontaneously contract was significantly reduced in the myometrium from post-menopausal women. Frequency of contraction of post-menopausal myometrium was shown to be increased however, not significance compared to pre-menopausal women. Amplitude of contraction is peak force (mN); Frequency of contraction is the number of contractions occurring in 30 minutes; Duration of contraction is duration of contraction (min) measured at the half maximal peak of contraction; AUC is a measure of the overall contractile activity occurring in 30 minutes, High $K^+$ response is the maximal force of contraction measured in mN and time to spontaneously contract measured in minutes. Average age and BMI in both groups are also found showing significant differences in the age of both groups.

### 3.4.3 Comparing the contractility of pre-menopausal women with post menopausal women excluding endometriosis patients.

Endometriosis did not make the tissue less likely to contract. However, next the contractility of the 10 women with endometriosis were removed to see if the contractility pattern changes and to see if the statistical differences remain. All the endometriosis patients are removed from the pre-menopausal group. The average age of the 30 women being assessed is 46.6 and the average BMI is 26.4, not much different to when the entire 40 women were examined.

**(a) Contraction amplitude**

The mean force amplitude of spontaneous contractions recorded for post-menopausal women (n=8) remained significantly reduced, 0.3±0.1 mN, compared to pre-menopausal women (n=22), 3.3±0.5 mN.

**(b) Frequency**

Frequency of contractions was greater for post-menopausal women, 8 ± 1.8 contractions per 30 minutes; pre-menopausal women: 5± 0.8 contractions per 30 minutes. Without endometriosis patients, frequency became closer to significance.

**(c) Duration**

The mean duration of contraction was shorter in post-menopausal women; 0.5 ± 0.2 minutes, pre-menopausal women; 1.2 ± 0.2 minutes. A student t-test showed this to be significantly shorter at $p<0.05$ level of significance without using endometriosis patients.

**(d) Area under the curve (AUC, 30 minutes)**

For post-menopausal women mean AUC calculated was 1.4± 0.6 a.u compared to 12.6± 2.2 a.u recorded for pre-menopausal women. Contractile activity of post-menopausal women remained significantly reduced compared to pre-menopausal women.

**(e) High $K^+$ depolarisation-induced contractions**

The high $K^+$ response achieved by post-menopausal myometrium was still significantly lower, 0.5± 0.2mN compared to the pre-menopausal myometrium 3.1± 0.5mN.

**(f) Time to commencement of spontaneous activity**

Spontaneous contractions occurred *in vitro***,** in pre-menopausal myometrium (n=22) after 45.7± 6.6 minutes compared with post menopausal (n=8) after 24.7± 7.4 minutes. This reduction in the time to spontaneously contract in the post menopausal women did not reach significance.

**Table 3.5** summarises the mean values of contraction amplitude, frequency, duration, AUC and High $K^+$ responses of spontaneous contractions as well as the mean time to spontaneously contract in pre and post-menopausal myometrium without endometriosis patients incorporated.

**Table 3.5 Summary of spontaneous contractile activity of pre and post menopausal non-pregnant myometrium without patients with endometriosis incorporated.**

| | Pre-menopausal | Post menopausal | *P*-value |
|---|---|---|---|
| **n- number** | 22 | 8 | --- |
| **Amplitude (mN)** | 3.3mN±0.5 | 0.3mN±0.1 | **0.001*** |
| **Frequency (no. Per 30 minutes)** | 5± 0.8 | 8 ± 1.8 | **0.072** |
| **Duration (minutes)** | 1.2 ± 0.2 | 0.5 ± 0.2 | **0.036*** |
| **AUC (30 minutes)** | 12.6± 2.2 | 1.4± 0.6 | **0.005*** |
| **High $K^+$ response (mN)** | 3.1± 0.5 | 0.5± 0.2 | **0.045*** |
| **Time to spontaneous activity (minutes)** | 45.7± 6.6 | 24.7± 7.4 | **0.087** |
| **Average age** | 40.6 | 63.2 | **$1.3x10^{-7}$*** |
| **Average BMI** | 26.3 | 26.7 | **0.794** |

*denotes significance recorded at p<0.05 level

Table 3.5: Student T-test identified that mean force amplitude of contraction, mean duration of contraction, AUC of contraction and mean High $K^+$ response was significantly reduced in the myometrium from post-menopausal women. Frequency of contraction of post-menopausal myometrium was shown to be increased however, this did not reach statistical significance compared to pre-menopausal women. Time to spontaneously contract did not reach significance either. Amplitude of contraction is peak force (mN); Frequency of contraction is the number of contractions occurring in 30 minutes; Duration of contraction is duration of contraction (min) measured at the half maximal peak of contraction; AUC is a measure of the overall contractile activity occurring in 30 minutes, High $K^+$ response is the maximal force of contraction measured in mN and time to spontaneously contract measured in minutes. Average age and BMI in both groups are also found showing significant differences in the age of both groups.

### 3.4.4 In vitro non-pregnant pre-menopausal women compared to post-menopausal women contractility, age and BMI-matched.

To investigate whether the changes **found** are age related or menopausal status related or both, a subset of samples from both the pre and post-menopausal groups were age and BMI matched as close as possible. With 9 women in the pre-menopausal group with a mean age of 46.4 years old and BMI 26.4 and 5 women in the post-menopausal with mean age of 58.4 years old and BMI 26.5 groups were compared. Representative age matched traces are seen in **Figure 3.2.** These data suggest that the changes we see are somewhat age related although there are not enough n numbers or younger post menopausal women to accurately assess. However, it was found that still there is a significant decrease in force amplitude in the post menopausal group, all other parameters were not significant as seen in **Table 3.6**.

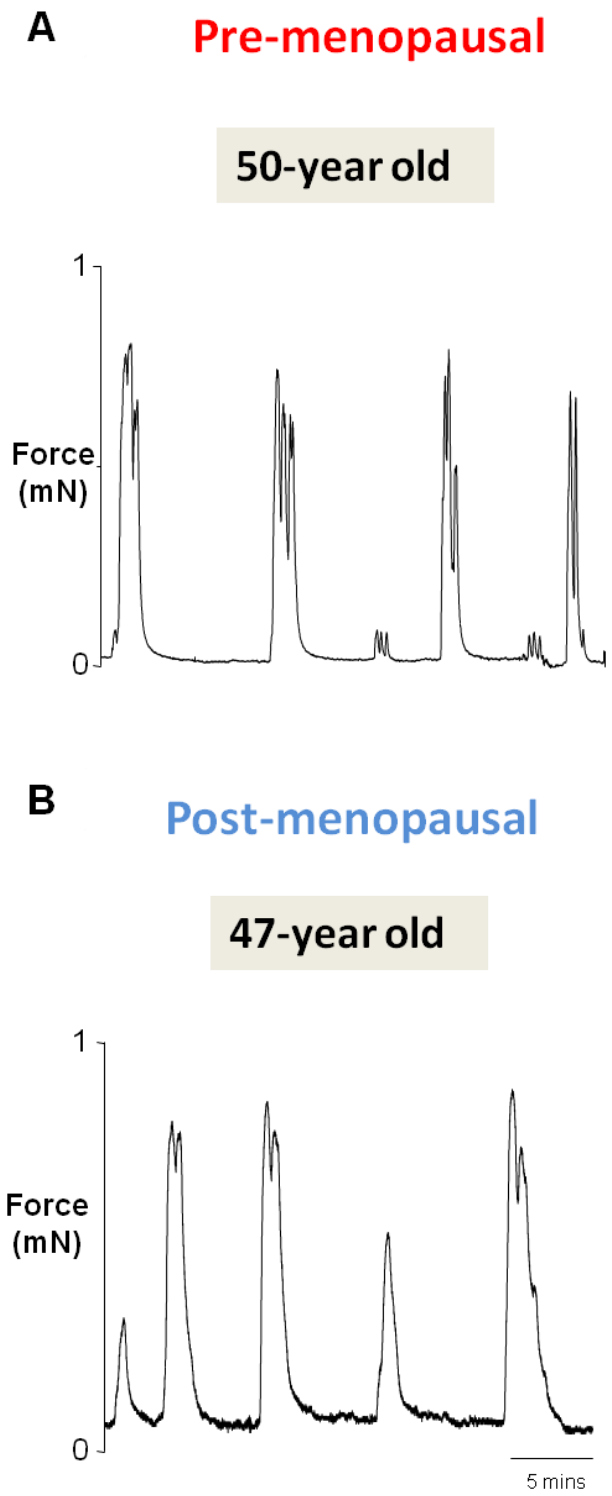



**Figure 3.2 Pre and Post-menopausal myometrial contractility age-matched.**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from a (A) 50-year old pre-menopausal women, and (B) 47-year old post menopausal woman. Strips were placed under a resting tension of 2 mN and superfused continually with physiological saline solution (pH 7.4) at 37 $^{o}$C.

**Table 3.6 Summary of spontaneous contractile activity of pre and post-menopausal non-pregnant myometrium age and BMI matched.**

| | Pre-menopausal | Post menopausal | *P*-value |
|---|---|---|---|
| **n- number** | 9 | 5 | --- |
| **Amplitude (mN)** | 3.6±0.8 | 0.4±0.1 | **0.017*** |
| **Frequency (no. Per 30 minutes)** | 5.3± 1.7 | 7.2 ± 1.6 | **0.493** |
| **Duration (minutes)** | 1.4 ± 0.3 | 0.7 ± 0.3 | **0.213** |
| **AUC (30 minutes)** | 13.5 ± 4.4 | 1.6± 0.9 | **0.030** |
| **High $K^+$ response (mN)** | 3.7± 0.9 | 0.5± 0.2 | **0.018** |
| **Time to spontaneous activity (minutes)** | 43.8±9.7 | 23.2±9.0 | **0.187** |

*denotes significance recorded at $p<0.05$ level

Student T-test identified that mean force amplitude of contraction was significantly reduced in the myometrium from post-menopausal women compared to pre-menopausal. All other parameters were not significantly changed. AUC and High $K^+$ were close to significance.

Amplitude of contraction is represented by force (mN); Frequency of contraction is represented by the number of contractions occurring in 30 minutes; Duration of contraction is represented by how long a single contraction lasts (min) and is measured at the half maximal peak of contraction; AUC is a measure of the overall contractile activity occurring in 30 minutes and High $K^+$ response is the maximal force of contraction measured in mN. Time to spontaneously contract measured in minutes.

### 3.4.5 How does non-pregnant contractility of the myometrium change with age?

Spontaneous contractions of non-pregnant human myometrium irrespective of menopausal state or presence of endometriosis was examined in relation to age. **Figure 3.3** shows four original contractility recordings of a non-pregnant women aged 32 (A), 48 (B), 62 (C) and 74 (oldest women in study) (D). A very clear loss of force is apparent with increasing age. The mean data shown in **Table 3.7** also supports this observation. There was no significant change in the time to commence contractions although time to commence contractions tended to be shorter in the patients >50, showing that the older uterus is not deterred in producing contractions. When contractions from all women are plotted on a scatter graph (**Figure 3.4**) this clear drop in force with age is seen ($r=-0.6969$, significant negative correlation, $p<0.0001$ , $n=40$). To investigate the effects of age on the non-pregnant uterus in more detail, the effects of stimulating the uterus with high $K^+$ (40 mM) which maximally activates functional voltage-gated L-type Ca channels inducing Ca entry and a large tonic contraction was examined (Wray 2001). For each tissue, spontaneous contractions were normalised to this maximal high $K^+$-induced contraction to determine the degree of activation of the contractile mechanisms. The results have been analysed for age cohorts of 25-29, 30-39, 40-49, >50 as shown in **Figure 3.5**.

The reduction in spontaneous force with age produced by the non-pregnant uterus was prominent (**Figure 3.5 A**) and is also seen when L-type Ca channels are maximally activated with high $K^+$ (**Figure 3.5 B**). These data suggest that there might be loss of functional muscular tissue (e.g. less muscle cells) or decreased expression of L-type Ca channels, with age (Floyd and Wray 2007; Taggart et al. 1997), although larger sample numbers are required before these suggestions can be tested. In **Figure 3.5 C** the spontaneous contractions are normalised to the high $K^+$ contraction to control for any effects of age on decreased muscle or L-type Ca expression. Relative to high $K^+$ the amplitude of spontaneous activity decreases with age, suggesting that the mechanisms generating spontaneous contractions are also affected. Thus in non-pregnant myometrium the suggestion is that force may decline as a consequence of muscle loss and/or decreased Ca entry and thus decreased

activation of the contractile proteins. Reduced levels of oestrogen with aging would be expected to cause atrophy of the myometrium, and thus explain some of the decreased strength of contractions. There is evidence that the L-type calcium channel subunits and function are hormonally regulated, with oestrogen associated with increasing, (Batra 1987) and progesterone decreasing (Tezuka et al. 1995) calcium channel expression. Thus as oestrogen decreases with aging, levels of L-type Ca channels will be expected to fall.

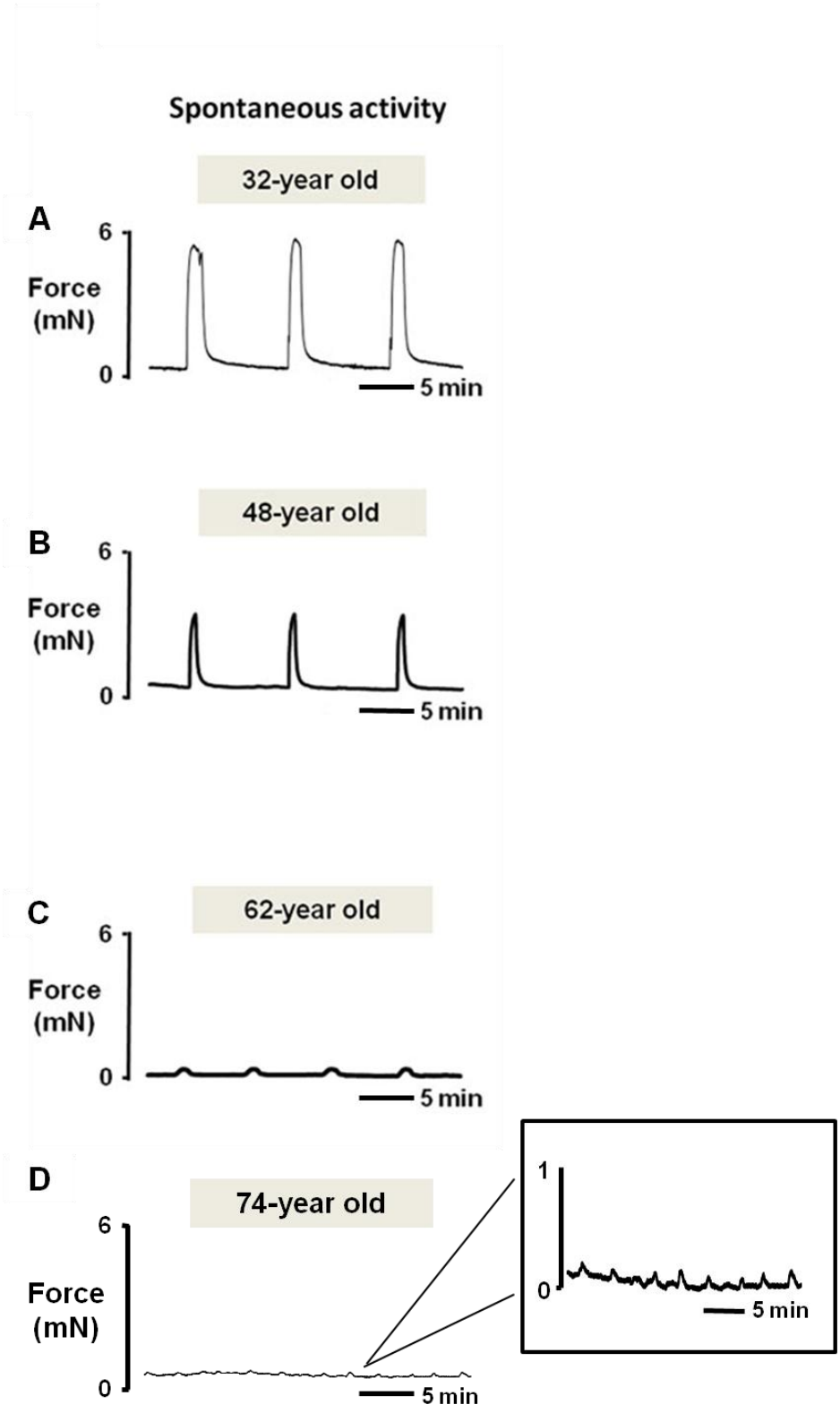



**Figure 3.3 Non-pregnant myometrial contractility in relation to age.**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from a (A) 32-year old, (B) 48-year old and (C) 62-year old woman (D) 74-year old woman undergoing hysterectomy (expanded version of contractions can also be seen within D). Strips were placed under a resting tension of 2 mN and superfused continually with physiological saline solution (pH 7.4) at 37 $^{o}$C.

**Table 3.7 Summary of spontaneous contractile activity of non-pregnant myometrium in relation to age.**

| | Age 25-29 | Age 30-39 | Age 40-49 | Age >50 |
|---|---|---|---|---|
| **n- number** | 3 | 9 | 19 | 9 |
| **Amplitude (mN)** | 6.4 ± 0.6 | 2.8± 0.4* | 2.2± 0.4* | 0.8± 0.5* |
| **Frequency (no. Per 30 minutes)** | 2.7± 0.7 | 8.3±4.0 | 5.7±0.9 | 6.7± 1.6 |
| **Duration (minutes)** | 1.1± 0.3 | 1.1± 0.3 | 1.1± 0.2 | 0.5± 0.2 |
| **AUC (30 minutes)** | 20.3± 0.9 | 12.4± 2.5 | 8.5±2.3* | 1.63±0.6* |
| **Time to spontaneous activity (minutes)** | 66.8± 27.9 | 43.5± 8.3 | 49.1± 5.8 | 23.1± 6.7 |

*denotes significance recorded at $p<0.05$ level when compared to age 25-29 group.

ANOVA with Bonferroni post hoc test identified that mean force amplitude of contraction, and AUC of contraction was significantly reduced in the myometrium from the age of over 40. Frequency of contraction, mean duration of contraction and time to spontaneously contract were not significantly different.

Amplitude of contraction is represented by force (mN); Frequency of contraction is represented by the number of contractions occurring in 30 minutes; Duration of contraction is represented by how long a single contraction lasts (min) and is measured at the half maximal peak of contraction; and AUC is a measure of the overall contractile activity occurring in 30 minutes. Time to spontaneously contract was measured in minutes. All data are expressed as mean ± S.E.M.

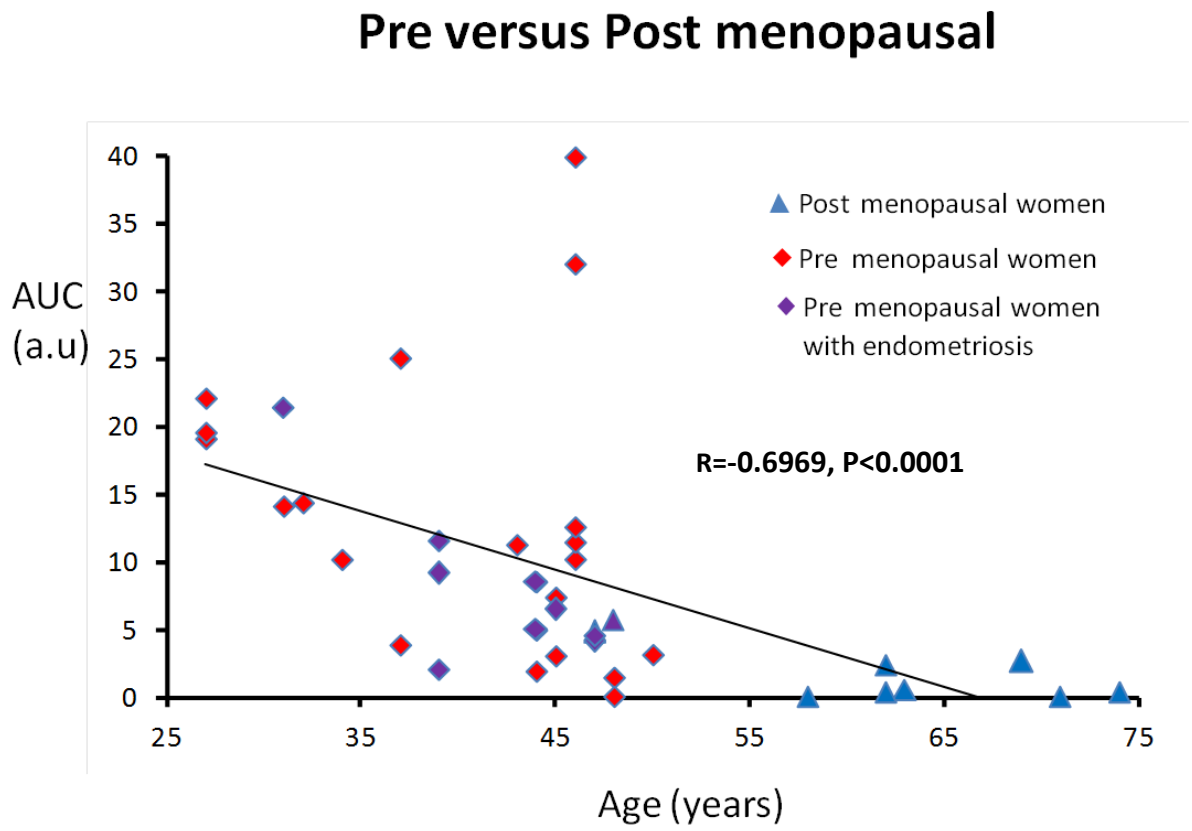



**Figure 3.4 Scatter graph to show the relationship between AUC and age**

Plot of spontaneous contractile activity (quantified as area under the contraction curve, (AUC, in arbitary units, au) of strips of non-pregnant myometrium obtained from women undergoing a hysterectomy, against age. Post menopausal women are denoted by blue triangles and pre-menopausal women are denoted by red diamonds (unless patient is pre-menopausal and suffers from endometriosis, these patients are denoted as purple diamonds). Spearman's rank test found a significant negative correlation between integral of force and age combining pre and post menopausal women (rs=-0.6969, significant negative correlation, p<0.0001, n=40).

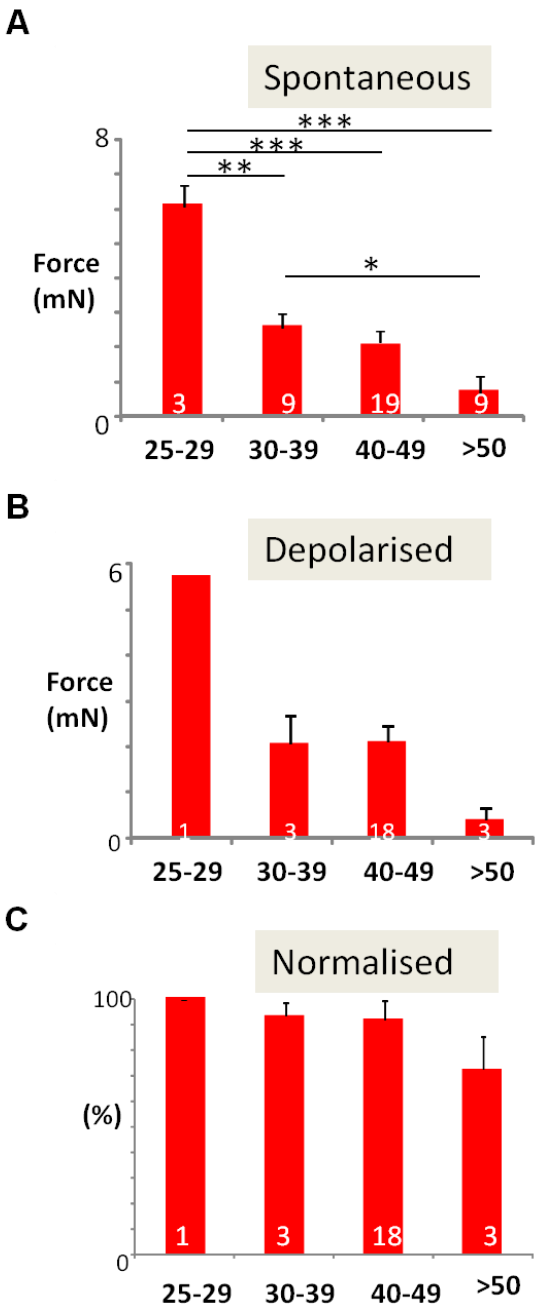



**Figure 3.5 Uterine activity with age of non-pregnant myometrium.**
A)Spontaneous force of contraction, B) force of contraction under high $K^+$ (40 mM) depolarisation and C) force of contraction normalised to high $K^+$ (where high $K^+$ equalled 100 %), according to age group. Mean force of contraction declined with increasing age group
(A) with little activity observed from myometrial strips from women over 50 years of age, in comparison to their younger counterparts. Similarly, maximal force achieved under high $K^+$ (B) was reduced in samples from older women, and normalised force shown in (C) also declined with advancing age. Values represent means ± SEM denoted by error bars, values in white indicate n-numbers. Anova with Bonferroni post hoc test was used, * represents $p<0.05$, **represents $p<0.01$, *** represents $p<0.001$.

### 3.4.6 How does non-pregnant contractility change with age excluding endometriosis patients?

Next the 10 endometriosis patients were taken out to see if the significant changes in contractility parameters and correlation in relation to age still remained. The endometriosis patients were only removed from ages 30-39 and 40-49. The mean data of 30 women without endometriosis separated by age can be found below in **Table 3.8**. The same age groups (30-39, 40-49, >50 when compared to 25-29 year olds) remain significant with and without the inclusion of patients with endometriosis. Frequency, duration and time to spontaneously contract remain not significantly changed. When contractions from these 30 women without endometriosis are plotted on a scatter graph (**Figure 3.6**), the significant negative correlation is still found in force with age. The correlation seen is slightly more negative at $r_s=-0.7214$ (significant negative correlation, $p<0.0001$ , $n=40$).

**Table 3.8 Summary of spontaneous contractile activity of non-pregnant myometrium in relation to age excluding patients with endometriosis.**

| | Age 25-29 | Age 30-39 | Age 40-49 | Age >50 |
|---|---|---|---|---|
| **n- number** | 3 | 5 | 13 | 9 |
| **Amplitude (mN)** | 6.4 ± 0.6 | 2.5± 0.3* | 2.5± 0.5* | 0.8± 0.5* |
| **Frequency (no. Per 30 minutes)** | 2.7± 0.7 | 4.9±1.2 | 5.4±1.3 | 6.7± 1.6 |
| **Duration (minutes)** | 1.1± 0.3 | 1.4± 0.4 | 1.2± 0.3 | 0.5± 0.2 |
| **AUC (30 minutes)** | 20.3± 0.9 | 13.5± 3.4 | 8.5±2.3* | 1.63±0.6* |
| **Time to spontaneous activity (minutes)** | 66.8± 27.9 | 30.8± 8.8 | 48.1± 6.0 | 23.1± 6.7 |

*denotes significance recorded at $p<0.05$ level when compared to age 25-29 group

Anova with bonferroni post hoc test identified that mean force amplitude of contraction, and AUC of contraction was significantly reduced in the myometrium from the age of over 40. Frequency of contraction, mean duration of contraction and time to spontaneously contract were not significantly different.

Amplitude of contraction is represented by force (mN); Frequency of contraction is represented by the number of contractions occurring in 30 minutes; Duration of contraction is represented by how long a single contraction lasts (min) and is measured at the half maximal peak of contraction; and AUC is a measure of the overall contractile activity occurring in 30 minutes. Time to spontaneously contract was measured in minutes.

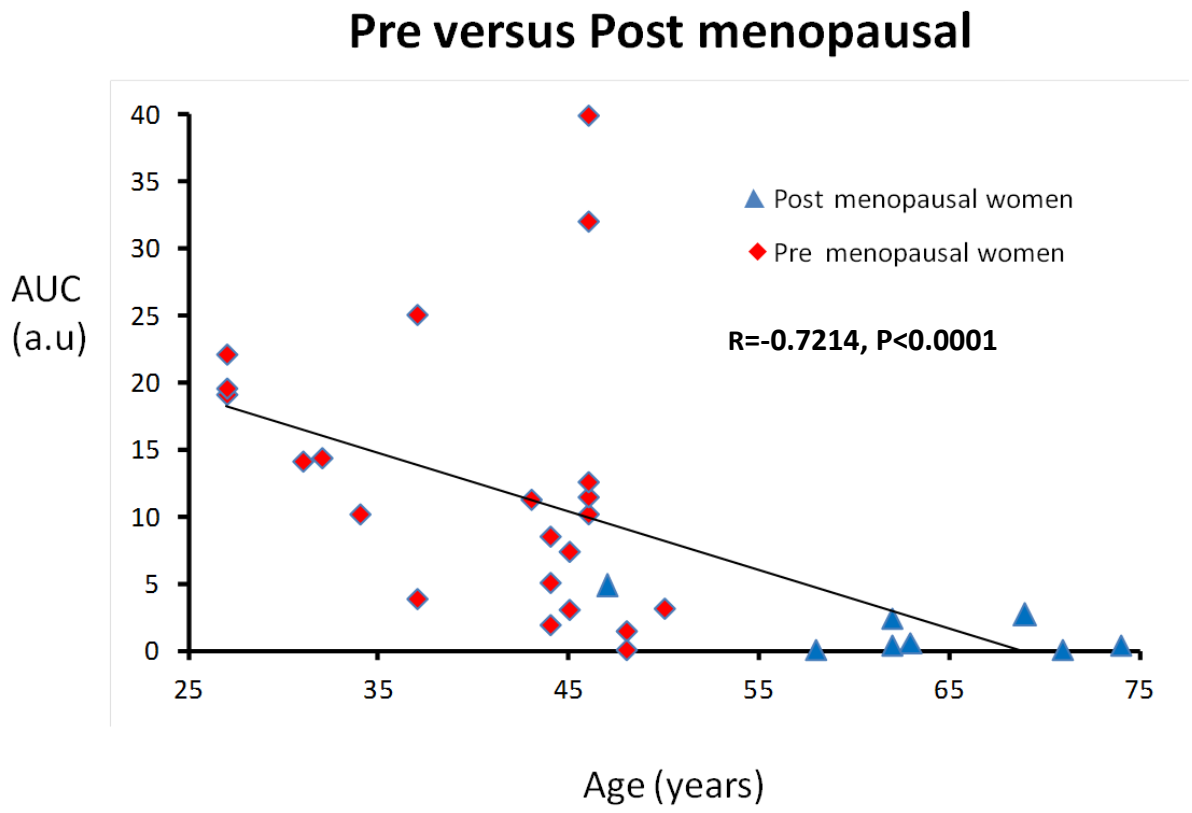



**Figure 3.6 Scatter graph to show the relationship between AUC and age without endometriosis patients included**

Plot of spontaneous contractile activity (quantified as area under the contraction curve, (AUC, in arbitary units, au) of strips of non-pregnant myometrium obtained from women undergoing a hysterectomy, against age. Post menopausal women are denoted by blue triangles and pre-menopausal women are denoted by red diamonds. Spearman's rank test found a significant negative correlation between integral of force and age combining pre and post menopausal women (rs=-0.7214, significant negative correlation, p<0.0001, n=30).

### 3.4.7 Myometrial contractility of women with endometriosis.

All endometriosis patients used within this thesis were pre-menopausal. Hence the control group used to compare the contractility of endometriosis patients were pre-menopausal women without endometriosis. Both groups were age and BMI matched as close as possible with the pre-menopausal group at an average age of 41.2 years old and average BMI 26.4 and with the endometriosis group at an average age of 42.0 years old and average BMI 28.0. Both BMI's are in the overweight category.

**(a) Contraction amplitude**

The mean force amplitude of spontaneous contractions recorded for women with endometriosis (n=10) was reduced, 2.1±0.4 mN, compared to pre-menopausal women (n=21), 3.2±0.5 mN. Representative recordings of spontaneously contracting myometrium from pre-menopausal women and a women suffering from endometriosis are shown in **Figure 3.7**.

**(b) Frequency**

Frequency of contractions was greater (**Figure 3.7**) for endometriosis women, 9.0 ±3.5 contractions per 30 minutes; pre-menopausal women: 5± 0.8 contractions per 30 minutes.

**(c) Duration**

The mean duration of contraction was shorter (**Figure 3.7**) in endometriosis women; 0.8± 0.1 minutes, pre-menopausal women; 1.2 ± 0.2 minutes.

**(d) Area under the curve (AUC, 30 minutes)**

For women with endometriosis mean AUC calculated was 7.9± 1.7 a.u compared to 12.3± 2.3 a.u recorded for pre-menopausal women. Contractile activity of endometriosis women was reduced compared to pre-menopausal women.

**(e) High $K^+$ depolarisation-induced contractions**

The high $K^+$ response achieved by the myometrium from women with endometriosis was lower, 1.8± 0.4mN compared to the pre-menopausal myometrium 3.0± 0.6mN.

**(f) Time to commencement of spontaneous activity**

Spontaneous contractions occurred *in vitro***,** in pre-menopausal myometrium after 45.7 ± 6.6 minutes compared with patients with endometriosis after 54.7± 6.7 minutes. This increase in the time to spontaneously contract in the women with endometriosis was not statistically different.

**Table 3.9** summarises the mean values of contraction amplitude, frequency, duration, AUC and High $K^+$ responses of spontaneous contractions as well as mean time to commence spontaneous contractions in pre-menopausal myometrium and myometrium from women with endometriosis.

All results did not reach significance. However as some of the endometriosis patients were on hormone therapy potentially affecting myometrial contractions, the contractility of women with endometriosis and on no medication other than mirena coil (a contraceptive) were compared.

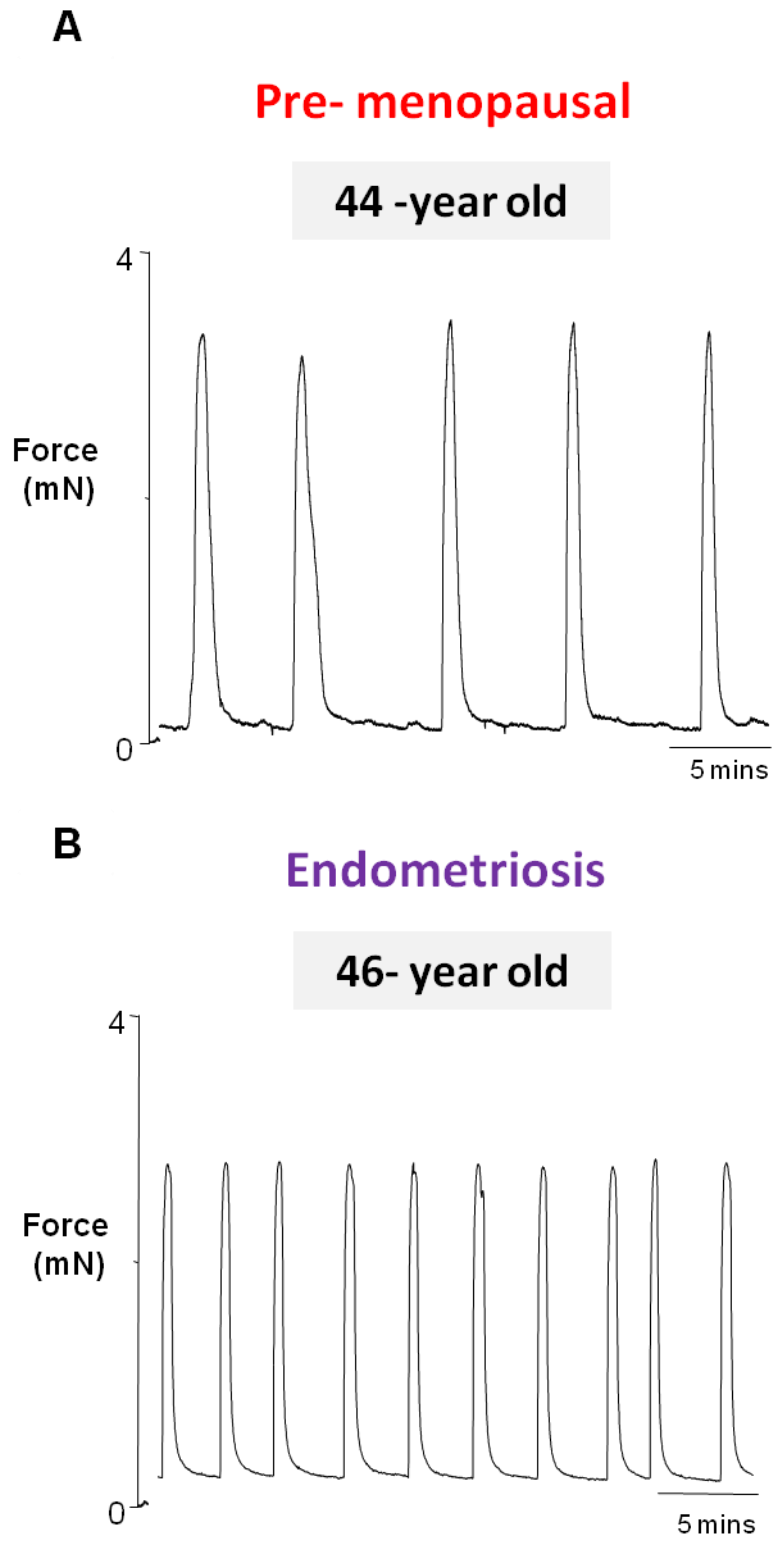



**Figure 3.7 Endometriosis and Pre-menopausal myometrial contractility.**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from a (A) 44-year old pre-menopausal women, and (B) 46-year old woman with endometriosis. Strips were placed under a resting tension of 2 mN and superfused continually with physiological saline solution (pH 7.4) at 37 $^{o}$C.

**Table 3.9 Summary of spontaneous contractile activity of women with endometriosis and pre- menopausal non-pregnant myometrium.**

| | Pre-menopausal | Endometriosis | *P*-value |
|---|---|---|---|
| **n- number** | 21 | 10 | --- |
| **Amplitude (mN)** | 3.2±0.5 | 2.1±0.4 | **0.168** |
| **Frequency (no. Per 30 minutes)** | 4.9± 0.8 | 9.0 ± 3.5 | **0.139** |
| **Duration (minutes)** | 1.2 ± 0.2 | 0.8 ± 0.1 | **0.162** |
| **AUC (30 minutes)** | 12.3± 2.3 | 7.9± 1.7 | **0.221** |
| **High $K^+$ response (mN)** | 3.1± 0.5 | 1.8± 0.4 | **0.238** |
| **Time to spontaneous activity (minutes)** | 45.7 ± 6.6 | 54.7± 6.7 | **0.411** |

*denotes significance recorded at p<0.05 level

All parameters were not significantly changed.

Amplitude of contraction is represented by force (mN); Frequency of contraction is represented by the number of contractions occurring in 30 minutes; Duration of contraction is represented by how long a single contraction lasts (min) and is measured at the half maximal peak of contraction; AUC is a measure of the overall contractile activity occurring in 30 minutes and High $K^+$ response is the maximal force of contraction measured in mN. Time to spontaneously contract measured in minutes.

### 3.4.8 Myometrial contractility of women with endometriosis and not on medication.

The pre-menopausal group included 13 patients not on any medication and 2 patients with mirena coil. The endometriosis group included 3 patients not on any medication and 3 patients with mirena coil. Average age and BMI for each group can be found in **table 3.10**.

**(a) Contraction amplitude**

The mean force amplitude of spontaneous contractions recorded for women with endometriosis (n=6) was reduced, 1.7±0.5 mN, compared to pre-menopausal women (n=13), 3.1±0.6 mN.

**(b) Frequency**

Frequency of contractions was significantly greater for women with endometriosis, 12 ± 5.6 contractions per 30 minutes; pre-menopausal women: 4± 0.4 contractions per 30 minutes. A student t-test at $p<0.05$ level of significance was used.

**(c) Duration**

The mean duration of contraction was shorter in endometriosis women; 0.7 ± 0.2 minutes, pre-menopausal women; 1.3 ± 0.2 minutes.

**(d) Area under the curve (AUC, 30 minutes)**

AUC of contractions, which is an index of the total work done by the tissue over a given time period, was calculated by measuring the area under the contraction curve in 30 minutes. For women with endometriosis mean AUC calculated was 7.8± 2.8 a.u compared to 13.7± 2.8 a.u recorded for pre-menopausal women. Contractile activity of endometriosis women was reduced compared to pre-menopausal women.

**(e) High $K^+$ depolarisation-induced contractions**

The high $K^+$ response achieved by myometrium from endometriosis women was lower, 1.8± 0.5mN compared to the pre-menopausal myometrium 3.0± 0.6mN.

**(f) Time to commencement of spontaneous activity**

Spontaneous contractions occurred *in vitro***,** in pre-menopausal myometrium (n=13) after 52.4± 6.8 minutes compared with patients with endometriosis on no medication (n=6) after 45.5 ± 7.9 minutes. No significant difference found.

**Table 3.10** summarises the mean values of contraction amplitude, frequency, duration, AUC and High $K^+$ responses of spontaneous contractions as well as mean time to commence spontaneous contractions in pre-menopausal myometrium and myometrium from women with endometriosis on no medication.

**Table 3.10 Summary of spontaneous contractile activity of women with endometriosis and not on medication and pre- menopausal non-pregnant myometrium.**

| | Pre-menopausal | Endometriosis | *P*-value |
|---|---|---|---|
| **n- number** | 13 | 6 | --- |
| **Amplitude (mN)** | 3.1mN±0.6 | 1.7mN±0.5 | **0.182** |
| **Frequency (no. Per 30 minutes)** | 4.1± 0.4 | 12.5 ± 5.6 | **0.040*** |
| **Duration (minutes)** | 1.3 ± 0.2 | 0.7 ± 0.2 | **0.169** |
| **AUC (30 minutes)** | 13.7± 2.8 | 7.8± 2.8 | **0.277** |
| **High $K^+$ response (mN)** | 3.0± 0.6 | 1.8± 0.5 | **0.316** |
| **Time to spontaneous activity (minutes)** | 52.4 ± 6.8 | 45.5± 7.9 | **0.550** |
| **Average age** | 43.1 | 42.2 | **0.755** |
| **Average BMI** | 27.8 | 27.8 | **0.998** |

*denotes significance recorded at $p<0.05$ level

Student's T-test identified that mean Frequency of contraction was significantly increased in the myometrium from women with endometriosis not on medication compared to the pre-menopausal women control group. All other parameters were not significantly changed.

Amplitude of contraction is represented by force (mN); Frequency of contraction is represented by the number of contractions occurring in 30 minutes; Duration of contraction is represented by how long a single contraction lasts (min) and is measured at the half maximal peak of contraction; AUC is a measure of the overall contractile activity occurring in 30 minutes and High $K^+$ response is the maximal force of contraction measured in mN. Average age and BMI in both groups are also found. Time to spontaneously contract measured in minutes.

### 3.4.9 Myometrial contractility of women with endometriosis and on medication.

The pre-menopausal group included 10 patients not on any medication, 2 patients with mirena coil, 1 patient on Tranexamic and mefenamic acid and 1 patient on Norethisterone until previous day. The endometriosis group included 2 patients on GnRHa, 1 patient on provera and the other on hormone therapy. Average age and BMI for each group can be found in **table 3.11**.

**(a) Contraction amplitude**

The mean force amplitude of spontaneous contractions recorded for women with endometriosis (n=4) was reduced, 2.8mN±0.8, compared to pre-menopausal women (n=14), 3.2mN±0.6. No significance was found.

**(b) Frequency**

No significant difference in frequency of contractions was found unlike in the women with endometriosis on no medication. Women with endometriosis, 3.8± 1.0 contractions per 30 minutes; pre-menopausal women: 4.2± 0.4 contractions per 30 minutes.

**(c) Duration**

The mean duration of contraction was shorter in endometriosis women; 0.8 ± 0.2 minutes, pre-menopausal women; 1.3 ± 0.2 minutes.

**(d) Area under the curve (AUC, 30 minutes)**

AUC of contractions, which is an index of the total work done by the tissue over a given time period, was calculated by measuring the area under the contraction curve in 30 minutes. For women with endometriosis mean AUC calculated was 7.9± 1.8 a.u compared to 13.8± 2.8 a.u recorded for pre-menopausal women. Contractile activity of endometriosis women was reduced compared to pre-menopausal women.

**(e) High $K^+$ depolarisation-induced contractions**

The high $K^+$ response achieved by myometrium from endometriosis women was not calculated as only one KCl response was recorded for the group.

**(f) Time to commencement of spontaneous activity**

Spontaneous contractions occurred *in vitro***,** in pre-menopausal myometrium (n=14) after 49.4.4± 6.3 minutes compared with patients with endometriosis on medication (n=4) after 68.5± 8.7minutes. No significant difference found.

Overall there was no significant difference in the contractility parameters of the pre-menopausal control group compared to the patients with endometriosis and on medication.

**Table 3.11** summarises the mean values of contraction amplitude, frequency, duration, AUC and High $K^+$ responses of spontaneous contractions in addition to mean time to commence spontaneous contractions in pre-menopausal myometrium and myometrium from women with endometriosis on medication.

**Table 3.11 Summary of spontaneous contractile activity of women with endometriosis and on medication and pre- menopausal non-pregnant myometrium.**

| | Pre-menopausal | Endometriosis | *P*-value |
|---|---|---|---|
| **n- number** | 14 | 4 | --- |
| **Amplitude (mN)** | 3.2mN±0.6 | 2.8mN±0.8 | **0.743** |
| **Frequency (no. Per 30 minutes)** | 4.2± 0.4 | 3.8 ± 1.0 | **0.692** |
| **Duration (minutes)** | 1.3 ± 0.2 | 0.8 ± 0.2 | **0.268** |
| **AUC (30 minutes)** | 13.8± 2.8 | 7.9± 1.8 | **0.345** |
| **Time to spontaneous activity (minutes)** | 52.4 ± 6.8 | 45.5± 7.9 | **0.154** |
| **Average age** | 49.4 | 48.5 | **0.903** |
| **Average BMI** | 27.5 | 28.4 | **0.528** |

*denotes significance recorded at $p<0.05$ level

All parameters were not significantly changed.

Amplitude of contraction is represented by force (mN); Frequency of contraction is represented by the number of contractions occurring in 30 minutes; Duration of contraction is represented by how long a single contraction lasts (min) and is measured at the half maximal peak of contraction; and AUC is a measure of the overall contractile activity occurring in 30 minutes. Average age and BMI in both groups are also found. Time to spontaneously contract measured in minutes.

## 3.5 Discussion

In 98% of non-pregnant biopsies worked on in this chapter, spontaneous contractions were established. The muscle bundles within the non-pregnant myometrium compared to the pregnant myometrium are more tightly bound, although dissection using a microscope and the tissue undergoing an endometrial scrape aided in the dissection of clear muscular tissue strips. It took an average of 44 minutes until spontaneous contractions occurred. Usually from my observations within the term pregnant human myometrium initiation of contractions takes around 70-90 minutes. In both tissue types, stable contractions were monitored for a period of 1- 2 hours.

Biopsies from non-pregnant women undergoing hysterectomy were taken immediately after the removal of the uterus from the lower half of anterior uterine wall, so as to approximate the area where the biopsy was removed from the pregnant uterus. For pregnant tissues, biopsies were taken after delivery from the middle of the upper edge, lower segment uterine incision at time of Caesarean section. Both lower and upper segments of the myometrium have been shown to have similar contractile profiles (Luckas and Wray 2000) All biopsies were collected and handled in the same way using a protocol to prevent tissue degradation and to ensure all conditions for experiments were the same, allowing for comparison of both tissues in proceeding chapters.

To the best of my knowledge, this chapter is the most extensive *in vitro* study of non-pregnant human myometrium and shows that good strong regular contractions do occur. It also demonstrates significant effects of aging, menopausal state and endometriosis on myometrial activity. The only findings from *in vitro* observations of non-pregnant human contractility without statistical evidence is that contractions of pre-menopausal myometrium contracted at a greater frequency, and were more frequent than post menopausal myometrium (Domali 2001). The only other study demonstrated smaller contractions were produced by the non-pregnant myometrium when compared to non labouring term pregnant myometrium while time between contractions was reduced in the non-pregnant myometrium (Rosenbaum 2012), discussed further below.

### 3.5.1 The effect of menopausal status on myometrial contractility

Once spontaneous activity was established, post menopausal myometrium had contractions that were lower in amplitude, shorter in duration but at a higher frequency than pre-menopausal myometrium. Overall, mean AUC of contraction confirmed the activity of the post-menopausal myometrium is significantly reduced compared to pre-menopausal myometrium. Although, when age and BMI are taken into account, only the significant decrease in force amplitude of contraction remains. This suggests age partially affects the results obtained. Thus, reduced uterine activity appears to be an inherent property of post menopausal women.

Interestingly contractility although low, is maintained well into post-menopause. This is consistent with an earlier *in vitro* study which found spontaneous activity in post-menopausal women (Domali 2001). These latter authors also demonstrated responsiveness to endothelin 1, which could be modified by ovarian steroids. This again emphasizes that post-menopausal human myometrium remains active and responsive to hormonal environment. Reduced contractile activity is the main finding in the post- menopausal myometrium, but what is the reason for this?

Uterine contractility is a directly related to underlying electrical activity in myometrial cells. Spontaneous contractions of the myometrium are produced by spontaneous changes in cell membrane potential towards a more positive potential (membrane depolarisation) which triggers the firing of a single action potential or bursts of action potentials (Kawarabayashi et al 1988), opening of L-type calcium channels and calcium influx (Wray 1993, Shmigol 1998). Contraction of the myometrium then transpires from the resulting myofilament cross-bridge cycling. The frequency of myometrial contractions is therefore directly related to changes in membrane potential and the frequency of bursts. The increase in contraction frequency recorded in post menopausal women in this study suggests that the cell membrane potential in these women is more excitable i.e. less negative than pre-menopausal women such that it would reach the threshold potential for action potential generation more readily than in pre-menopausal myometrium. This helps to explain why as women reach post menopause they spontaneously contract quicker than in the pre menopausal women. Instead, the increased frequency of

contraction may also be due a faster repolarisation or recovery of the membrane to resting potential.

However given the data in my study illustrating reduced contraction amplitude and duration for post menopausal myometrium, it would indicate that despite the higher frequency of reaching threshold for activation in these tissues, the mechanical activity produced by the change in membrane potential is still reduced. This could be due to poor propagation of calcium signalling events in the muscle cells of post menopausal myometrium and less recruiting of muscle cells or muscle bundles to contract, or alternatively reduced smooth muscle with advancing menopausal state and therefore less L-type calcium channels and reduced calcium entry resulting in the reduced force produced. Reduced levels of oestrogen with reaching post menopause could directly affect L-type expression as already discussed below.

### 3.5.2 The effect of aging on non-pregnant myometrial contractility

The results of this study are the first to show aging decreases non-pregnant uterine contractility. It also suggests that in the non-pregnant uterus, the majority of the decline may occur by the age of 35 years. Suggested mechanisms for this underlying decrease in contractility with age could be through morphological changes or hormonal changes of the myometrium. However data on how the myometrium changes with age are scarce. Whereas the morphology of endometrial tissue has been shown to change as a result of reductions in steroid hormones, how aging affects the morphology of the myometrium has not yet been examined, although studies on the microanatomy of the aging reproductive tract have been published (Heyn 2005). Changes in the biophysical properties of the myometrium with age also appear to have been neglected. It would be interesting to examine how myocytes size, muscle mass and connective tissue content are affected by aging.

Within this chapter the negative relationship showing a decrease in contractility with age, correlates with the poorer obstetric outcomes noted with advancing age which in turn may attribute to the greater incidence of high risk medical conditions that are associated with older women. Interestingly, we find that upon increasing age (25-43 years old) within the pregnant myometrium this significant correlation is lost

(Arrowsmith 2012). This more or less maintained force in pregnant women up to around age 40, suggests that if a woman becomes pregnant at this age, the uterus will respond to the hormonal environment of pregnancy, and increase its muscle mass, and hence contractile potential, removing any age-related differences (Wu et al. 2008). This suggestion is consistent with the uterine responses of women in their 50s and 60s to hormonal treatment for IVF and the uterus safely bearing these pregnancies to term. Oxytocin however, may have reduced efficacy in the myometrium of older women which may have implications clinically such as in cases of poor labour progression. Further studies should investigate the details of the underlying mechanisms.

Aging produces changes in metabolism including an increase in cholesterol and LDLs. Body mass index (BMI) also increases as women age. Both cholesterol and BMI have been shown to depress myometrial contractility (Zhang 2007a, b), and could therefore contribute to a reduction in function. Conversely, removal of cholesterol using cholesterol sequestering agents enhances contractile activity, thus in the uterus, cholesterol inhibits function. Partially, due to effects on K channel activity (Shmygol 2007; Noble 2006). Furthermore, myometrial strips with high cholesterol content (measured using thin layer chromatography) contracted more poorly in the laboratory than those with a lower cholesterol content (Noble 2009).

Overall this reduction in spontaneous force with age produced by the non-pregnant uterus was prominent and is also seen in the responses to high $K^+$. Hence these data support the theory that there might be loss of functional muscular tissue (e.g. less muscle cells) or decreased expression of L-type Ca channels, with age (Floyd and Wray 2007; Taggart et al. 1997), although larger sample numbers are required and morphological studies before these suggestions can be tested. Spontaneous contractions were normalised to the high $K^+$ contraction to control for any effects of age on decreased muscle or L-type Ca expression. Relative to high $K^+$ the amplitude of spontaneous activity decreases with age, suggesting that the mechanisms generating spontaneous contractions are also failing. Thus in non-pregnant myometrium the suggestion is that force may decline as a consequence of muscle loss and/or decreased Ca entry and thus decreased activation of the contractile

proteins. Reduced levels of oestrogen with aging would be expected to cause atrophy of the myometrium, and thus explain some of the decreased strength of contractions. There is evidence that the L-type calcium channel subunits and function are hormonally regulated, with oestrogen associated with increasing, (Batra 1987) and progesterone decreasing (Tezuka et al. 1995) calcium channel expression. Thus as oestrogen decreases with aging, levels of L-type Ca channels will be expected to fall.

### 3.5.3 Does endometriosis affect myometrial contractility?

Assessing the spontaneous contractility of non-pregnant myometrium from women with endometriosis shows differences in the contractility profile when compared to their pre-menopausal healthy counter parts. These data show an increase in frequency of contractions. This parameter reaches significance when patients with endometriosis and on medication are removed, a trend decrease in all other parameters of contraction is found. The women on medication removed were on hormone therapy. This study suggests that perhaps hormone therapy is dampening the increase in frequency found. The high $K^+$ data demonstrates a decrease in high K+ response within the endometriosis women possibly due to a decrease in the L-type calcium channels. The overall data obtained from this study supports the previous findings, of increased frequency as observed in early *in vivo* intrauterine pressure recordings and videosonography of uterine peristalsis (Leyendecker 2004, Bulletti 2002). The increase in frequency of contraction *in vivo* was two times greater than there healthy counterparts were as *in vitro* the frequency was three time greater than when compared. Bulletti et al compared the contractility of infertile women with and without endometriosis., Thus the differences in frequency found may be accounted for by fertility status. The differences in contractile amplitudes between this study and Bulleti *et al* may also be attributed to the fact that they were monitoring whole uterine activity *in vivo* whereas we are looking at the contractility of myometrial tissue strips *in vitro*. The theory of retrograde menstruation (Sampson, 1922) proposes that retrograde bleeding occurs at the time of menses in women with endometriosis. This results in transtubal migration of viable

endometrial cells that attach and implant in the pelvic cavity, to ultimately develop into endometriotic implants (Bulletti 2002). It is also found that there is a much higher incidence of these retrograde contractions in women with endometriosis. Overall these findings support a causal role of alterations in myometrial contractility in the genesis or continuation of endometriosis. This also suggests that this change in contractility could have an important effect on the uterine retrograde transport capacity and consequently fertility. This was highlighted in hysterosalpingoscintigraphy showing that transport of labelled inert particles is drastically increased during the early and mid-follicular phases of the cycle, but the directed transport of the particles into the tube ipsilateral to the dominant follicle is absent in the periovulatory phase, concluding in women with endometriosis directed sperm transport is impaired (leyendecker 2004).

### 3.5.4 Conclusion

The results of this study are novel. In this chapter I have compared myometrial contractility from pre-, and post-menopausal women as well as from women with endometriosis. This is also the first study to look at the effect of advancing age on the non-pregnant myometrium. The data demonstrates clear effects of age and menopausal state upon myometrial contractility. It shows that aging significantly decreases uterine contractility as does progressing menopausal state, which is not surprising as there is a strong relationship between the two. Endometriosis also shows an increase in frequency of contractions and a non-significant but consistent decrease in the other parameters of contractility. Consistent with an earlier *in vitro* study which had found spontaneous activity in post-menopausal women, and earlier *in vivo* studies showing more frequent contractions within the myometrium with endometriosis (Domali 2001, Bulletti 2002), these changes in the myometrial contractions of women with endometriosis could be implicated as having a causal role in the genesis and continuation of the disease.

The reasons behind this impaired contractility may be related to alterations in calcium signalling in the post - menopausal and women with endometriosis. Reduced calcium transients are indicative of less calcium entry through L-type voltage calcium

channels or perhaps alterations in the expression of L-type calcium channels, which is supported by the High $K^+$ data I presented in this chapter, where High $K^+$ contractions were reduced in the post menopausal women as well as a trend decrease in women with endometriosis. Reduced muscle content could also be contributing to the reduced contractility found in this chapter, especially with advancing age. Further work needs to be performed to investigate this.

In summary, contractions of the non-pregnant myometrium can be measured *in vitro* and are stable allowing for investigation of the effects of $H_2S$ compared with the term pregnant myometrium as discussed next in **Chapter 4**.

### 3.5.5 Limitations of Study

One of the main issues to be addressed for future work is the low n numbers as this will allow for further insight to be given with respect to contractility differences in response to the different medications the women are on such as HRT, GnRH, progestagens as well as to define further any differences in contractility in response to endometriosis.

# Chapter 4

## Investigating the role of $H_2S$ within the myometrium through $H_2S$ producers NaHS and novel slow releasing H2S-generating compound, GYY4137.

### 4.1 Abstract

Better tocolytics are required to help prevent preterm labour. The gasotransmitter Hydrogen sulfide ($H_2S$) has been shown to reduce myometrial contractility and thus is of potential interest. However previous studies used NaHS, which is toxic and releases $H_2S$ as a non-physiological bolus and thus alternative $H_2S$ donors suitable for drug development are sought. A new $H_2S$ generating compound, GYY4137, developed to slowly release $H_2S$ and thus better reflects physiological conditions, appears to be such a drug.

The effects of GYY4137 were examined on contractility and compared to NaHS, in human and rat myometrium. As it is unknown how the effects of $H_2S$ vary with gestation, its involvement in the mechanism for transition to labour, and its effects in the presence of oxytocic drive, this was also investigated, to increase understanding of its physiological importance. The effects on contractility in response to GYY4137 (1nM-1mM) and NaHS (1mM) were examined on myometrial strips from, biopsies of women undergoing elective caesarean section or hysterectomy, and from non-pregnant, 14, 18, 22 day (term) gestation or labouring rats.

In pregnant rat and human myometrium dose-dependent and significant decreases in spontaneous contractions were seen with increasing concentrations of GYY4137, which also reduced underlying Ca transient, measured using Indo-1 fluorescence. NaHS (1mM) also significantly decreased contraction. Both $H_2S$ producers significantly reduced force in oxytocin-stimulated preparations and their inhibitory effects increased as gestation advanced, but were abruptly reversed in labour. The effects of GYY4137 and NaHS also occurred in depolarized preparations. Glibenclamide, an inhibitor of ATP-sensitive potassium ($K_{ATP}$) channels, abolished the inhibitory effect of GYY4137.

These data suggest (i) $H_2S$ contributes to uterine quiescence from mid-gestation until labour, (ii) that $H_2S$ affects L-type calcium channels and $K_{ATP}$ channels reducing Ca entry and thereby myometrial contractions, (iii) add to the evidence that $H_2S$ plays a physiological role in relaxing myometrium, and thus (iv) $H_2S$ is an attractive target for therapeutic manipulation of human myometrial contractility and that drugs such as GYY4137 may be effective.

## 4.2 Introduction

$H_2S$ is a gaseous signalling molecule that has been implicated in several physiological and pathophysiological processes from long term potentiation (kimura 2002) and inflammation (Li 2005), to smooth muscle contractility (Teague 2002, Zhao 2009, Dhaese 2009, Sidhu 2001). Two cytosolic enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Patel 2009) act on the sulphur containing amino acids, cysteine and homocysteine, to produce $H_2S$ (Li 2011, Hughes 2009). At least two enzymes have been identified that degrade $H_2S$ to thiosulfate and sulphate, thiosulfate sulphur transferase and Thiol S- methyltransferase (Teague 2002, Li 2011, Ramasamy 2006). Thus $H_2S$ will be physiologically regulated within cells and rapid rates of turnover enable it to function as a signalling molecule (Vitvitsky 2012).

The effects of $H_2S$ have been examined in several smooth muscles and decreased contraction is the most common finding e.g. vas deferens,(Teague 2002)**,** blood vessels (Webb 2008) GI tract (Teague 2002, Zhao 2009, Dhaese 2009)**.** The mechanism by which $H_2S$ produces its effects on smooth muscle contractility is not clear, although $K_{ATP}$ channels have been implicated in some studies (Dawe 2008, Distrutti 2006, Tang 2005, Zhao 2001). Other studies however have found no role for $K_{ATP}$ channels (Boyarsky 1978, Dhaese 2009, Kubo 2007). In the myometrium although $K_{ATP}$ channels are expressed (Curley 2002) they so far appear to have only a limited functional importance compared to voltage dependent K channels (Heaton 1993, Aaronson 2006,Longo 2003 ) , thus other targets for $H_2S$ may be important in the myometrium. Changes in intracellular [$Ca^{2+}$] are known to underlie contractility changes in response to agonists and tocolytics in myometrium (Longo 2003, Szal 1994, Wray 2005). Recently, a study in cardiomyocytes suggested, $H_2S$ might inhibit L-type [$Ca^{2+}$] channels through sulfhydration as NaHS decreased the functional free sulfhydryl groups available in the L-type [$Ca^{2+}$] channel (Zhang 2012). In non-contracting (butanedione monoxime treated) cerebral artery, Tian et al, (Tian 2012) used fluo-4 and showed decreases in $Ca^{2+}$ levels as NaHS was increased from 0.1 to 1 mM, and suggested that NaHS relaxes these vessels by reducing L-type $Ca^{2+}$ current. There have however been no simultaneous measurements of the changes of intracellular $Ca^{2+}$ that occur when changes in contraction result with $H_2S$ production in any tissue, and hence

its role in the mechanisms of $H_2S$'s effects is unclear. Understanding how $H_2S$ affects $Ca^{2+}$ signalling in smooth muscle will provide further insight into how $H_2S$ can affect force

There is a pressing need to better understand how uterine contractility is controlled and to develop better tocolytics to reduce the morbidity and mortality associated with pre-term delivery (Goldenberg 2002, Wray 2008). Thus an endogenous molecule that can reduce contractility is of interest. It has already been shown that the uterus possesses the enzymes to produce $H_2S$ from L-cysteine, and reports have shown $H_2S$ to be able to reduce contractions of myometrium from rat and human (Patel 2001, Hu 2011, You 2011). Thus alterations of $H_2S$ levels may be an attractive target for therapeutic manipulation in problematic labours. It is not clear however if the effects of $H_2S$ are gestationally dependent, which would indicate that $H_2S$ is part of the mechanism maintaining uterine quiescence and governing the switch to labour onset, or if it remains at an unchanged constitutive level in myometrium.

The previous studies investigating $H_2S$ in myometrium used addition of NaHS as a means of producing $H_2S$. This will produce $H_2S$ in a large, rapid bolus and thus it may be questioned how well this simulates the physiological condition. In addition because of its potential lethality, it is unlikely that NaHS will be a useful therapeutic tool. Recently a novel $H_2S$ generating compound, GYY4137 (morpholin-4-ium 4 methoxphenyl (morpholino) phosphinodithionate) has been developed. It slowly releases $H_2S$, both *in vitro* and *in vivo* (Li 2008), and has been shown to slowly relax aortic rings and *in vivo* to cause vasodilation and act as an anti-hypertensive (Li 2008). To the best of our knowledge this more physiological approach to the study of $H_2S$ in myometrium has not been examined. This in turn limits information on which to judge the clinical potential/ usefulness of $H_2S$ manipulation in controlling uterine activity. In order to increase mechanistic understanding of how $H_2S$ reduces uterine contractility, simultaneous measurements of changes in intracellular Ca and force were also made (Kupittayanant 2002).

The aims of this chapter were therefore to determine: (1) the effects of GYY4137 on contractions of human and rat myometrium, (2) how responses of the myometrium to

$H_2S$ vary with gestational state, (3) the effects of glibenclamide on GYY4137-induced changes in contractility; (4) the effects of $H_2S$ produced via NaHS and GYY4137 on spontaneous, oxytocin and high $K^+$ depolarization stimulated contractions, and (5) the effect of GYY4137 on myometrial Ca signals.

## 4.3 Methods

### 4.3.1 Tissue

Strips of longitudinal myometrium (~ 1x5mm) were dissected from the uterus of humanely killed non-pregnant, 14 day, 18 day and 22 day gestation and labouring Wistar rats (Wray 2008) The gestation of the rat was defined from day 0, when the male was placed in the cage to mate. Human myometrial strips were dissected from biopsies obtained with informed consent and ethical approval from women undergoing an elective term caesarean section (means gestational age 39 weeks, mean maternal age, 31; range 22-41 years, N=15 ) or pre-menopausal hysterectomy (mean age, 40; range 27- 48 years, N=12). Indications for caesarean section included maternal request, previous traumatic vaginal delivery, previous caesarean section or breech presentation. None of the women included in this study had any underlying diseases (hypertension, diabetes, pre-eclampsia, intrauterine growth restriction etc.). Indications for hysterectomy were menorrhagia, fibroids or prolapse. Biopsies were obtained from the upper lip of the lower segment uterine incision at caesarean section (Luckas 2000) and from corresponding macroscopic normal area of the uterus at hysterectomy.

### 4.3.2 Solutions

All chemicals were produced as described in **chapter 2**. All chemicals were obtained through Sigma (UK), apart from GYY4137, which was obtained from Santa Cruz biotechnology, USA, NaHS, obtained from Alfa Aesar, uk and Indo-1, Invitrogen, uk. The composition of Physiological Saline Solution (PSS) was as follows (mM): 154 NaCl, 5.1 KCl, 0.12 $MgSO_4 7H_2O$, 10.9 HEPES, 8 Glucose, 2 $CaCl_2$, pH 7.4. In some experiments to depolarize the tissue, the KCl in the PSS was increased to 40 mM and NaCl reduced equivalently. In some experiments, 0.5 nM oxytocin was added to the PSS to study oxytocin induced contractions. The $H_2S$ forming solutions were made in

PSS at 1mM for NaHS and 1n M, 1 µM, 0.1 and 1 mM for GYY4137, pH readjusted to 7.4. (Olson 2012). Both GYY4137 and NaHS were made and incubations performed in a fume cupboard at 37$^{o}$C. Glibenclamide was used at 10µM. Diamide and DTT were used at 1mM.

### 4.3.3 $Ca^{2+}$ and Force Measurements:

Tissue preparation and measurements of calcium and tension are the same as described in **Chapter2**, general materials and methods.

### 4.3.4 Incubation protocol

Contractile activity was seen in all myometrial strips within 60 mins for rat and 3 hours for human after perfusion with PSS (Noble 2006). The strips were allowed to contract spontaneously and an equilibrium period of at least 30 minutes with stable contractions was obtained before incubation in any chemical. After recording control activity, the effect of 45 minutes exposure to NaHS, GYY4137 or control (PSS) solution on uterine activity was examined by placing the strip in an Eppendorff with the agent, at 37$^{o}$C within a fume hood due to the toxicity of $H_2S$. The tissues were then carefully re-attached to the tension transducer, superfused with PSS and contractility again recorded. The same was performed for glibenclamide experiments only the control activity was exposed to 10µM glibenclamide as well as during incubation in GYY4137 1 mM or PSS. Each concentration of drug was obtained on a separate strip of myometrium. Control incubation traces can be found in **chapter 2** (page 91).

### 4.3.5 Statistics

Contractions were analysed for amplitude, frequency, and area under the curve, (AUC, in arbitrary units, au) for; 10 minutes, rat data; 30 min, human data (to accommodate the slower rate of contractions), and; 15 minutes, high $K^+$, before and after $H_2S$ forming solution incubation, using Origin 8 (Shmigol 1998). Each strip tested for the effect of each $H_2S$ producer, had a paired control response in PSS rather than test solution. After incubation the contractions were assessed 5 minutes after re-attachment. Diamide and DTT were added directly to the bath to examine their effects comparing 10 minutes control and Diamide or DTT exposed in the same

way. Student's t tests were performed to compare two groups. Anova with Bonferroni post hoc tests were used to compare more than two groups. P was taken as showing a significant difference when $P<0.05$. Each n was from a different biopsy or rat.

## 4.4 Results

### 4.4.1 Establishment of spontaneous contractions and control incubation protocol

All tissues were superfused with physiological saline salt solution (PSS) at 37$^{o}$C, pH 7.4 until spontaneous contractile activity was established. The commencement of spontaneous contractile activity of rat myometrium was always within an hour were as spontaneous contractile activity of human myometrium was within 2 hours of continuous superfusion with PSS at 36-37$^{o}$C. Where spontaneous activity did not occur following 2 hours, the strip was subjected to a 1 (rat) or 2 minute (human) high $K^{+}$ and the strip was further monitored for spontaneous contractions between 30 minutes and 1 hour. If no further activity was recorded the strip was discarded and another strip tried. All samples included in this study were spontaneously active.

Due to the great toxicity of $H_2S$ the tissue strips with the $H_2S$ producing solutions, were incubated in a fume cupboard and then re-attached via their clips, to the tension transducer at the end of the incubation period. It was therefore necessary to show that under control conditions i.e. incubation with PSS and re-attaching, no significant changes in contractile parameters were found when contractions were re-established. **Figure 2.7** within **chapter 2** shows that this was the case. Analysis of 4 strips (non-pregnant rat), 6 strips (14 day gestation rat), 7 strips (18 day gestation rat), 7 strips (22 day, term pregnant rat), 4 strips (labouring rats), 6 strips (non-pregnant human) and 6 strips (pregnant human) showed that there were no significant changes to any of the parameters of contractions (**Table 4.1**).

**Table 4.1. Changes in contractile parameters of myometrial tissue in response to control incubations**

| | NP | 14 day | 18 day | 22 day (term) | Labouring | Non-pregnant Human | Term pregnant human |
|---|---|---|---|---|---|---|---|
| **n-number** | 4 | 6 | 7 | 6 | 4 | 7 | 4 |
| **Force Amplitude (%)** | 96±6% | 104±1.9% | 101±4% | 99 ±2% | 102±4% | 97±3% | 90±7% |
| **Frequency (%)** | 89±13% | 100.4±7.6 % | 92±3% | 100±7% | 95±9% | 93±6% | 106±5% |
| **Duration (%)** | 101±9% | 97.0±6% | 105±4% | 99±4% | 90±5% | 94±6% | 102±7% |
| **AUC (%)** | 85±12% | 107±3% | 97.1±7.3 % | 95±5% | 104±4% | 88±9% | 104±6% |

Rat myometrial or human myometrial strips were studied from 4-7 animals. After baseline values were obtained (10 minute period immediately before incubation in experimental solutions,100 %), tissues were incubated in either physiological saline (control) for 45 minutes and then the parameters of contraction re-measured, and expressed as the percent of baseline values (i.e. paired data).Values are means ± s.e.m.

No Significant differences were found between any of the values.

### 4.4.2 Dose dependent effects of GYY4137 on spontaneous contractions in non-pregnant rat myometrium

Having established a robust protocol (**section 4.4.1**) I proceeded to determine the effects of GYY4137 on myometrial contractility. Concentrations of GYY4137 from 1nM to 1mM were examined in non-pregnant rat myometrium. Typical results for each concentration are shown in **figure 4.1A**. The mean data is presented within **figure 4.1 B**. As seen in the original traces, no significant changes in non-pregnant contractility profiles where seen upon increasing doses up to 1mM GYY4137. At the highest concentration 1mM GYY4137 the force remained unaffected (100.8±7% relative to control of 100%, n=4). Even integrating the differences in force, duration and frequency by measuring the AUC, at 1mM GYY4137 showed no significant change (93.6±28% relative to control period of 100%, n=4).

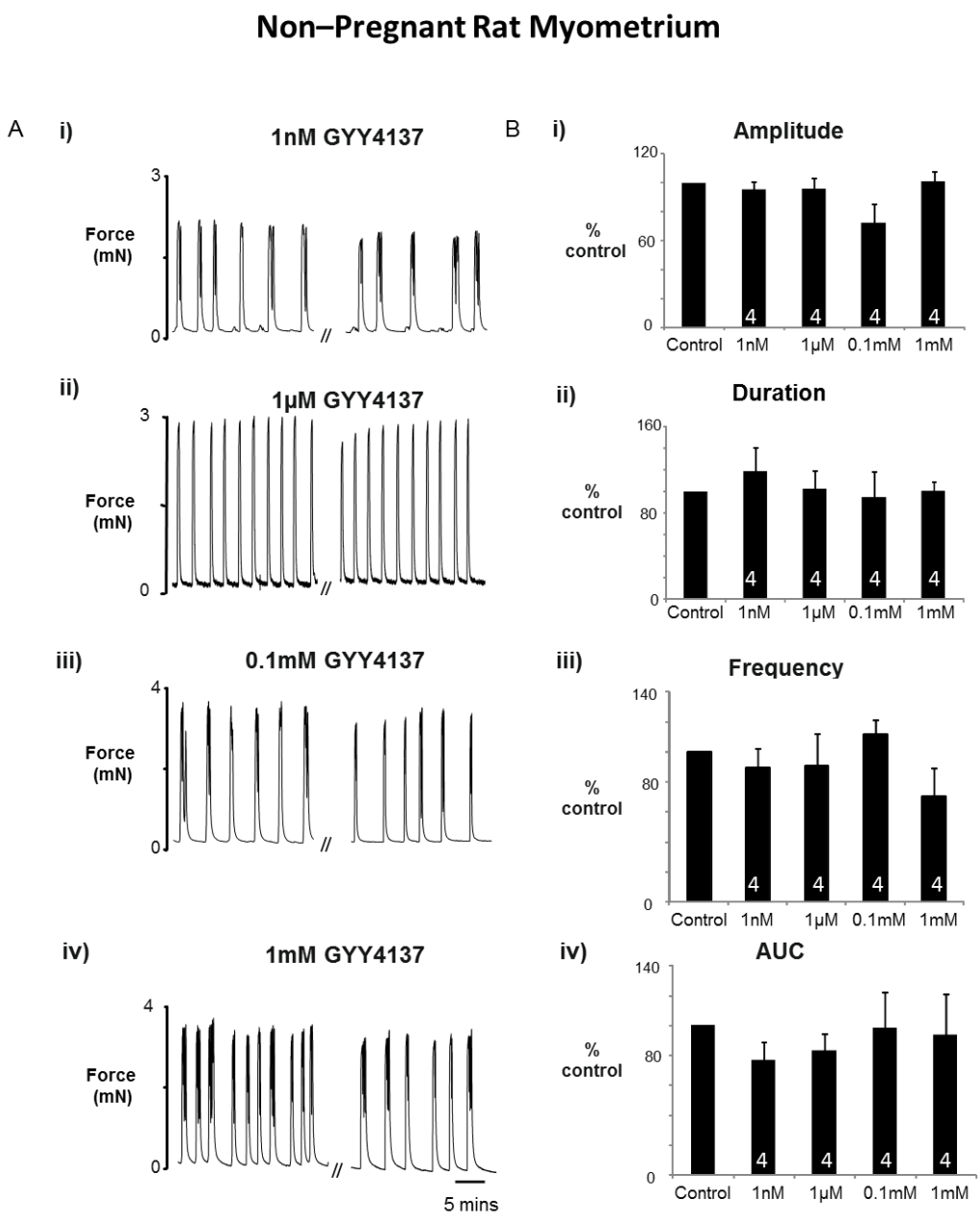



**Figure 4.1: Dose dependency of GYY4137 in non-pregnant rat.**

(A) Representative isometric recordings of spontaneously contracting myometrial strips obtained from non-pregnant rat, before and after 45 minute incubations in i) 1nM, ii)1μM, iii) 0.1mM, iv) 1mM GYY4137. (B) Mean data± s.e.m, denoted by error bars, showing the dose dependent decrease in i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to GYY4137. Values within bars indicate n-numbers. No significant differences in contractile activity were found, using Anova with Bonferroni *post hoc* tests.

### 4.4.3 Dose dependent effects of GYY4137 on spontaneous contractions in term pregnant rat myometrium

Exposure of contracting term pregnant rat myometrium to increasing concentrations of GYY4137 from 1nM to 1mM caused a dose dependent decrease in the amplitude of force of contractions (**figure 4.2**). Representative traces of each concentration are illustrated in **Figure 4.2 A**. The mean data presented in graphical form can be found in **figure 4.2B**.

The decrease in force was most marked at the highest concentration of GYY4137 1mM (34.0±6% relative to control period 100%, n=6). Statistical analysis showed a significant decrease in contractility from 1nM to 1mM (p=0.003) and from 1nM to 0.1mM (p=0.030). The decrease in frequency of contractions was significant at concentrations from 1nM and 1µM to 1mM GYY4137 (p=0.002, p=0.042 respectively). There was no significant effect on duration of contraction after increasing concentrations of GYY4137. However, by integrating the differences in force, duration and frequency by measuring the AUC, showed a dose dependent decrease with increasing concentrations of GYY4137 which was most marked at the highest concentration of GYY4137, 1mM (22.0±10% relative to control period 100%, n=6). With significance at concentrations of 0.1mM and 1mM (p=0.014, p=0.003 respectively). These results show that GYY4137 causes a dose dependent decrease in myometrial contractility in pregnant rat myometrium.

A dose response curve for the effect of GYY4137 on the force of myometrial contractions showing the logEC50 is shown in **Figure 4.3**.

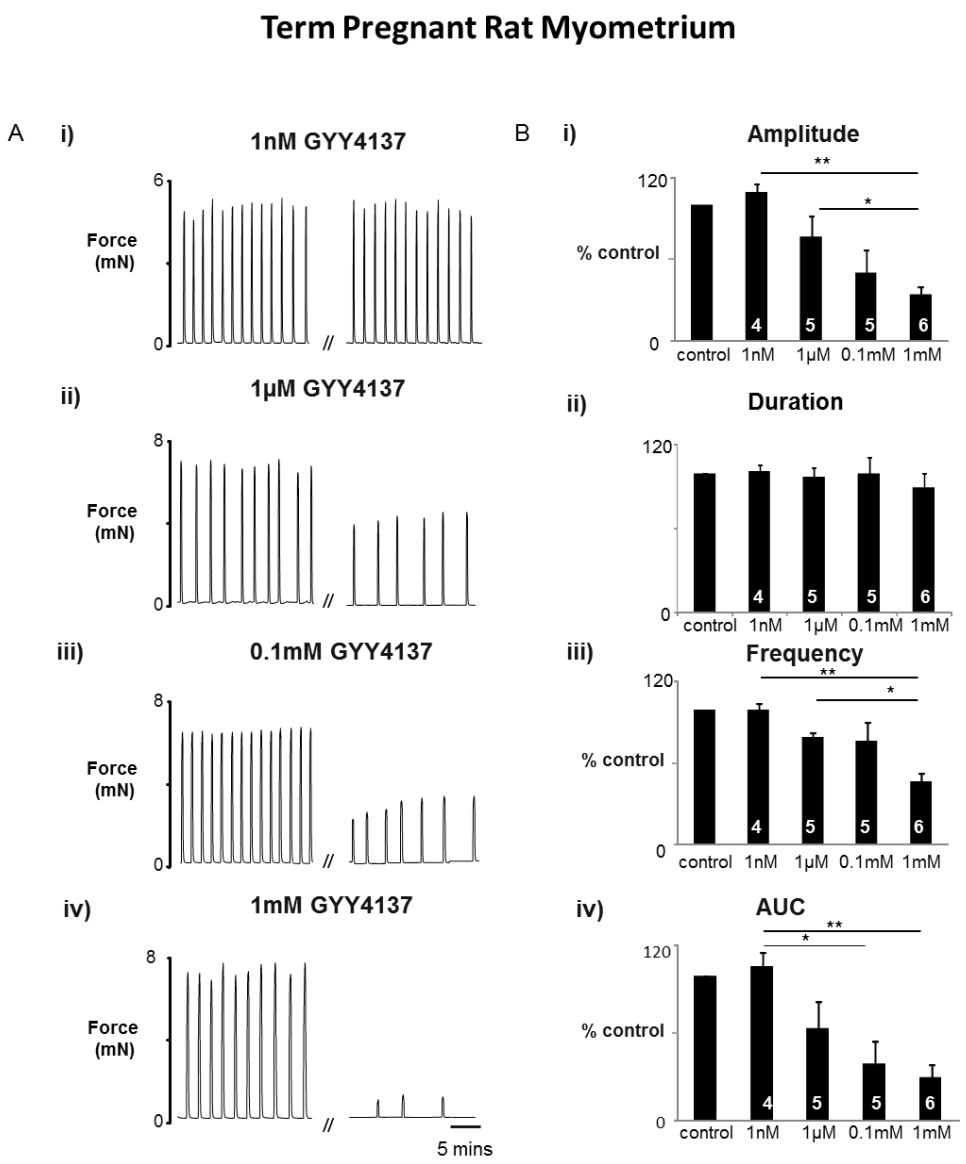



**Figure 4.2: Dose dependency of GYY4137 in term pregnant rat.**

(A) Representative isometric recordings of spontaneously contracting myometrial strips obtained from 22 day gestation rat, before and after 45 minute incubations in i) 1nM, ii)1µM, iii) 0.1mM, iv) 1mM GYY4137. (B) Mean data± s.e.m, denoted by error bars, showing the dose dependent decrease in i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to GYY4137. Values within bars indicate n-numbers. * represents P<0.05, ** represents P<0.01, using Anova with Bonferroni *post hoc* tests.

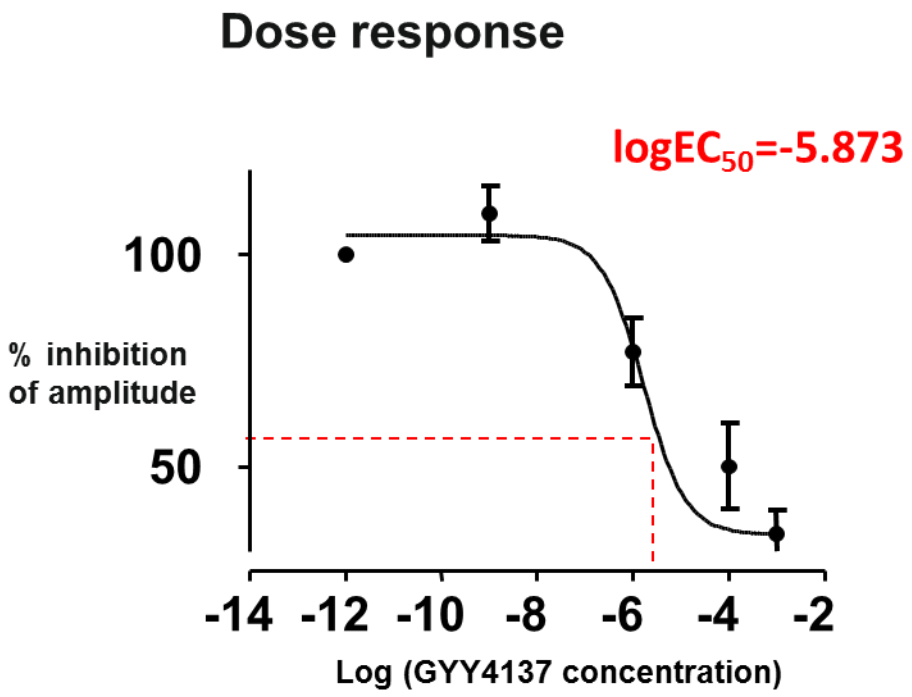



**Figure 4.3 LOG dose response curve for pregnant rat myometrium**

Log dose response curve for the effect of GYY4137 on rat myometrial contractility, showing sigmoidal relationship. The EC50 value of -5.873 depicts the concentration required to reduce myometrial contractility by 50% compared to minimum and maximum values.

### 4.4.4 Gestational dependent effects of GYY4137 and NaHS

Having shown that GYY4137 can reduce contractions of term pregnant myometrium, I next examined if its efficacy varied throughout pregnancy, and as no data were available on this point for NaHS, it was also investigated. Typical examples of the effects of 1mM GYY4137 (n =4-7) and NaHS (n =4-7) from non-pregnant, 14, 18 and 22 day pregnant and labouring rat myometrial tissue are shown in **Figures 4.4A** and **4.5A** respectively.

Exposure of non-pregnant, 14 day, 18 day, 22 day and labouring contracting myometrial tissue to 1mM GYY4137 caused a gestational dependent decrease in the amplitude of force of contractions (**figure 4.4**). Increasing effects of GYY4137 on contractility as gestation progressed were found (**Figure4.4Aii-iv**). It can be seen that GYY4137 has no significant effect on non-pregnant myometrium (**Figures 4.4A and Bi**). As shown in the mean data, (**Figure 4.4B**), amplitude, frequency of contractions and AUC are progressively reduced by GYY4137 from mid-gestation up until labour onset. No significant differences were found at day 14 as effects were small. Effects increased as gestation advanced with significant decline in force of contraction at day 18 and day 22 (23.3±12.7% and 34.4±5.6% respectively). There were no significant changes to frequency, or duration, However by integrating the differences in force, duration and frequency by measuring the AUC, showed large decreases at day 18 and day 22 (21.4±12.3% and 21.7±10.3% respectively). There was a marked reversal of the inhibitory effect of GYY4137 once labour was initiated. No effect on any of the parameters of spontaneous contractions of labouring tissue in response to GYY4137 was found (**Figure 4.4Av**). Statistical analysis showed a significant increase in the force and AUC of labouring contractions in response to GYY4137 when compared to day 18 and day 22 (106.1±4.8%, 103±10% respectively).

As revealed in **Figure 4.5A** the effects of NaHS also increased as gestation advanced. As with GYY4137 there was no significant effect on the non-pregnant (**Figure 4.5i**) or labouring (**Figure 4.5v**) myometrium. The mean data for the effects of NaHS throughout gestation are shown in **Figure 4.5B** and the significant decrease on force compared to non-pregnant myometrium can be seen at 22 days gestation (14.9±8.0%). Significant decreases on AUC in response to NaHS were also seen at day 18 and day 22

(40.9±9.3% and 16.6±13.5% respectively). Once labour was initiated, statistical analysis showed a significant increase in the force and AUC of labouring contractions in response to NaHS when compared to day 18 and day 22 (99.1±6.7%, 99.3±5.7% respectively).

**Figure 4.4 and 4.5iv)** shows a term pregnant myometrial strip which had been incubated in 1mM GYY4137 and NaHS respectively, clear effects on contraction are apparent. The mean data for contraction amplitude, Duration, frequency and AUC measured over 10 minutes, after incubation with GYY4137 and NaHS, compared with the immediate control period are shown in **Table 4.2**; significant reductions in all parameters of contraction occurred except for changes to duration. **Figure 4.4 and 4.5iv)** also shows the gradual recommencement of force after return to control solutions.

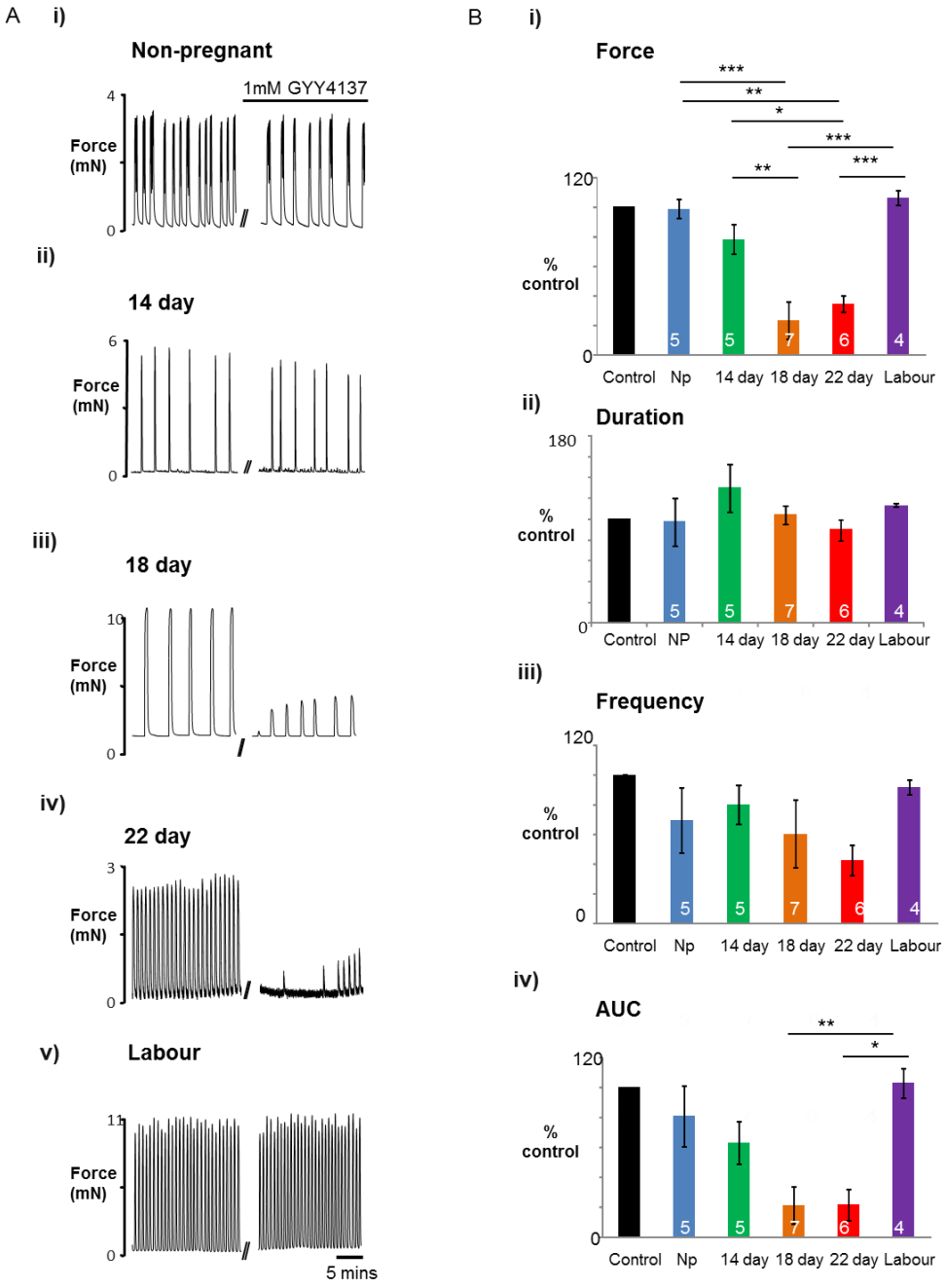



**Figure 4.4: Rat myometrial contractility changes over gestation in response to 1mM GYY4137.**

**(A)** Representative isometric recordings of spontaneously contracting myometrial strips obtained from i) non-pregnant (NP), ii) 14 day, iii) 18 day, iv)22 day gestation and v) in labour rats before and after incubation in 1 mM GYY4137. **(B)** Mean data ± s.e.m, denoted by error bars, of the gestational dependent decrease in i) Amplitude, ii) Duration, iii) Frequency, iv) AUC in response to GYY. Values represent Means ± s.e.m, denoted by error bars. Values within bars indicate n-numbers. * represents P<0.05, ** represents p<0.01, *** represents p<0.01, using Anova with Bonferroni *post hoc* tests.

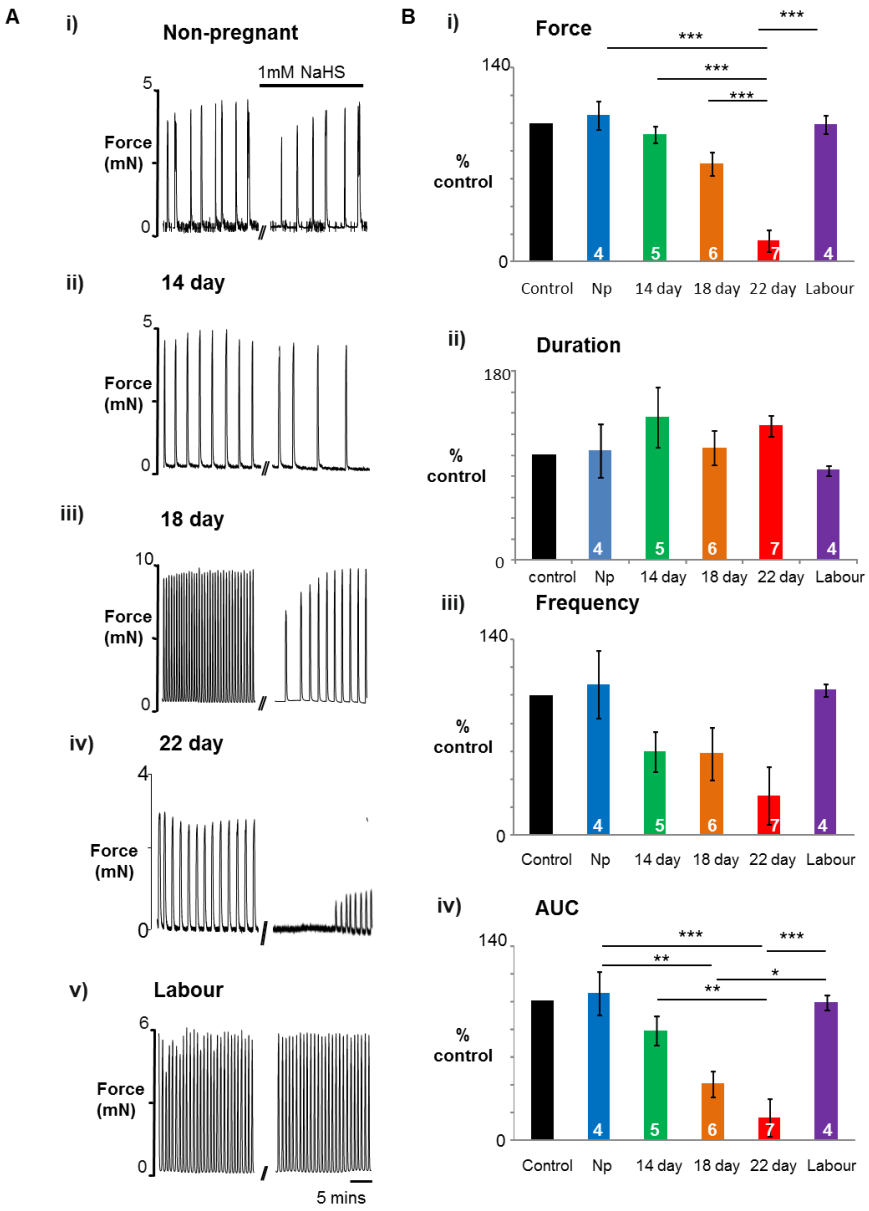



**Figure 4.5: Rat myometrial contractility changes over gestation in response to 1mM NaHS.**

**(A)** Representative isometric recordings of spontaneously contracting myometrial strips obtained from i) non-pregnant (NP), ii) 14 day, iii) 18 day, iv) 22 day gestation and v) in labour rats. **(B)** Mean data ± s.e.m, denoted by error bars, of the gestational dependent decrease in i) Amplitude, ii) Duration, iii) Frequency, iv) AUC in response to NaHS. Values within bars indicate n-numbers. * represents P<0.05, ** represents p<0.01, *** represents p<0.01, using Anova with Bonferroni *post hoc* tests.

**Table 4.2. Changes in contractile parameters in response to 1mM NaHS and GYY4137 in term pregnant rat myometrium**

| Parameter measured | Control (% ± SE, n=7) | NaHS incubated (% ± SE, n=7) | GYY4137 incubated (% ± SE, n=6) |
|---|---|---|---|
| **Contraction Amplitude** | 99 ±2% | 15±8%* | 34±6%* |
| **Frequency** | 100±7% | 23±9%* | 42±10%* |
| **Duration** | 99±4% | 59±28% | 121±35% |
| **AUC** | 95±5% | 16±13%* | 22±10%* |

Term (day 22) rat myometrial strips were studied from 4-7 animals. After baseline values were obtained (10 minute period immediately before incubation in experimental solutions,100 %), tissues were incubated in either physiological saline (control) or the solutions indicated, for 45 minutes and then the parameters of contraction re-measured, and expressed as the percent of baseline values (i.e. paired data).Values are means ± s.e.m.

***** represents significant differences in contractility compared to preceding control period ($p<0.05$, t-test). AUC; area under the curve.

### 4.4.5 Recovery periods and times to contract within the rat myometrium.

The length of time it took for the strips incubated in GYY4137 and NaHS to start contracting after the 45 minute incubation was monitored to see whether this was affected in response to $H_2S$. There was no significant differences, however in **Figure 4.6** you can see that with GYY4137 incubations (**Figure 4.6A**) and NaHS incubations (**Figure 4.6B**) there is a trend of an increase in the length of time to contract up to term which is reversed upon labour onset.

The maximum recovery amplitude of contraction after incubation was examined for both GYY4137 (**Figure 4.7A**) and NaHS incubated (**Figure 4.7B**) strips where possible. This showed no significant differences over gestation using Anova with bonferroni post hoc testing illustrating significant recovery was achieved. Presumably the $H_2S$ had volatilised and oxidized. It can still be seen that the most significantly affected gestations 18 day and 22 day recovered the least.

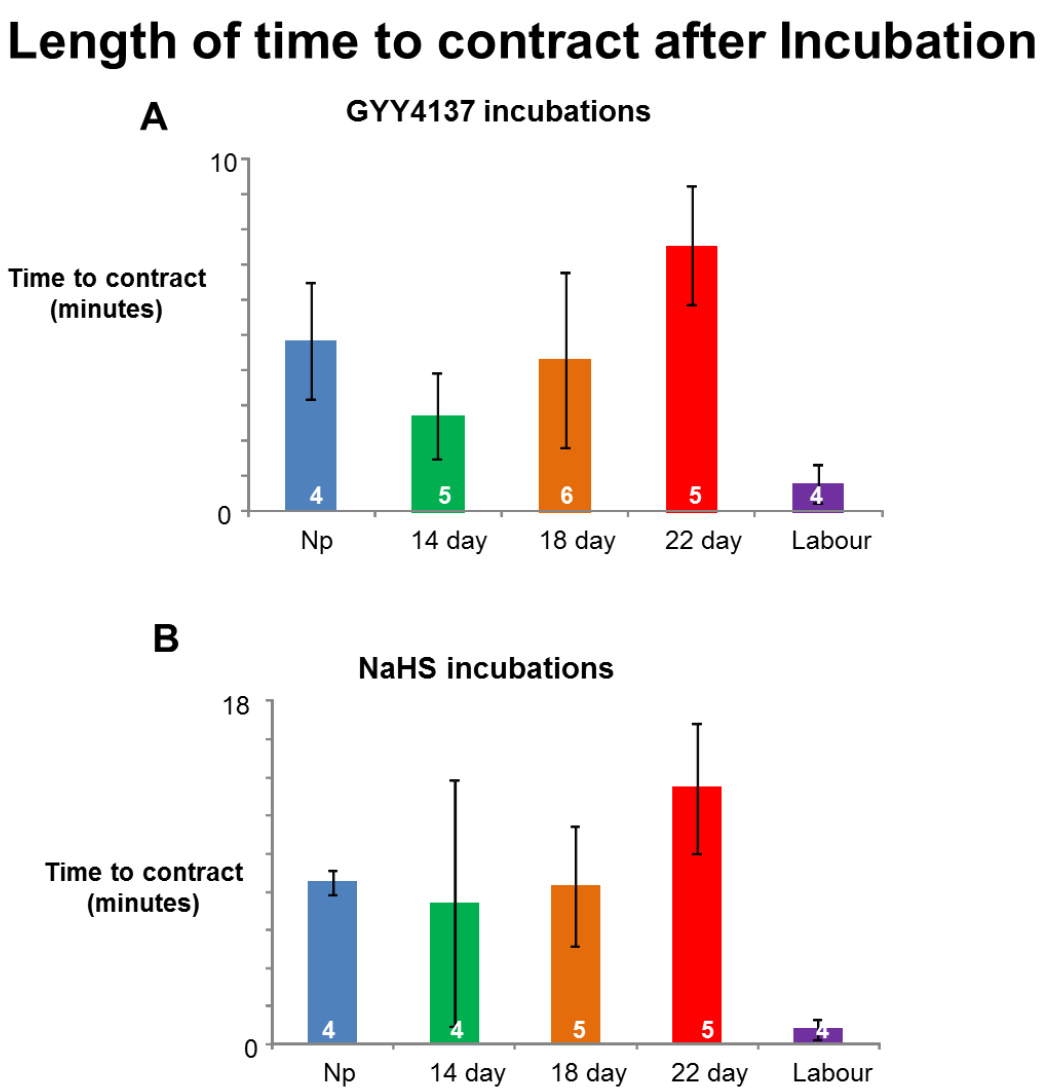



**Figure 4.6 Times to contract after incubation of rat myometrium over gestation.**

Mean data ± s.e.m, denoted by error bars, of the times to start contracting after 45 minute incubations in (A) GYY 4137 (B) NaHS. A trend of increased time to contract can be seen up until 22 day gestation, which disappears upon labour onset. Values within bars indicate n-numbers. No significant differences were found using Anova with Bonferroni *post hoc* tests.

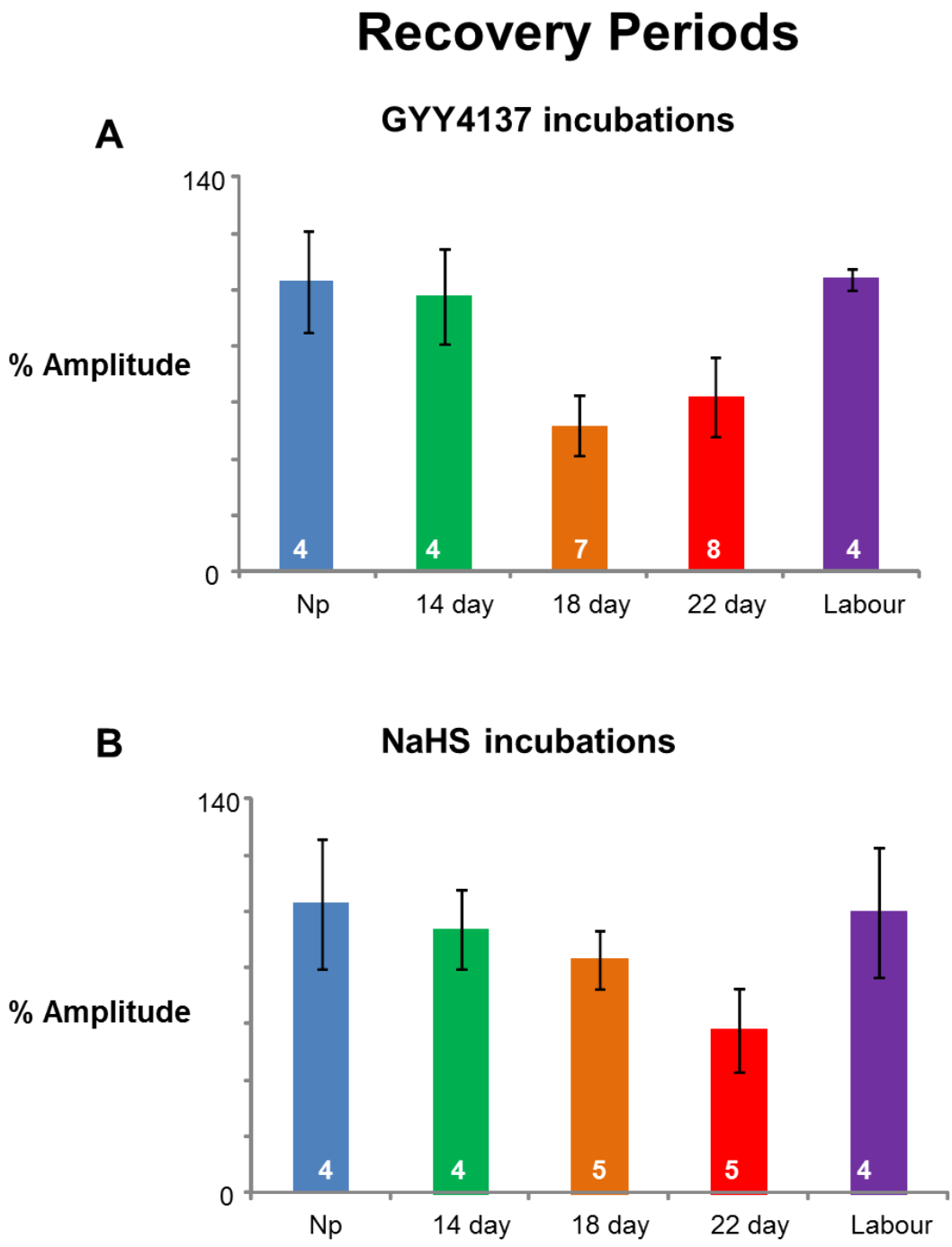



**Figure 4.7 Amplitude of contraction recovery in response to GYY4137 and NaHS of rat myometrium over gestation.**

Mean data ± s.e.m, denoted by error bars, of the maximum recovery amplitude of contraction after 45 minute incubations in (A) GYY 4137 (B) NaHS. Values within bars indicate n-numbers. No significant differences were found using Anova with Bonferroni *post hoc* tests.

### 4.4.6 Effects on calcium entry and Calcium signalling

As the above data show very clear reductions in the strength of myometrial contractions in the presence of $H_2S$, it was next determined if $Ca^{2+}$ channels and transients are affected by $H_2S$ producers in two ways. Firstly, high $K^+$ was used to depolarize the myometrium and open voltage gated $Ca^{2+}$ channels (Noble 2006). If the response to depolarization is unaltered by the $H_2S$ donors then this would indicate that their effects were on the normal processes leading to membrane depolarization. Secondly, direct measurements of intracellular $Ca^{2+}$ simultaneously with force in the absence and presence of GYY4137 were made. If $Ca^{2+}$ entry is decreased, then this should be apparent in the associated $Ca^{2+}$ transients (Shmigol 1998).

**High $K^+$ depolarisation**

Depolarisation using 40mM High $K^+$ for 15 minute was examined before and after 45 minutes incubation in 1mM NaHS (**figure 4.8B**, n=6) and GYY4137 (**figure 4.8C,** n=8). In day 22 pregnant rats, the two successive applications of high $K^+$, produced very similar contractile responses; a rapid rise in force which plateaus and shows only a small decrement until the tissue is returned to control solution (**Figure 4.8**). As shown in **Figures 4.8B and C**, both $H_2S$ producers inhibited the amplitude of the High $K^+$ contractions significantly (43±10%, NaHS 82±6% GYY4137) and AUC (34±4%, NaHS 84±7% GYY4137) compared to control (95±5%, 105±3% respectively, **figure 4.8A**).

**Calcium signalling**

In a subset of studies described within this chapter term pregnant myometrial tissue was loaded with intracellular calcium indicator indo-1. Strips loaded and incubated in control solution, PSS, showed no changes in force and underlying calcium transients (**figure 4.9A**). An overlay of both force and calcium under control conditions (PSS, **figure 4.9Bii**) shows that changes in calcium precedes the changes in force. The effects of 1mM GYY4137 on $Ca^{2+}$ signalling on day 22 of gestation were examined. **Figure 4.9B** clearly shows spontaneous Ca transients (indo-1 fluorescence) underlie the phasic contractions of the myometrium. As before, GYY4137 produced significant decreases in contraction amplitude, which as can be seen in **Figure 4.9B**,

are accompanied by a significant decrease in the amplitude of $Ca^{2+}$ transients to 76 ± 8%, (*n* = 5). **Figure 4.9Bii** shows an overlay of force and $Ca^{2+}$ in the presence of control and GYY4137 conditions, illustrating the decline in force and calcium in the presence of GYY4137.

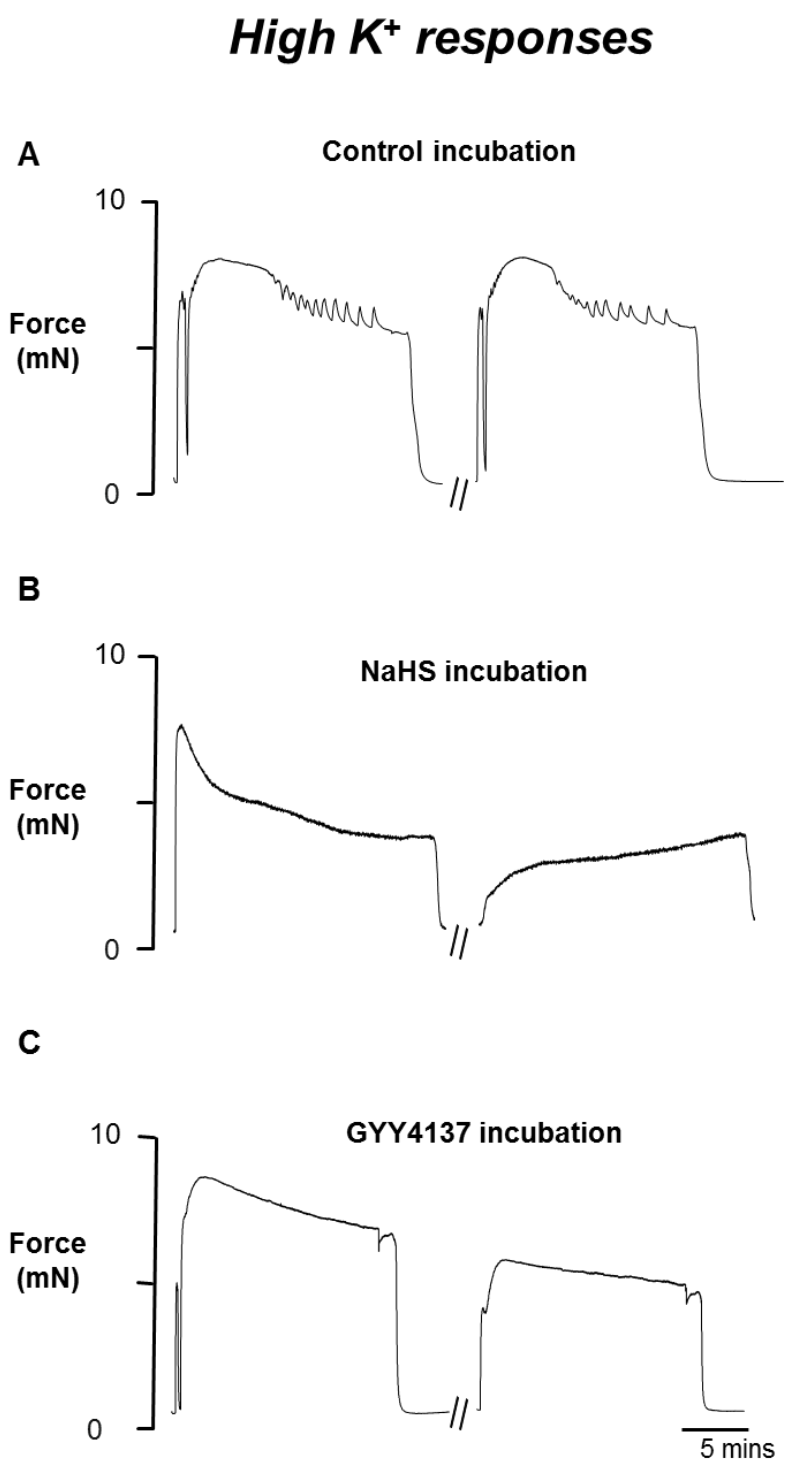



**Figure 4.8: Effects of NaHS and GYY4137 on High $K^+$ depolarisation.**

High $K^+$ (40 mM) depolarisation, 15 mins, of term pregnant rat myometrial strips and the effects in the presence of A) physiological saline solution (PSS), B) 1mM NaHS (n=6) C) 1mM GYY4137 ( n=8). All solutions were used at 37$^{o}$C and pH 7.4.

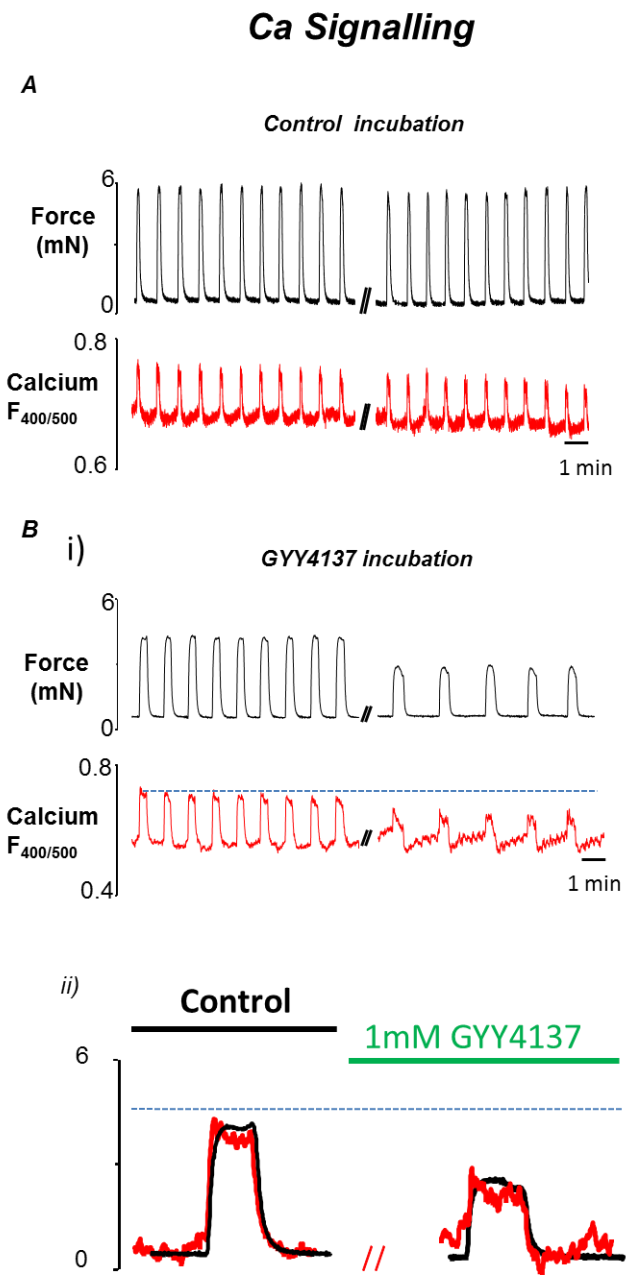



**Figure 4.9: Effects of GYY4137 on calcium signalling.**

Recording of force and intracellular Ca (from Indo-1–emitted fluorescence F400:500), measured simultaneously in spontaneously contracting myometrial strips dissected from term rat myometrium before and after incubation in A) Control (PSS, n=3), Bi) GYY 1mM (n=5). Bii) an overlay of both force and calcium showing that the change in calcium precedes the changes in force as well as showing the decrease in contractions in the presence of GYY4137 1mM compared to control. All solutions were used at 37$^{o}$C and pH 7.4.

### 4.4.7 Effects of $K_{ATP}$ channel inhibition on GYY4137-induced changes in contractility.

Studies have demonstrated that $K_{ATP}$ channels are involved in $H_2S$ modulation of smooth muscle tone (Tang 2005, Zhao 2001) but this has not been studied in rat uterus. In 4 paired experiments the effects of GYY4137 in the presence of glibenclamide, (10 μM) a blocker of $K_{ATP}$ channels was investigated. As previously found (Heaton 1993) glibenclamide had little effect on spontaneous contractions shown in **Figure 4.10A.** The mean data for the effects of Glibenclamide, Glibenclamide & GYY4137 as well as GYY4137 incubations is shown in **Figure 4.10C and Table 4.3**. Incubation of term myometrial tissue in GYY4137 in the continued presence of glibenclamide had little effect on the parameters of contraction within rat myometrium when compared to GYY4137 alone (**Figure 4.8B**). As can be seen in **Table 4.3**, GYY4137 did not produce any significant effects when glibenclamide was present on force, frequency or AUC when compared to GYY4137 alone (73±19%, 101±28%, 72±14% compared to 34±6%, 42±10% 22±10% respectively relative to paired control of 100% (normalised). There was no significant change to duration.

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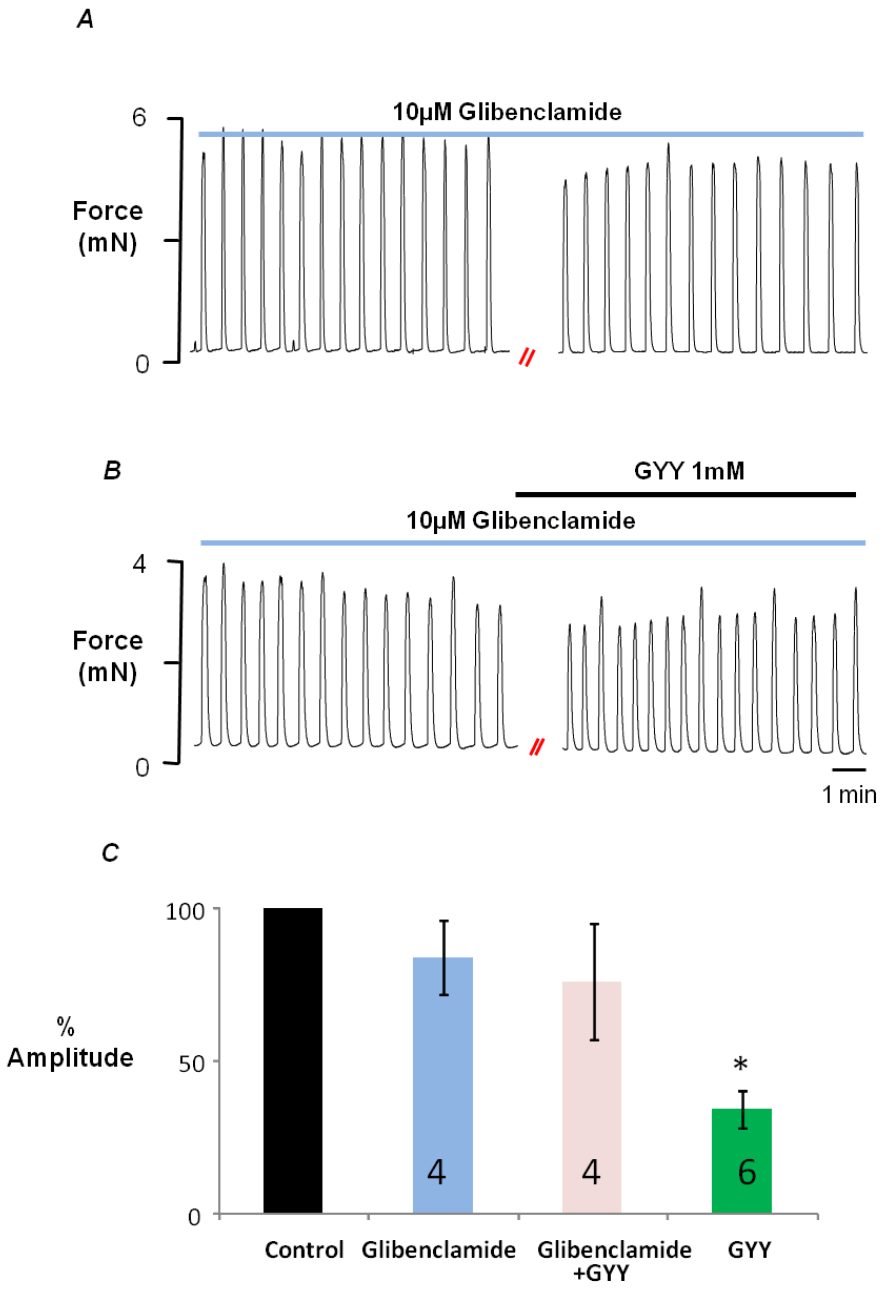


**Figure 4.10: Effects on calcium entry and calcium signalling.**

$K_{ATP}$ channel inhibitor glibenclamide (10µM) was applied to rat myometrial strips 20 minutes before and during the 45 minute A) control incubation (PSS) and B) GYY4137 (1mM, n=4) incubation period. All solutions were used at 37°C and pH 7.4. C) Illustrates the mean data± s.e.m, denoted by error bars. Values within bars indicate n-numbers. * represents P<0.05

**Table 4.3. Changes in contractile parameters in response to GYY4137, Glibenclamide and GYY4137 with Glibenclamide, in term pregnant rat myometrium**

| Parameter measured | Control (PSS) | Glibenclamide (% ± SE, n=4) | GYY4137+ Glibenclamide (% ± SE, n=4) | GYY4137 incubated (% ± SE, n=6) |
|---|---|---|---|---|
| **Contraction Amplitude** | 100% | 81±12% | 73±19% | 34±6%* |
| **Frequency** | 100% | 99±13% | 101±28% | 42±10%* |
| **Duration** | 100% | 92±15% | 124±16% | 121±35% |
| **AUC** | 100% | 84±21% | 72±14% | 22±10%* |

Term (day 22) rat myometrial strips were studied from 4-6 animals. After baseline values were obtained (10 minute period immediately before incubation in experimental solutions,100 %), tissues were incubated in either physiological saline (control) or the solutions indicated, for 45 minutes and then the parameters of contraction re-measured, and expressed as the percent of baseline values (i.e. paired data).Values are means ± s.e.m.

***** represents significant differences in contractility compared to control period ($p<0.05$). AUC; area under the curve.

### 4.4.8 Effects of sulfhydryl-modifying reagents Diamide (DM) and Dithiothreitol (DTT) on contractions of term rat myometrium

Sulfhydryl modifiers can help to simulate or prevent S-sulfhydration. DM is an oxidizing sulfhydryl modifying substance converting SH bonds to disulfide bonds reducing the amount of free sulfhydryl groups simulating the sulfhydration mechanism. Examining diamides response on contractility (**Figure 4.11A**) shows similar responses to GYY4137 and NaHS, i.e. a significant decrease in amplitude, frequency and AUC of contraction (all $p<0.05$, $n=5$) with no significant change to duration (**Table 4.4**). A representative trace is shown in **Figure 4.11A**. DTT is a reducing sulfhydryl modifying reagent which transforms disulfide bridges into sulfhydryl groups in cysteine-containing proteins. This had no significant effect on contractility as illustrated in **Figure 4.11B** and summarised in **table 4.4,** this highlights free sulfhydryl groups produce no significant effect on contractility.

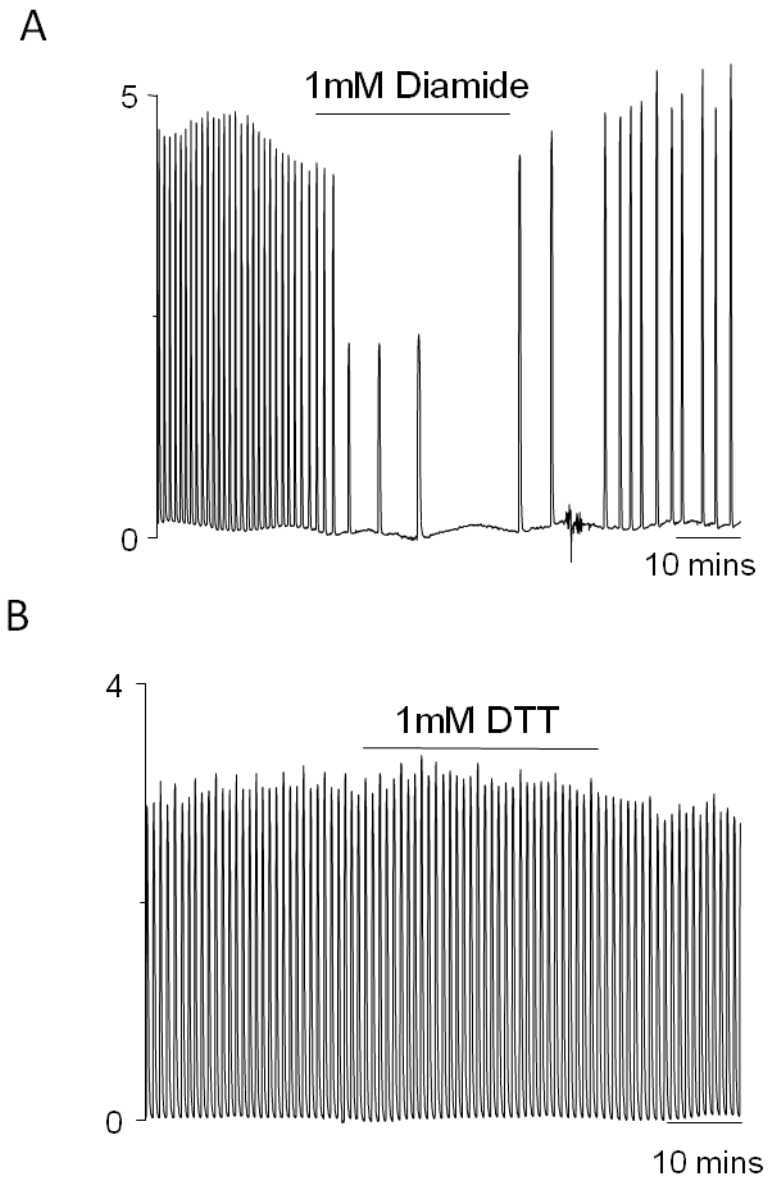


**Figure 4.11: Term rat myometrial contractility changes to sulfhydryl modifiers Diamide and Dithioreitol.**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from 22 day gestation animals in response to (A) Diamide (DM, 1mM) an oxidant sulfhydryl modifier and (B) Dithioreitol (DTT, 1mM), a reductant sulfhydryl modifier. Diamide significantly reduced amplitude, frequency and area under the curve similar to NaHS and GYY4137 ($p<0.05$, $n=5$). DTT caused no changes to contractility ($n=3$). Student t-tests were performed.

**Table 4.4. Changes in contractile parameters in response to Dithioreitol and Diamide compared to control, in term pregnant rat myometrium**

| | Control (PSS) | Dithioreitol (DTT, % ± SE, n=3) | Diamide (DM, % ± SE, n=5) |
|---|---|---|---|
| **Force** | 100% | 89±11% | 13±12%* |
| **Frequency** | 100% | 122±16% | 39±35%* |
| **Duration** | 100% | 143±15% | 21±16% |
| **AUC** | 100% | 144±26% | 6±5%* |

Term (day 22) rat myometrial strips were studied from 3-5 animals. After baseline values were obtained (10 minute period immediately before superfusion in experimental solutions,100 %), tissues were superfused in either physiological saline (control) or the solutions indicated, for 30 minutes and then the parameters of contraction re-measured, and expressed as the percent of baseline values (i.e. paired data). Values are means ± s.e.m.

***** represents significant differences in contractility compared to control period ($p<0.05$). AUC; area under the curve.

### 4.4.9 Effects of GYY4137 and NaHS on non-pregnant human myometrium

Having established in rat that GYY4137 and NaHS could significantly reduce force in term but not non-pregnant myometrium, next their affect on non-pregnant human myometrium were investigated. Contractions were monitored and analysed 30 minutes before and after incubation in the solution of interest. As revealed in rat myometrium there were no significant changes in force, duration, frequency, or AUC of control contractions, the same was found upon control incubations of non-pregnant human myometrium in PSS (97±3%, 94±11%, 93±6%, and 88±9%,respectively, n=7, **Figure 4.12A**).

Exposure of spontaneous by contracting non-pregnant human myometrium with NaHS (**Figure 4.12B**, n=6) showed no significant effects on force, duration, or AUC of contractions (90±9%, 99±13%, 80±10% respectively relative to control 100%). However, frequency was significantly decreased (60±16%). Exposure of spontaneously contracting non-pregnant human myometrium with GYY4137 (**Figure 4.12C**, n=5) showed no significant effects on force, duration, frequency or AUC contractions (84±10%, 82±14%, 116±10%, and 88±19%, respectively relative to control).

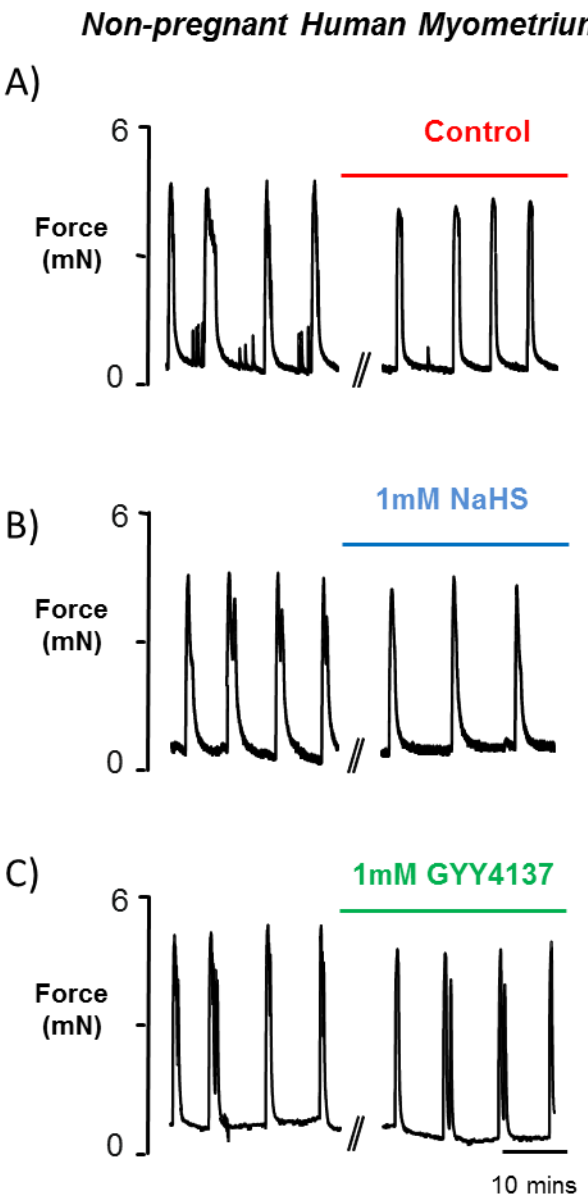


**Figure 4.12 Effects of NaHS and GYY4137 on non-pregnant human myometrium**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from non-pregnant human tissue. Strips were placed under a resting tension of 2mN and superfused continually with physiological saline solution (pH 7.4) at 37°C before and after 45 minute incubations in (A) physiological saline solution (control, represented in red), (B)1mM NaHS (blue), (C)1mM GYY4137 (green) all at a pH 7.4 and at 37°C.

### 4.4.10 Effects of GYY4137 and NaHS on term pregnant human myometrium

In contrast to the non-pregnant human myometrium, as shown in **Figure 4.13B and 4.13C**, both $H_2S$ producers generated clear effects on term human myometrium and significant reductions in force, as seen in **Table 4.5**. Statistical analysis showed significant decreases in force, frequency and AUC of contraction after incubation in NaHS 1mM ($p=0.014$, $p=0.004$, and $p=0.020$ respectively, $n=6$) and GYY4137 1mM ($p=0.008$, $p=0.038$, and $p=0.010$ respectively, $n=7$). Duration of contractions did not reach significance for either $H_2S$ producer ($p=0.055$, NaHS and $p=0.070$). These results show that GYY4137 and NaHS significantly inhibit contractions of term pregnant human myometrium and that contractions of non-pregnant human myometrium are not significantly affected. All except for frequency in the presence of NaHS were a reduction is found in both tissues (non-pregnant and pregnant).

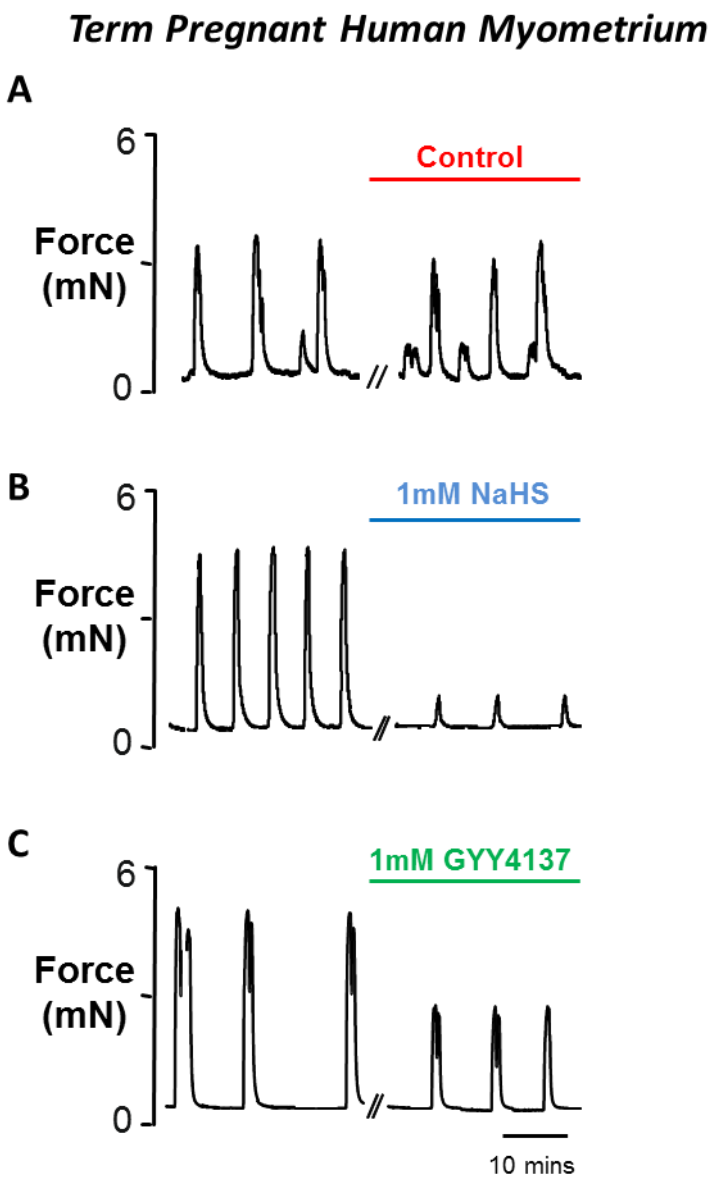


**Figure 4.13 Effects of NaHS and GYY4137 on term pregnant human myometrium**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from term pregnant non-labouring human tissue. Strips were placed under a resting tension of 2mN and superfused continually with physiological saline solution (pH 7.4) at 37°C before and after 45 minute incubations in (A) physiological saline solution (control, represented in red), (B)1mM NaHS (blue), (C)1mM GYY4137 (green) all at a pH 7.4 and at 37°C.

**Table 4.5: Changes in pregnant human myometrial contractile parameters in response to NaHS, and GYY4137 incubations with and without oxytocin.**

| Parameter measured | Control (%) | NaHS incubated (% ± SE, n=7) | NaHS +Oxytocin (%± SE, n=7) | GYY4137 incubated (%± SE, n=6) | GYY4137 +Oxytocin (%± SE, n=5) |
|---|---|---|---|---|---|
| **Contraction Amplitude** | 100 | 10±4%*** ‡** | 40±9%***** | 35 ±14%***** | 33±14% |
| **Frequency** | 100 | 23±9%*** ‡** | 50±10%***** | 48±19%***** | 76±26% |
| **Duration** | 100 | 52±19% | 71±25% | 51±18% | 68±17% |
| **AUC** | 100 | 3±2%*** ‡** | 22±7%***** | 23 ±10%***** | 29±12%* |

After baseline values were obtained (30 minute period immediately before incubation in experimental solutions,100 %), tissues were incubated in either physiological saline (control) or the solutions indicated, for 45 minutes and then the parameters of contraction re-measured, and expressed as the percent of baseline values (i.e. paired data).Values are means ± s.e.m. ***** represents significant differences in contractility compared to preceding control period.($p<0.05$, t-test). **‡,** represents significant reduction in spontaneous contractility when compared to in the presence of oxytocin (0.5nM) along with either GYY4137 or NaHS. AUC; area under the curve.

### 4.4.11 Effects of GYY4137 and NaHS on term pregnant human oxytocin-stimulated contractions

*In vivo* term human myometrium will be stimulated by circulating oxytocin thus it could be posited that this stimulation prevents the effects of $H_2S$, hence I investigated if GYY4137 and NaHS could reduce contractility in term myometrium stimulated by oxytocin. Oxytocin produces a clear increase in phasic contractions in pregnant myometrium, upon which the effects of, NaHS, (n=7) and GYY4137 (n=5) were tested (**Figure 4.14B and 4.14C**). Both compounds reduced significantly the parameters of contraction in all samples, but as can be seen in **Table 4.5** the effects were not as potent as found for spontaneous activity. For incubations in NaHS 1mM, force, frequency and AUC of contractions were significantly reduced at 40±9%, 50±10% and 22±7% respectively under the presence of oxytocin. Although not as significantly diminished as the response to NaHS 1mM, on force, frequency and AUC of spontaneous contractions, 10±4%,, 23±9%, 3±2% respectively. For incubations in GYY4137 1mM, force, frequency and duration of contractions were decreased but not significantly in the presence of oxytocin at 33±14%, 76±26% and 68±17% respectively. Although integrating force, duration and frequency to attain the AUC still showed a significant decrease in contractions (29±12%).

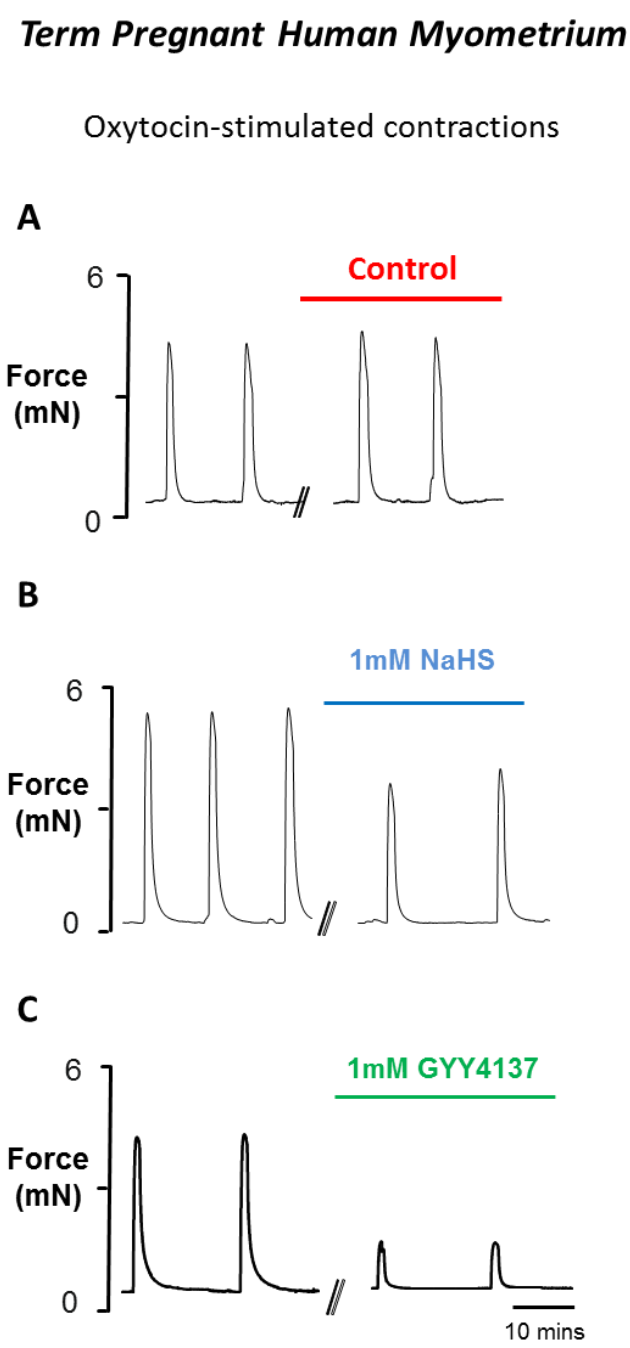


**Figure 4.14 Effects of NaHS and GYY4137 on term pregnant oxytocin-stimulated human myometrium**

Representative isometric recordings of oxytocin-stimulated (0.5nM) spontaneously contracting myometrial strips obtained from term pregnant non-labouring human tissue. Strips were placed under a resting tension of 2mN and superfused continually with physiological saline solution (pH 7.4) at 37°C before and after 45 minute incubations in (A) physiological saline solution (control, represented in red), (B) 1mM NaHS (blue), (C) 1mM GYY4137 (green) all at a pH 7.4 and at 37°C.

## 4.5 Discussion

The present study is the first to investigate the effects of the novel $H_2S$ generator GYY4137 in a non-vascular smooth muscle, the myometrium. The effects in rat and human myometrium were studied and I found that: i) GYY4137 causes a concentration-dependent reduction in contractility of myometrium, ii) The ability of $H_2S$ to inhibit contraction is not constant but rather is greatest close to term before disappearing during labour iii) GYY4137 and NaHS significantly reduced contractility in pregnant but not non-pregnant human myometrium, iv) $H_2S$ significantly reduced tonic force produced by high-K depolarization and oxytocin-stimulated contractions, and v) GYY4137 reduced the intracellular Ca transients underlying contractions and inhibition of $K_{ATP}$ channels prevented the effects of GYY4137. Together these data suggest $H_2S$ affects both membrane potential and L-type Ca channels to relax myometrium and that physiologically, $H_2S$ levels may be altered during gestation to contribute towards myometrial quiescence until labour. This suggestion is supported by recent findings reporting that $H_2S$ production is decreased within human term labouring myometrium compared to non labouring myometrium (You 2011).

### 4.5.1 Experimental conditions and protocols

GYY4137, like NaHS, releases $H_2S$ when in aqueous solutions such as PSS, but was developed to release it with a slower and more prolonged time course than that obtained with sulfide salts (Li 2008, Wang 2012). Measurements of $H_2S$ *in vivo* and *in vitro* confirmed a release of $H_2S$ with GYY4137 taking several minutes to peak, whereas NaHS produces a larger, more or less instantaneous release of $H_2S$ (Lee 2011). In subsequent work it was confirmed that $H_2S$ release from GYY4137 was 10% of that observed with NaHS, but was sustained, (Lee 2011) and that a structural analogue, ZYJ1122, which lacked sulphur, was without effect. Our incubations were performed in a fume cupboard and tissue then transferred to the experimental rig for force and other measurements. I waited five minutes after re-attachment of the tissue to the force transducer, to allow the tissue to settle and wash off of the $H_2S$ producing compounds. This is likely therefore to have resulted in an under-estimation of the effects of $H_2S$, and suggests *in vivo* that GYY4137 will be more potent than measured

in our *in vitro* studies. As seen in many of the figures, force builds up throughout the period after incubation, presumably as the $H_2S$ is volatilized and oxidized (Olson 2012). Notwithstanding these experimental conditions clear effects of GYY4137 are apparent. Consistent with previous work (Hu 2011, Sidhu 2001) within this thesis it was found that NaHS decreased or even abolished spontaneous contractility in rat and human myometrium.

### 4.5.2 GYY4137 and smooth muscle

In the only other study on smooth muscle function, Li et al showed GYY4137 could relax contraction of aortic rings with an $EC_{50}$ of 115 µM. In the uterus I found contractions to be relaxed with an $EC_{50}$ of 1.6 µM. NaHS has been reported to relax different vascular tissues with $EC_{50}$ of 1-300 µM (Olson 2010), thus it is apparent that there is considerable inter-tissue differences in $EC_{50}$ values, although experimental differences may account for much of the variation.

As mentioned earlier, studies in both animal and human tissues over the last few years have demonstrated a role for $H_2S$ within smooth muscle. While many have reported relaxation (Webb 2008, Dhaese 2009, Teague 2002, Zhao 2009), some have found increased contraction or different effects dependent upon $H_2S$ concentration (Webb 2008, Zhao 2001). It has been suggested that these differences may be due to the lower conversion efficacy of NaHS to $H_2S$ at high concentrations (Tian 2012). Such dual responses were not found by us with NaHS in pregnant rat myometrium, consistent with previous findings in the pregnant human myometrium (Hu 2011). *In vivo* data points to relaxation being the predominant effect of $H_2S$ in the vasculature. Mice lacking CSE, the biosynthetic enzyme for $H_2S$, are hypertensive and their blood vessels do not relax to acetylcholine, and administration of NaHS to animals causes vasodilation (Yang 2008, Ishii, 2010).

### 4.5.3 Effects of $H_2S$ change with gestational state

Striking differences in the response of the myometrium were found, both in rat and human tissue, to GYY4137 and NaHS depending upon the gestational state of the tissue. No significant effects on contractions in the non-pregnant tissue to addition of

either $H_2S$ source were found but clear effects by mid-gestation. The inhibitory effect on contraction then further increased until term. These data suggest that the relaxant effects of $H_2S$ are increased as pregnancy advances. The most striking effect however was the abrupt transition upon labour; $H_2S$ was without effect as seen by the data for both compounds. This suggests that $H_2S$ contributes to uterine quiescence in late pregnancy and that the myometrium can rapidly change its responses to $H_2S$.

### 4.5.4 Mechanism of $H_2S$ effects in myometrium

There are many suggestions for the mechanism by which $H_2S$ exerts its effects and as with NO, it is likely that there will be many targets (Li 2011). The main mechanism appears to be due to $H_2S$ modifying cysteine residues in many proteins through S-sulfhydration (Mustafa 2011) i.e. cysteine's covalent modification by which -SH groups on cysteine residues of a protein are converted to –S-SH, via addition of sulphur from $H_2S$ (Mustafa 2009). This molecular mechanism is similar to the S-nitrosylation effect of NO, however, unlike S-nitrosylation, S-sulfhydration activates rather represses, its target proteins (Gallyas 2012). The data in this thesis using DTT, a reductant sulfhydryl modifier and Diamide, a oxidant sulfhydryl modifier supports this showing that firstly free sulfhydryl groups have no effect on contractility and that disulphide bonds cause a similar effect to NaHS and GYY4137. Although it would be interesting to see whether using DTT could reverse GYY4137 and NaHS effects on force and calcium as in Zhang et al 2012 where it could markedly reverse the $H_2S$ donor-induced inhibition of $I_{Ca,\,L}$ (L-type calcium current) in cardiomyocytes. Illustrating that if $H_2S$ targets on the crucial free-sulfhydryl groups on the L-type $Ca^{2+}$ channel and inhibits the L-type calcium current, the inter-chain disulfide bond linkages would be rapidly reduced by DTT, and therefore the inhibition would be reversed (Zhang 2012).

The most widely researched effect of $H_2S$ is on $K_{ATP}$ channels within smooth muscle. In vascular smooth muscle cells $H_2S$ stimulated single-channel activity of $K_{ATP}$ channels by directly increasing their opening probability (Wang 2012). Recent work has made progress in identifying which residues in the channel are affected by $H_2S$, with Cys 6 and 26 on the extracellular N terminal of the SUR1 subunit of the channel being identified (Jiang 2010). $K_{ATP}$ channels have been suggested as one of the targets of

$H_2S$ that lead to reduced myometrial contractility (Hu 2011). In contrast, other studies showed that the $K_{ATP}$ channels are not involved in $H_2S$ relaxation in smooth muscle tissues including vascular, bronchial, and gastrointestinal smooth muscle (Kubo 2007, Teague 2002, Lee 2007). Our data with glibenclamide would support a role for these channels in the mechanism of $H_2S$ effects in myometrium. GYY4137 had no significant effect on myometrial contractility when $K_{ATP}$ channels had been incubated with glibenclamide. As opening of these channels will cause hyperpolarisation, and this in turn decreases the opening of L-type Ca channels, this suggests that $K_{ATP}$ are a target in myometrium. Hyperpolarization and relaxation induced by $Na_2S$ has been directly measured in arterioles (Liang, 2012). However as this hyperpolarization was shown to be due to activation of Ca sparks and opening of Ca-activated K (BK) channels, and Ca sparks are not present in myometrium (Burdyga 2007), this cannot account for hyperpolarization in the myometrium .

### 4.5.5 Changes in intracellular Calcium

The above suggests that L-type Ca entry will be reduced by $H_2S$ sources in the uterus. There are however few studies measuring the effects of $H_2S$ on Ca in any tissues, and none have done so simultaneously with contraction. Reduction of Ca by $H_2S$ has previously been demonstrated in non-contractile arterial segments (Tian 2012). Our simultaneous measurements of intracellular Ca and contractions show a $H_2S$-dependent reduction in intracellular Ca accompanies the decrease in amplitude of the phasic contractions. To the best of our knowledge these are the first measurements directly demonstrating that the effects of $H_2S$ in producing reduction in force are due to decreased Ca transient amplitude.

### 4.5.6 Effects of GYY4137 on depolarized and oxytocin-stimulated contraction.

This study also shows in pregnant rat myometrium that the tonic force produced by depolarization with high $K^+$, used to directly open L-type calcium channels, is reduced by GYY4137. This suggests that mechanisms beyond membrane potential changes are also a feature of the $H_2S$ relaxation mechanism in the uterus. There is now mounting evidence that the L-type Ca channels themselves are targets of $H_2S$. Sun et al, (Sun 2008) in cardiac myocytes were the first to show that $H_2S$ can inhibit L-type Ca

channels. Recently others have shown inhibition of these channels by NaHS also occurs in vascular smooth muscle (Tian 2012, Al-Magableh 2011) and Zhang et al (Zhang 2012) have gone on to show that this is dependent upon the protein sulfhydryl state of the channel. An increase in resting $Ca^{2+}$ was also found in endothelial cells with NaHS (Bauer 2010), thought to be due to store operated $Ca^{2+}$ entry. Thus direct effects on $Ca^{2+}$ entry via L-type $Ca^{2+}$ channels and other channels, also contributes to the relaxant effects of $H_2S$. This inhibition of $Ca^{2+}$ channels will also explain why in some tissues inhibition with glibenclamide of $K_{ATP}$ channels often does not fully prevent the relaxant effects of $H_2S$ donors. There is evidence from gastric fundus and distal colon, where glibenclamide is without effect, that $H_2S$ may affect $Ca^{2+}$ sensitization of the contractile machinery (Dhaese 2009, Dhaese 2010), but as $Ca^{2+}$ sensitization plays little role in spontaneous activity of myometrium (Kupittayanant 2001), this is unlikely to be contributing to the data obtained within this chapter.

Our data clearly show that $H_2S$ donors can reduce contractility even when stimulated by oxytocin in the pregnant myometrium. Oxytocin increases $Ca^{2+}$ within the myometrium, partly by depolarization and increasing L-type $Ca^{2+}$ channel entry (Wray 2007). Thus the mechanisms by which $H_2S$ suppresses spontaneous contractions are likely to also feature in the effects on oxytocin-induced contractions. As shown in Table 4.5, the effects of NaHS were less in the presence of oxytocin compared to spontaneous activity, presumably due to the increased contractile drive with oxytocin stimulation. This was not the case for GYY4137 perhaps as the $H_2S$ produced by GYY4137 outlasts the instantaneous $H_2S$ produced by NaHS and this allows the contractile drive to have more of an effect on NaHS incubated strips (Lee 2011).

### 4.5.7 Gestational changes and $H_2S$ mechanism of action

The above gives insight into the mechanism of action of $H_2S$ but does not explain the reasons for susceptibility changes over gestation. Suggestions include (i) increased vulnerability to sulfhydration of L-type calcium channels as L-type calcium channel subunits increase toward term (Collins 2000); (ii) reduction in kir6.1 and 6.2 $K_{ATP}$ subunits once myometrium is labouring, as before labour $H_2S$ exerts its effects on these subunits (Xu 2011); (iii) up regulation of the $H_2S$ breakdown enzymes with

gestation, or (iv) changes in uterine environment with labour, such as hypoxia and pH changes, (Quenby 2004, Wray 1992) may result in faster breakdown of $H_2S$, but this remains controversial, (Olson 2012, Doeller 2005). The lack of specific inhibitors of these enzymes and the difficulty of accurately measuring $H_2S$ in tissues, hinders further study of these last two points. The differences between non-pregnant and term pregnant human and rat contractility responses could also be attributed to changes in suceptability of L-type ca channels and Katp channels to be sulfhydrated, down regulation of H2S breakdown enzymes at term or uterine environment changes where by $H_2S$ breakdown is hindered at term.

### 4.5.8 GYY4137 and tocolysis

The synthesis of GYY4137 and its cardiovascular effects in rats were first reported by Li et al 2008) (Li 2008). As pointed out by these authors, while much data were being obtained showing the importance of $H_2S$, studies were limited by the lack of a compound to better mimic the endogenous release of $H_2S$ in cells. The commonly used NaHS or $Na_2S$ release $H_2S$ instantaneously in aqueous solutions, producing very large and transient increases in its concentration. GYY4137's potential as a slow-releasing $H_2S$ compound with effects on vascular smooth muscle *in vitro* and *in vivo* were shown, its time scale of $H_2S$ production measured and its lack of toxicity to aortic cells shown (Li 2008). Subsequent work has supported low toxicity (Yu 2010) and also indicated anti-cancer properties (Lee 2011), anti inflammatory activity (Li 2009), and anti apoptotic (Lavu 2011) activity of GYY4137. Thus GYY4137 or subsequent compounds, (Predmore 2012) may well be suitable for a variety of patho-physiological conditions, including tocolysis in threatened preterm labour, i.e. to stop the onset of labor, although further work, including studies on labouring samples, are needed to develop this suggestion. Recent studies of interactions between the enzymes producing or destroying $H_2S$ and their inhibitors, also represent another way of manipulating its effects in the uterus (Sun 2009) The finding that free $H_2S$ values are up to 100-fold higher in smooth muscle (aorta) compared to liver, blood, heart and kidney, (Levitt 2011) also encourages these approaches.

### 4.5.9 Summary

In conclusion, NaHS and GYY4137 relax term pregnant human and rat myometrium. Within the rat myometrium we show increased potency as term approaches is shown; an effect that is rapidly reversed as labour starts. GYY4137 can reduce force produced spontaneously, by oxytocin or high $K^+$ depolarization. The mechanism involves both $K_{ATP}$ channels and importantly, L-type Ca channels. GYY4137 reduces the intracellular Ca transients that underlie spontaneous contractions. Our data and that of previous studies suggest $H_2S$ could contribute to uterine quiescence and that increasing its level in myometrium could be an attractive target for therapeutics to inhibit the onset of labor. Increased understanding of the mechanisms for transition to labor should also follow from obtaining a better understanding of $H_2S$ in the myometrium.

### 4.5.10 Limitations of study

Limitations of study include:

- No direct evidence of sulfhydration. Furture work using a biotin switch assay investigating sulfhydration of L-type and Katp channels in the uterus needed as using DTT and DM effect many mechanisms including oxidation of glutathione.
- No work on human labouring samples, need to investigate human labouring samples to see whether the effect seen in the rat can directly relate. Difficult samples to attain.
- When thinking about tocolytics many factors have to be borne in mind, including effects on other tissues, the fetus, tolerance, side effects, formulations and desensitization. However this is a worthwhile goal, as illustrated by the successful introduction of atosiban, the Oxytocin receptor anatagonist.

# Chapter 5

## Investigating the role of $H_2S$ through L-cysteine and expression of the enzymes producing and breaking down $H_2S$.

## 5.1 Abstract

$H_2S$ is produced *in vivo* from L-cysteine, by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). The enzyme rhodanese has an isoform, TST involved in the breakdown of $H_2S$. Thus $H_2S$ will be regulated within cells. In the previous chapter it has been clearly shown that $H_2S$ producing compounds NaHS and GYY4137 have a relaxant effect on the myometrium and reduce Ca transients at term. However, is the same true for endogenous L-cysteine? This chapter investigates L-cysteine's effects on simultaneous force and calcium measurements within non-pregnant and term pregnant rat (22 day) myometrium. L-cysteine's effects were also examined in non-pregnant and term pregnant human myometrium. The characteristics of spontaneous and high $k^+$ depolarisation induced contractions are discussed. Another question addressed by determining enzyme expression levels, is could the differences in contractility in non-pregnant compared to pregnant myometrium (**Chapter 4)** in response to $H_2S$, be a result of differences in $H_2S$ production or removal rates within the uterus?

There is no previous account of the effects of L-cysteine on non-pregnant and term pregnant rat myometrium and its effects are therefore unknown. In this chapter I therefore determined its effects and possible mechanisms of action, by studying changes in underlying calcium, intracellular pH, and changes in response to BayK 8644 and high $K^+$. L-cysteine and the contribution of its chemical effect were also assessed using D-cysteine, the stereoisomer of L-cysteine as well as, L-serine and sodium pyruvate both by-products of $H_2S$ production through L-cysteine. In addition inhibitors of both $H_2S$ producing enzymes were examined to determine whether changes to the L-cysteine responses occurred. Finally, as $H_2S$ can be naturally produced by garlic, I also monitored changes in contractility in response to garlic.

The distribution of CBS, CSE and TST were assessed using immunohistochemistry and/or Western blotting in NP, 10 day, 14 day, 18 day, and 22 day gestation rat

myometrium as well as within non-pregnant and term pregnant human myometrium. L-cysteine applied to term pregnant rat and human myometrium, dose-dependently and significantly decreased spontaneous contractions, and also reduced the underlying Ca transient (Indo-1 fluorescence). The frequency and AUC of contraction increased. The intracellular pH, measured via carboxy SNARF-1 fluorescence, declined in L-cysteine. L-cysteine had no effect in depolarized preparations. BayK8644 reduced the effects of L-cysteine although the initial increase in contractions once L-cysteine was applied remained significant.

This data also shows only increases in frequency of contraction were found upon application of L-cysteine within the non-pregnant rat and human myometrium. Inhibitors of $H_2S$ production reduced or blocked the relaxant effect produced by L-cysteine although stimulant effects remained. Garlic also produced similar responses to L-cysteine with decreases in amplitude as well as an overall increase in frequency and AUC.

Both $H_2S$ production enzymes, but not TST, were present in all myometrium examined. Closer examination of the non-pregnant and term pregnant human tissue through western blotting illustrated that within the term pregnant tissue significantly less amounts of both enzymes are present.

In conclusion L-cysteine caused an increase in frequency and decline in force amplitude. The effects on amplitude can be explained by L-cysteine affecting Ca transients and L-type calcium channels similar to GYY4137 and NaHS. However, the stimulatory effect caused by L-cysteine remains to be elucidated. Preliminary experiments with garlic shows tocolytic effects but remain to be further investigated, as does the $H_2S$ enzyme distribution decline from non-pregnant to term to allow for the onset of labour.

## 5.2 Introduction

$H_2S$ has been around for millions of years and is well known for its smelly odour and toxicological effect (Reiffenstein 1992). However in recent years it has been recognized for its physiological role as a gaseous signalling molecule. Tissues produce $H_2S$ via two enzymatic pathways, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) and L-cysteine is the substrate these enzymes use to produce $H_2S$. Therefore as both enzymes are present in the myometrium (Patel 2009, You 2011), it is capable of producing $H_2S$ from the precursor L-cysteine. CSE and CBS are cytosolic enzymes. CBS is a homotetramer consisting of 551-amino-acid subunits with a subunit molecular weight of ~63KDa and CSE is a 405-amino-acid protein consisting of a tetramer formed by two homodimers, with active and stable dimer of ~45KDa (Sun 2009, Whiteman 2011 Singh 2009, & You 2011). An enzyme called rhodanese has been implicated in the breakdown of $H_2S$, specifically its TST isoform. However, neither $H_2S$ producing enzyme has been looked for in the non-pregnant myometrium or at different stage gestations in the rat and TST has not been examined in the uterus at all. The main finding on application of $H_2S$ through $H_2S$ donors is that it has a relaxant effect on smooth muscle, which has been clearly shown in **Chapter 4**, this stimulated the examination of L-cysteine's effect on force and calcium of the myometrium as well as the presence of the $H_2S$ producing and breakdown enzymes with view to help develop new physiological and naturally occurring tocolytics and to help explore the physiological relevance of $H_2S$ in the myometrium.

There have been few studies examining the effect of L-cysteine on uterine contractility. One study investigated the effect of increasing concentrations of L-cysteine up to 1mM on 19 day rat myometrium, finding a dose dependent decrease in contractile activity (Sidhu 2001). L-cysteine on term non labouring and labouring human myometrium also reduced contraction amplitude, but also increased frequency. This study also found that myometrial strips treated with glibenclamide an inhibitor of $K_{ATP}$ channels abolished the effects of L-cysteine and that L-cysteine's effects were less potent in the term labouring human strips (You 2011). The latter observations ties in with **Chapter 4** were glibenclamide abolished the effects of NaHS

and GYY4137 and that both $H_2S$-producer effects were eliminated upon labour onset.

$H_2S$ is also naturally produced by garlic (*Allium Sativum*). Garlic consumption has been correlated with the reduction in multiple risk factors associated with cardiovascular diseases such as increased reactive oxygen species, and high blood pressure to high cholesterol (Banerjee 2002, Benavides 2007). Within isolated hearts, aortic rings, tracheas and intestines, garlic juice inhibited smooth and cardiac muscle contractions (Aqel MB 1991). Given the need to find more efficacious tocolytics to prevent pre-term labour, the suggestion that $H_2S$ can relax uterine smooth muscle is important and if verified could form the basis of new methods to control uterine activity and maybe aid in the understanding of quiescence. It would be even more beneficial if this tocolytic could be naturally occurring from the food that we eat and this is why I have investigated the effect of garlic on myometrial contractility. If similar effects to GYY4137 and NaHS are found, then its use clinically will be of interest.

As there is little mechanistic insight into the effects of L-cysteine on myometrial contractility, this was examined. This chapter aims to demonstrate the contractile responses of L-cysteine within non-pregnant and term pregnant rat and human myometrium. Characteristics of spontaneous and high $K^+$ depolarisation-induced contractile activities are discussed. Further investigation into the mechanism of L-cysteine with respect to BAYK 8644, $Ca^{2+}$ signalling, intracellular pH and use of $H_2S$ producing enzyme inhibitors are also discussed. I also explored the contractility changes of the myometrium in the presence of garlic which naturally produces $H_2S$.

The enzymes CBS and CSE are widely distributed in tissues. In the cardiovascular system the expression of CSE is much higher compared to CBS, though the opposite is found in brain (Lowicka 2007, Awata 1995). Expression of both CBS and CSE have been documented in smooth muscle tissue. CSE seems to be the predominant enzyme responsible for $H_2S$ production in vasculature smooth muscle (Zhao 2001) were as in the gastrointestinal and penile smooth muscle both contribute (Fiorucci 2005, De'Emmanuele Di Villa Bianca 2009). In the myometrium, Patel et al found the

presence of both $H_2S$ producing enzymes in rat non-pregnant and 19 day gestation uterus as well as human term pregnant myometrium. You et al further examined the presence of both enzymes in term non labouring and labouring human myometrium at the protein and mRNA level, finding both present and during labour $H_2S$ enzymes are down-regulated. No work has been undertaken to look at the expression of both enzymes within different stage gestation rat myometrium or looked at the presence in non-pregnant human versus term pregnant myometrium hence this was researched.

TST is the isoform of rhodanese that detoxifies $H_2S$ to sulfite and sulfate from thiosulfate. (Caliendo 2010). TST is a mitochondrial matrix enzyme and ~33-kDa in weight (Ramfrez 2004). TST was shown to detoxify $H_2S$ in human colon but has yet to be looked at in the myometrium. Could TST have a role in the differences found in contractility? Does its presence differ at different stages of gestation in rat myometrium and in non-pregnant versus term pregnant human myometrium?, this will be investigated.

## 5.3 Methods

### 5.3.1 Tissue

Strips of longitudinal myometrium (~ 1x5mm) were dissected from the uterus of humanely killed non-pregnant, and 22 day gestation (equivalent to term) Wistar rats (Wray 2008). The gestation of the rat was defined from day 0, when the male was placed in the cage to mate. Human myometrial strips were dissected from biopsies obtained with informed consent and ethical approval from women undergoing an elective term caesarean section (mean gestational age 39 weeks, mean maternal age, 29; range 27-37 years, N=8 ) or pre-menopausal hysterectomy (mean age, 38.2; range 27- 50 years, N=5). Indications for caesarean section included previous caesarean section or breech presentation. None of the women included in this study had any underlying disease (hypertension, diabetes, pre-eclampsia, intrauterine growth restriction etc.). Indications for hysterectomy were menorrhagia or irregular bleeding. Biopsies were obtained from the upper lip of the lower segment uterine incision at caesarean section (Luckas 2000) and from corresponding macroscopic normal area of the uterus at hysterectomy.

### 5.3.2 Solutions

All chemicals were produced as described in **Chapter 2**. All chemicals were obtained through Sigma (UK), apart from garlic, which was obtained from RAJAH finest authentic foods, UK. The composition of Physiological Saline Solution (PSS) was as follows (mM): 154 NaCl, 5.1 KCl, 0.12 $MgSO_4 7H_2O$, 10.9 HEPES, 8 Glucose, 2 $CaCl_2$, pH 7.4. In some experiments to depolarize the tissue, the KCl in the PSS was increased to 40 mM and NaCl reduced equivalently. L-cysteine was made in PSS at 1n M, 1 µM, 0.1 and 1 mM, then pH readjusted to 7.4. (Olson 2012). Garlic powder (Rajah), was diluted in PSS at 0.01, 0.2, 0.7 and 1 mg/ml and also pH readjusted to 7.4. Allicin (diallyl thiosulfinate), the main organosulfur compound, is produced from the amino acid alliin by action of the enzyme alliinase when garlic is crushed to a powder. Allicin, unstable in aqueous solution, rapidly decomposes mainly to diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS),and ajoene (Amagase 2006). BayK was used at 0.1µM, sodium pyruvate, L-serine and D-cysteine were used at

1mM. Enzyme inhibitors, propylargylglycine (PAG), *O*-(carboxymethyl)hydroxylamine hemihydrochloride (AOAA) and hydroxylamine (HA) were used at 0.1mM. All solutions were applied directly to the bath at 37$^{o}$C.

### 5.3.3 $Ca^{2+}$ and Force Measurements:

Tissue preparation and measurements of calcium and tension are the same as described in **Chapter2**, general materials and methods.

### 5.3.4 pHi and Force Measurements:

Tissue preparation and measurements of pHi and tension are the same as described in **Chapter2**, general materials and methods.

### 5.3.5 Statistics

Contractions were analysed for amplitude, frequency, duration and area under the curve, (AUC, in arbitrary units, au) for; 10 minutes, at the beginning and end of response to L-cysteine and garlic rat data, 30 minutes, high K, using origin 8. L-cysteine's response within human data was assessed over a 30 minute period to accommodate the slower rate of contractions. Each strip tested for the effect of L-cysteine or garlic had a paired control response in PSS rather than test solution. Student's t tests were performed to compare two groups. Anova with Bonferroni post hoc tests were used to compare more than two groups. P was taken as showing a significant difference when $P<0.05$. Each n was from a different biopsies or rat. In some cases results are expressed as percentage of control contractions were control is 100%.

### 5.2.6 Immunohistochemistry

Sections of 4μm thick were cut in the pathology department and mounted onto glass slides. Sections were cut from 6 non-pregnant and 6 pregnant women as well as non-pregnant, 10, 14, 18, and 22 day rat myometrium. The sections were stained as described in Chapter 2 section 2.6. Briefly sections from human myometrium were labelled appropriately before combining in a metal rack were rehydrated and placed into boiling 10mM Sodium Citrate buffer pH6.0 for antigen retrieval. Endogenous

peroxidise activity was blocked with 3% hydrogen peroxide before incubation non specific block 5% Bovine Albumin Serum (BSA) overnight incubation with 1:50 CBS and 1:150 CSE antibodies. Beta actin (Abcam) was used as an antibody control and Aorta and brain were used as a positive control for CSE and CBS, respectively. The following day the sections were incubated with secondary antibody before developing with 3, 3'-Diaminobenzidine (DAB) (Sigma). All slides from rat were developed for the same time to reduce any bias in the experiments. All slides from non- pregnant and term pregnant myometrium were developed for the same time to reduce any bias in the experiments. 10 images were photographed from each sample (6 non-pregnant and 6 term pregnant human myometrium as well as non-pregnant, 10, 14, 18, and 22 day rat myometrium) using a 40x objective. (Nikon Biophot Microscope, Nikon Corporation, Tokyo 100-8331, Japan, Eclipsenet software, developed by Laboratory Imaging s.r.o. for Nikon Instruments Europe B.V., 1170AE Badhoevedorp, The Netherlands).

### 5.2.7 Western Blotting

Protein was extracted, quantified and Western blots run as described in Chapter 2 section 2.7. Briefly a small section of myometrium was dissected from non-pregnant and term pregnant myometrial biopsies, diced with a scalpel before placing into protein extraction buffer. The tissue was then homogenised, centrifuged and the supernatant removed and stored at - 20°C until use. Once quantified 25µg of protein was prepared in 3x loading buffer, boiled at 100°C. 10% acrylamide gels were cast and 20µl of the prepared proteins were added to the wells. The gels were run at 150 volts for 90 mins before transfer to nitrocellulose membranes at 40V for 1hour and 10minutes at room temperature. Once transferred the nitrocellulose membranes were placed into 5% BSA before overnight incubation at 4°C with primary antibody. The primary antibodies used were CBS at 1:250 (Abnova), CSE at 1:200 (Abnova), TST at 1:1000 (Abcam) and loading control Actin 1: 5000 (Abcam). The following day the primary antibody was removed and the membranes were incubated with the appropriate secondary antibody either Goat Anti Mouse, Goat Anti Rabbit (Thermo Fisher Scientific) before developing with SuperSignal West Pico Chemiluminescent

Substrate (Thermo Fisher Scientific). Analysis of Western blots was carried out in ImageJ using densitometry.

## 5.4 Results

### 5.4.1 The effect of L-cysteine on term pregnant rat myometrial contractility

Exposure of contracting rat myometrium to increasing concentrations of L-cysteine from 1nM to 1mM caused a dose dependent decrease in the amplitude of force of contractions. Typical force traces are shown in **figure 5.1i)-iv)** and the mean data for amplitude, duration, frequency and AUC are presented in graphical form in **Figure 5.2** showing the results in response to L-cysteine in the first (**Figure 5.2A**) and last (**Figure 5.2B**) 10 minutes of perfusion. Looking at initial 10 minutes response to L-cysteine (**Figure 5.2 Ai-iv**) showed no significant change until 1mM when a stimulatory effect on contractility was found. A significant increase in force amplitude, frequency and AUC, was seen at 1mM L-cysteine (p=0.007, 0.00009, 0.005 respectively). There was no change in the duration of contractions.

Examining the last 10 minutes of the response on application of L-cysteine (**Figure 5.2Bi-iv**) highlighted a decrease in force amplitude, most marked at the highest concentration of L-cysteine 1mM (67±6%) relative to control period 100% (n=8). Statistical analysis showed a significant decrease in contractility from 1nM to 1mM (p=0.019). Increased frequency of contractions achieved highest significance at 1mM L-cysteine (p=0.002) as well as duration after increasing concentrations of L-cysteine, however by integrating the differences in force, duration and frequency by measuring the AUC, showed the most marked increase at the highest concentration of L-cysteine 1mM (180±28%) relative to control period 100% (n=8) and significant at 1mM concentration (p=0.037). These results showed effects most prominent at 1mM L-cysteine, myometrial contractility after 30 minutes showed a decrease in force amplitude with overall increase in AUC.

A dose response curve for the final 10 minute effect of L-cysteine on the force of term pregnant rat myometrial contractions showing the logEC50 is illustrated in **Figure 5.3**.

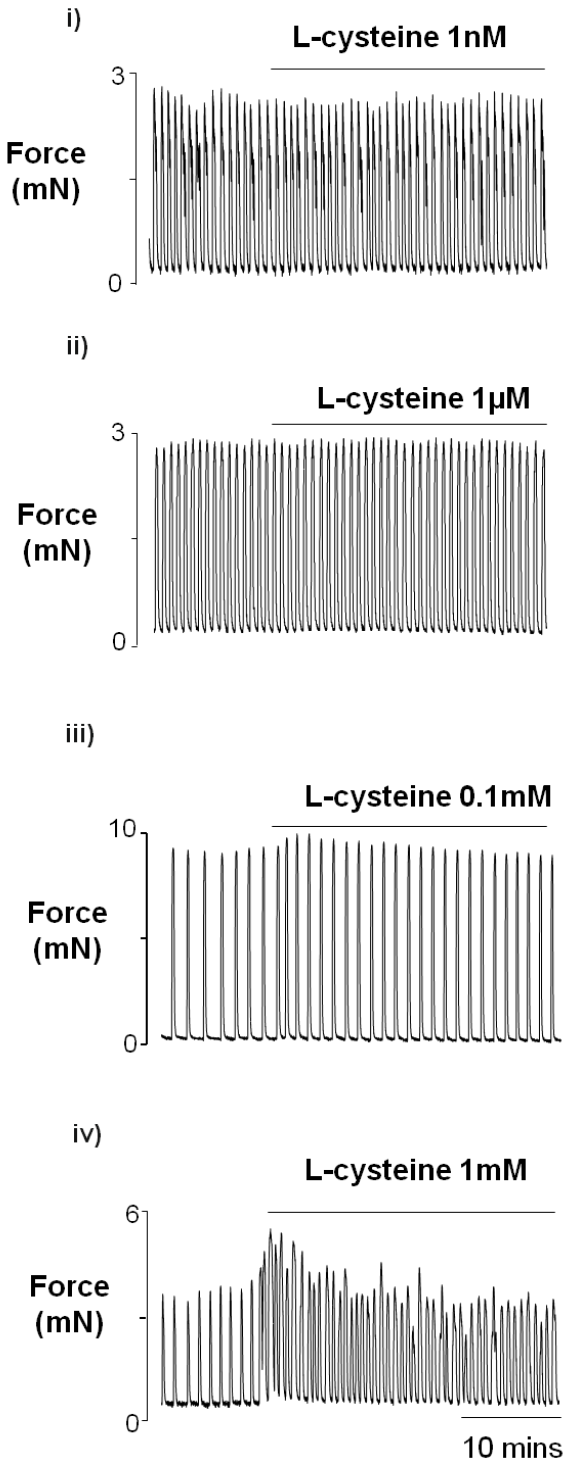


**Figure 5.1: Dose dependency of L-cysteine in term pregnant rat myometrium.**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from term pregnant rat (22 day gestation). Each myometrial strip was perfused with either i) 1nM, ii)1µM, iii) 0.1mM, iv) L-cysteine.

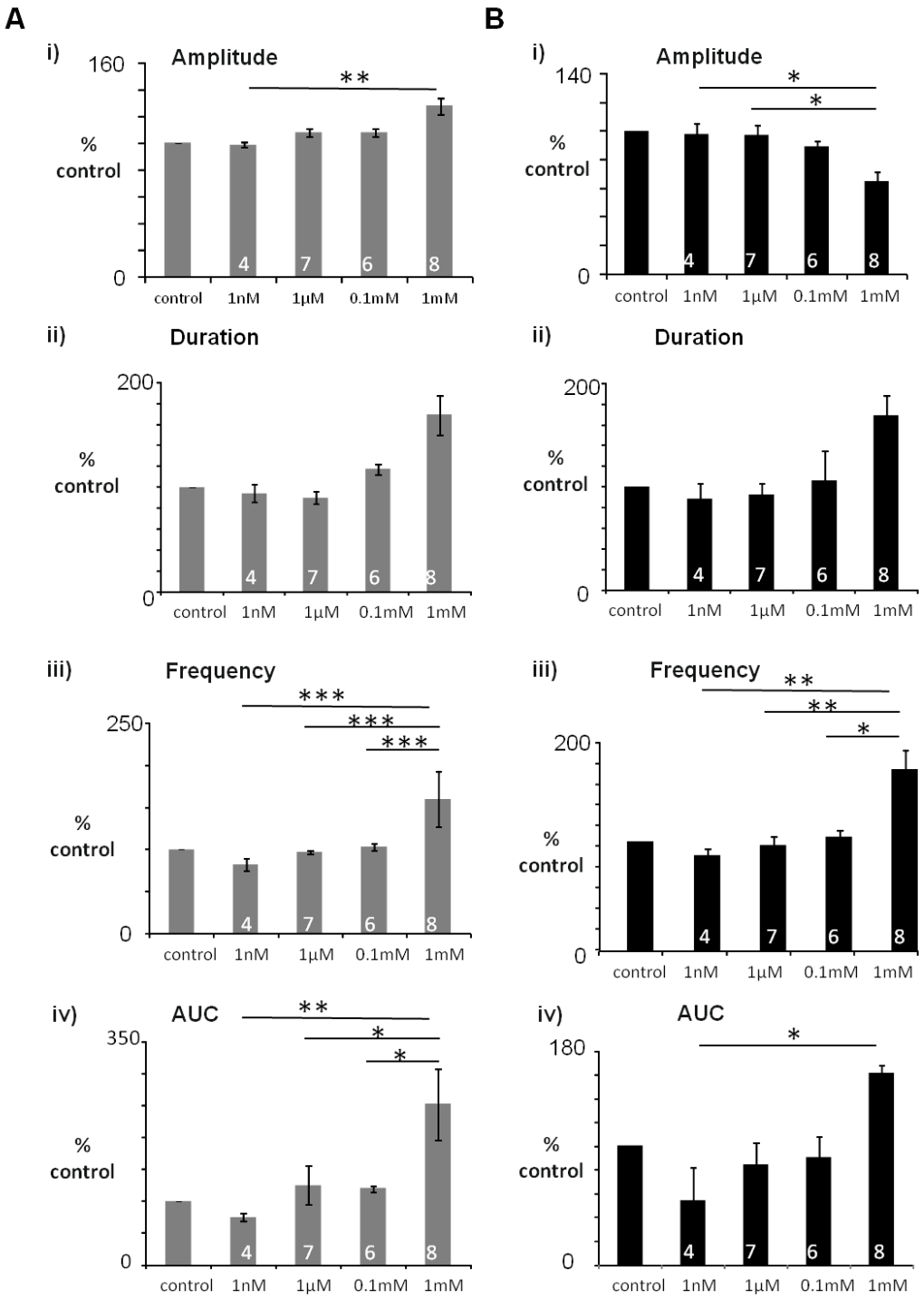


**Figure 5.2: Mean data of dose dependency of L-cysteine in term pregnant rat myometrium.**

Mean data± s.e.m, denoted by error bars, showing the first 10 minutes (A) and the last 10 minutes (B) of applications of L-cysteine. The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is $p<0.05$. ** is $p<0.01$, ***$p<0.001$ using Anova with Bonferroni *post hoc* tests. (A) shows a dose dependent increase in contractility as does (B) only with a significant dose dependent decrease in amplitude.

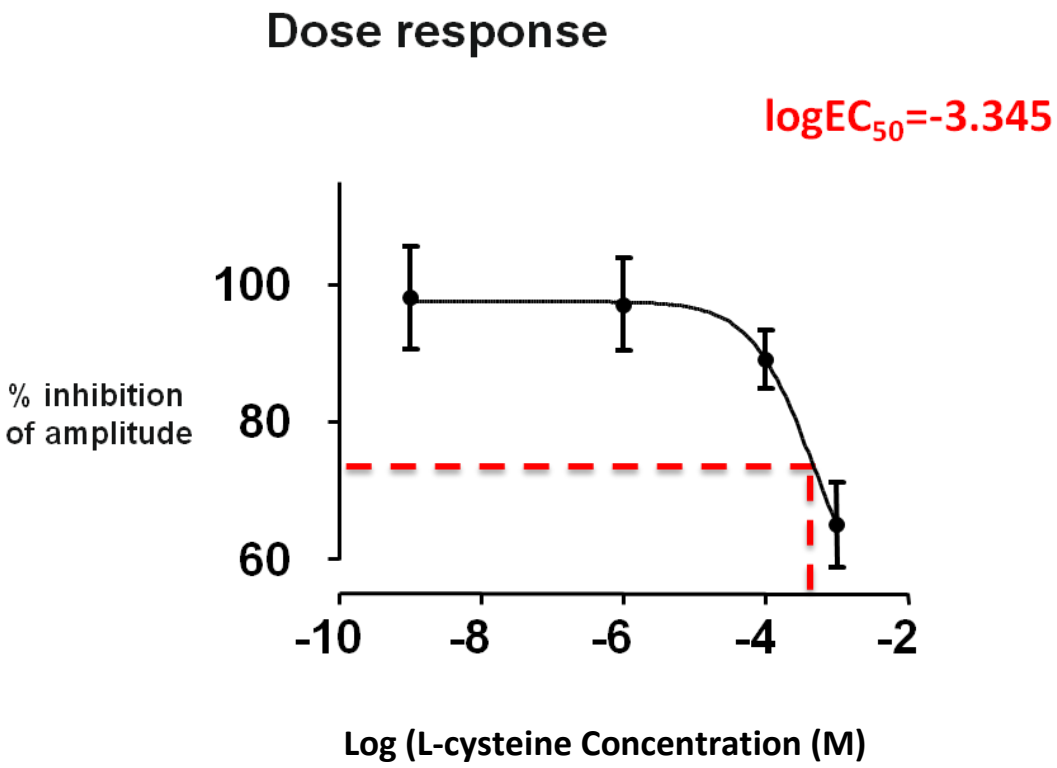


**Figure 5.3 LOG dose response curve for pregnant rat myometrium**

Log dose response curve for the effect of L-cysteine on rat myometrial contractility, showing the sigmoidal relationship. The log EC50 value of -3.345 depicts the concentration required to reduce myometrial contractility by 50% compared to minimum and maximum values.

### 5.4.2 The effect of L-cysteine on non-pregnant rat myometrial contractility

Concentrations of L-cysteine from 1nM to 1mM were examined in non-pregnant rat myometrium, original traces from each concentration are illustrated in **Figure 5.4**. L-cysteine within non-pregnant myometrium, in the initial 10 minute (**Figure 5.5A i-iv**) period highlighted a dose dependent increase in contractile profile upon increasing doses. The stimulation seen only became significant in increasing the amplitude of contraction at 1mM ($p= 0.003$, $n=10$). No significance in duration and frequency was found. Were as integrating the differences in force, duration and frequency by measuring the AUC significance was found between 1nM and 1µM to 1mM ($p=0.005$, $p=0.012$ respectively) showing a dose dependent increase with increasing concentrations of L-cysteine which was most marked at the highest concentration of L-cysteine 1mM (198±17%).

Studying the last ten minutes of the response to L-cysteine (**Figure 5.5B i-iv**) demonstrated a trend increase in all parameters with no significance. Frequency of contractions at 1mM L-cysteine showed a significant increase ($p=0.013$, $n=10$).

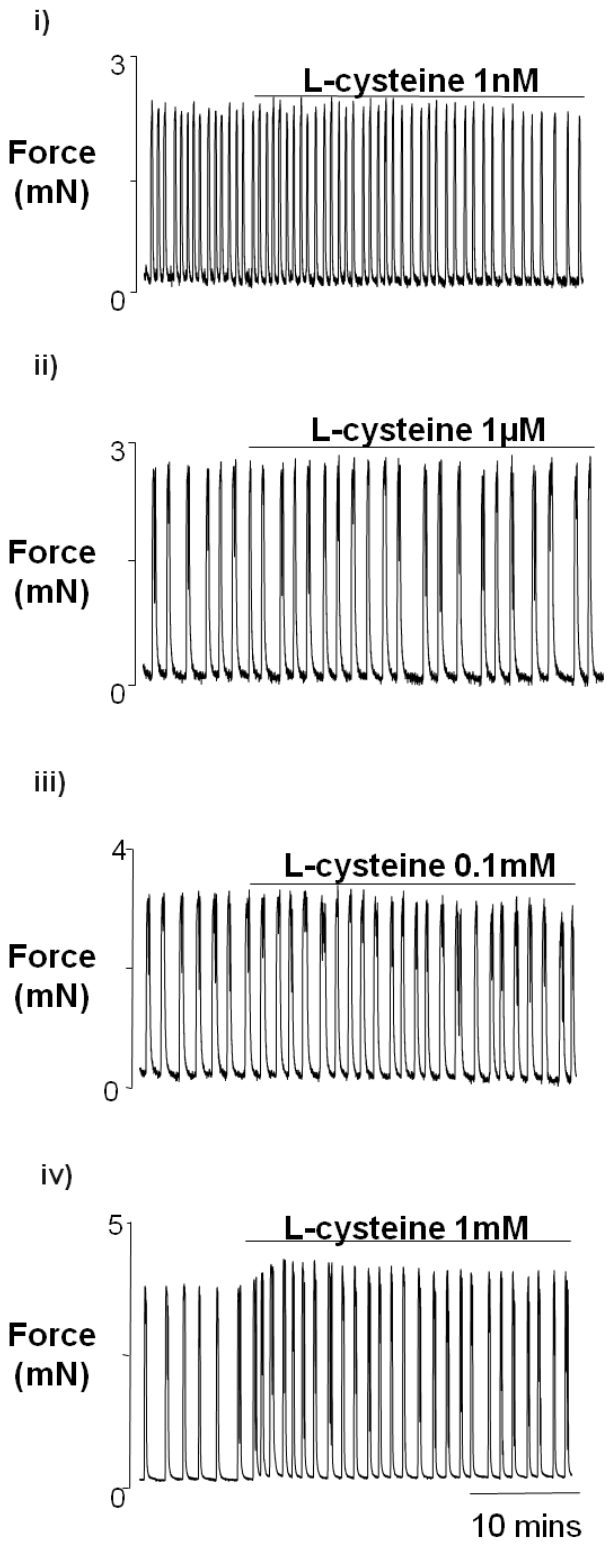


**Figure 5.4: Dose dependency of L-cysteine in non-pregnant rat myometrium.**

Representative isometric recordings of spontaneously contracting myometrial strips obtained from non-pregnant rats. Each myometrial strip was perfused with either i) 1nM, ii)1µM, iii) 0.1mM, iv) L-cysteine.

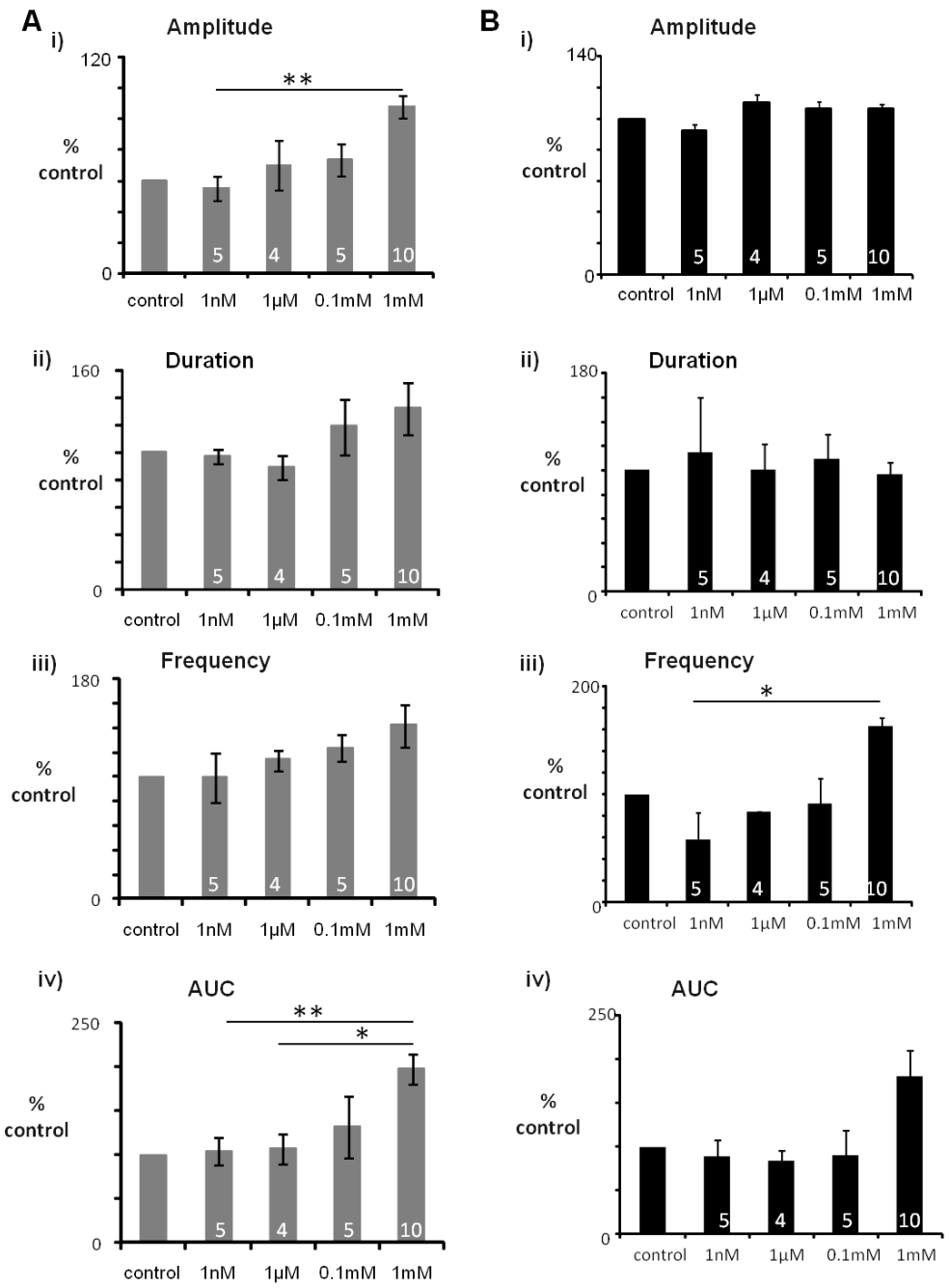


**Figure 5.5: Mean data of dose dependency of L-cysteine in non-pregnant myometrium.**

Mean data± s.e.m, denoted by error bars, showing the first 10 minutes (A) and the last 10 minutes (B) of applications of L-cysteine. The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is $p<0.05$. ** is $p<0.01$, ***$p<0.001$ using Anova with Bonferroni *post hoc* tests. (A+B) show a dose dependent increase in contractility.

### 5.4.3 Comparing the response of 1mM L-cysteine within non-pregnant and term pregnant rat myometrium

The first and last ten minutes of responses to 1mM L-cysteine were compared in both term and non-pregnant myometrium. For the first ten minutes there was no difference in the parameters of the stimulation found. At the last ten minutes of activity the mean force amplitude of spontaneous contractions within term pregnant myometrium were smaller at 67±3% (n=8) compared to non-pregnant at 93±11% (n=10). The mean duration, frequency and AUC of contraction were not significantly elevated when compared to the non-pregnant. The overall mean data for all parameters can be found in **figure 5.6**, student t-tests were performed to look for significance at $p<0.05$. Therefore in conclusion the changes in contractile activity on application of L-cysteine to non-pregnant and pregnant myometrium were not significantly different apart from a remaining significant decrease in amplitude of pregnant myometrial contractions.

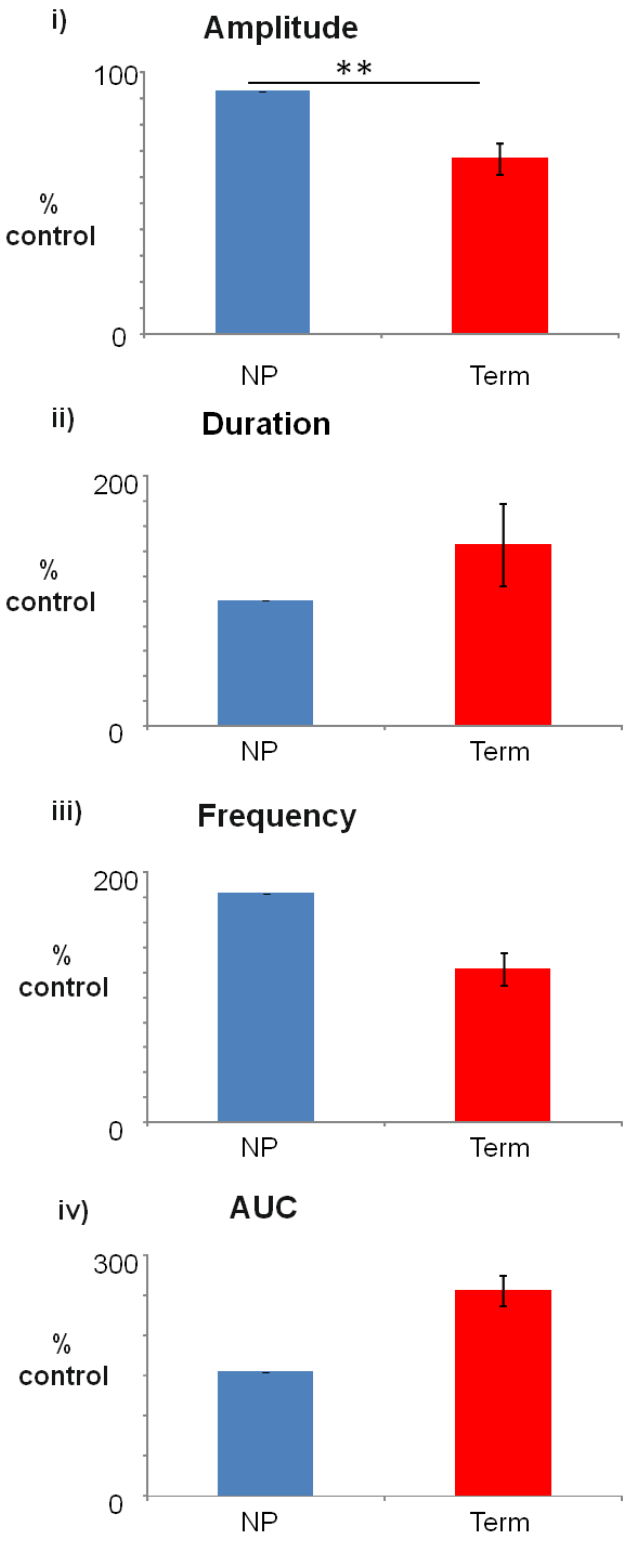


**Figure 5.6: Comparison of rat non-pregnant and term pregnant L-cysteine responses.**

Mean data± s.e.m, denoted by error bars, showing non-pregnant (NP) and term pregnant differences in final ten minute perfusion with L-cysteine. The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is $p<0.05$. ** is $p<0.01$, ***$p<0.001$ using student ttests.

### 5.4.4 Effects on calcium entry and Calcium signalling

The data above very clearly shows increased frequency of contractions that progress to a reduction in the amplitude of term pregnant rat myometrium in the last 10 minutes of L-cysteine application which could be attributed to the effects of $H_2S$ produced by L-cysteine as found on incubation with $H_2S$ producers NaHS and GYY4137 (**chapter 4**). Next it was determined if $Ca^{2+}$ channels and transients were affected upon application of L-cysteine. Firstly, high $K^+$ was used to depolarize the myometrium and open voltage gated $Ca^{2+}$ channels (Noble 2006). Secondly, direct measurements of intracellular $Ca^{2+}$ simultaneously with force in the presence of L-cysteine. If $Ca^{2+}$ entry is decreased, then this should be apparent in the associated $Ca^{2+}$ transients (Shmigol 1998). Thirdly, Bay K 8644 was used to investigate whether upon opening L-type calcium channels changes the response of L-cysteine. As Bay K 8644 opens L-type $Ca^{2+}$ channels and leads to a rise in cytosolic $Ca^{2+}$, however the mechanism by which the sustained elevation in cytosolic calcium results in the generation of phasic contractile activity is unclear (chien 1996).

Depolarisation using high $K^+$ for 30 minutes was examined with and without 1mM L-cysteine (n=5). In day 22 pregnant rats, the two consecutive applications of high K, with and without L-cysteine, produced very similar contractile responses; a rapid rise in force which plateaus (**Figure 5.7A**). No significant differences in AUC were found.

The effects of 1mM L-cysteine on $Ca^{2+}$ signalling in day 22 of gestation rat myometrium were examined. As **Figure 5.7B** clearly shows spontaneous $Ca^{2+}$ transients (indo-1 fluorescence) underlie the phasic contractions of the myometrium. L-cysteine produced significant decreases in $Ca^{2+}$ amplitude to 75±5%, shown in **figure 5.7B** with an overall increase in the frequency of Ca transients to 129±25%. (p=0.021, p=0.004 respectively, *n* = 5).

Consistent with previous studies upon application of 0.1µM BayK (**Figure 5.8**) increased the intensity and frequency of smooth muscle contractions (Knot 1991, Sato 1988, Chien 1996). The effects of L-cysteine in the presence of BayK were not as pronounced, the decrease in contractility found after the 30 minute L-cysteine incubation did not reach significance (p=0.06). For duration, frequency and AUC

there were also no significant changes. Although, the AUC for the initial 10 minutes of L-cysteine incubation remained significantly increased.

Overall L-cysteine affects Ca entry as it significantly decreases $Ca^{2+}$ transient amplitude after 30 minute incubations as well as increases frequency. KCL and BayK 8644 results show that the effects of L-cysteine are eliminated in the presence of KCl and partially inhibited in the response to bayK 8466.

### 5.4.5 Examining the chemical effect of L-cysteine through use of D-cysteine and sodium pyruvate.

To investigate whether the difference seen in responses to L-cysteine are not just simply a chemical effect D-cysteine, the stereo isomer of L-cysteine as well as sodium pyruvate and L-serine, by products formed when the CSE and CBS enzymes use L-cysteine to produce $H_2S$, were studied (**Figure 5.9**). L-serine is an analogue of L-cysteine. The optimum concentration of L-cysteine used was 1mM and this was the concentration used within this section to directly compare responses of D-cysteine, sodium pyruvate and L-serine.

There was no effect on contractility in the presence of D-cysteine (n=4, **Figure 5.9A**) also previously found by sidhu et al 2001 (sidhu 2001). Both sodium pyruvate (n=4) and L-serine (n=2) produced a similar increased contractile profile upon initial application. No statistical analysis was performed on L-serine but sodium pyruvate showed a significant increase in amplitude of initial contractions once sodium pyruvate had been added at 113±3% (p=0.03). This lead me to the question could the initial stimulation of force in the presence of L-cysteine be due to an intracellular pH change?

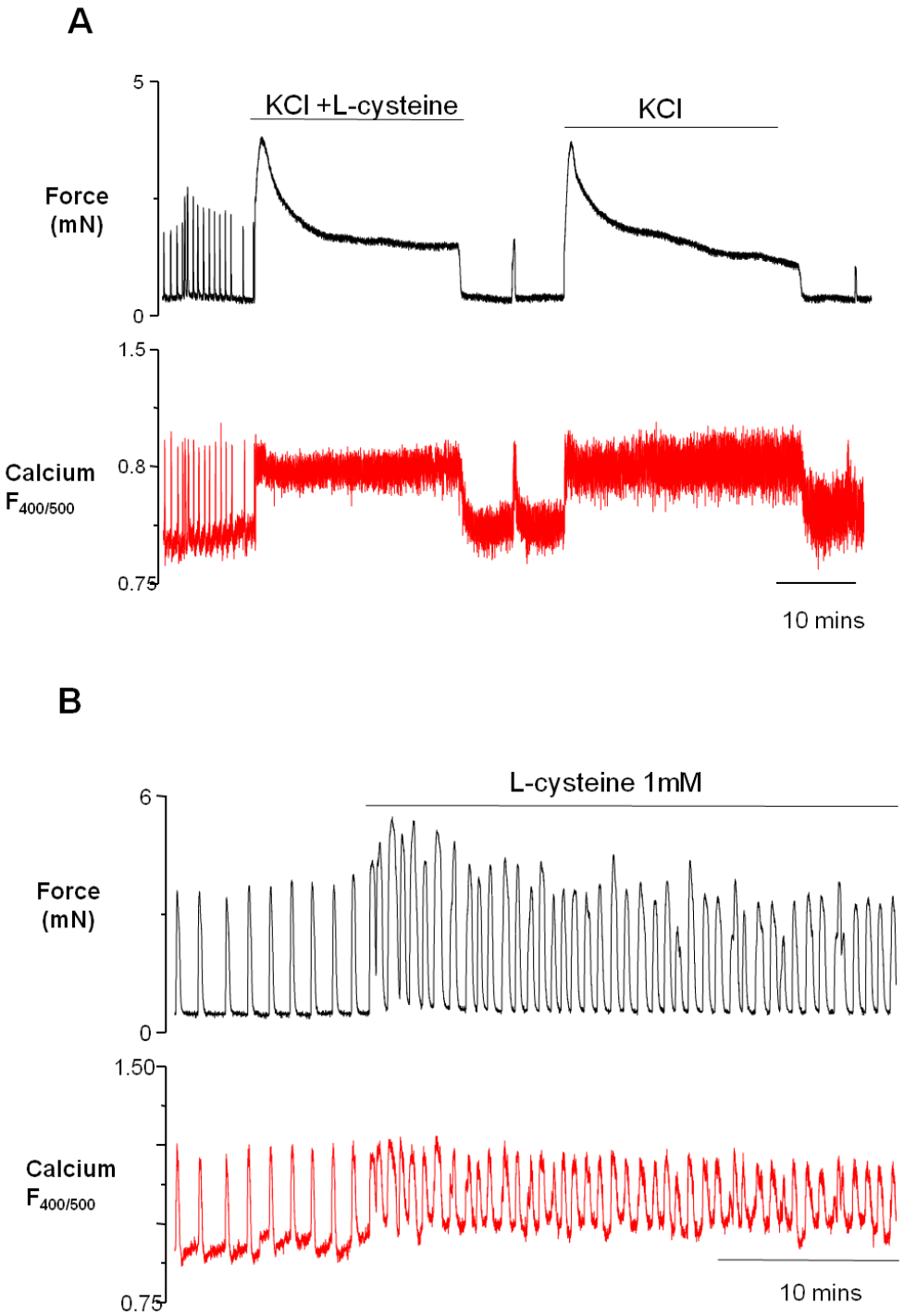


**Figure 5.7: Effects on calcium entry and calcium signalling.**

**(A)** High K (40 mM) depolarisation, 30 mins, of term pregnant rat myometrial strips and the effects in the presence of 1mM L-cysteine (n=5)**. (B)** Recording of force and intracellular Ca (from Indo-1–emitted fluorescence F400:500), measured simultaneously in spontaneously contracting myometrial strips dissected from term rat myometrium in the presence of L-cysteine (n=5). All solutions were used at 37$^{o}$C and pH 7.4.

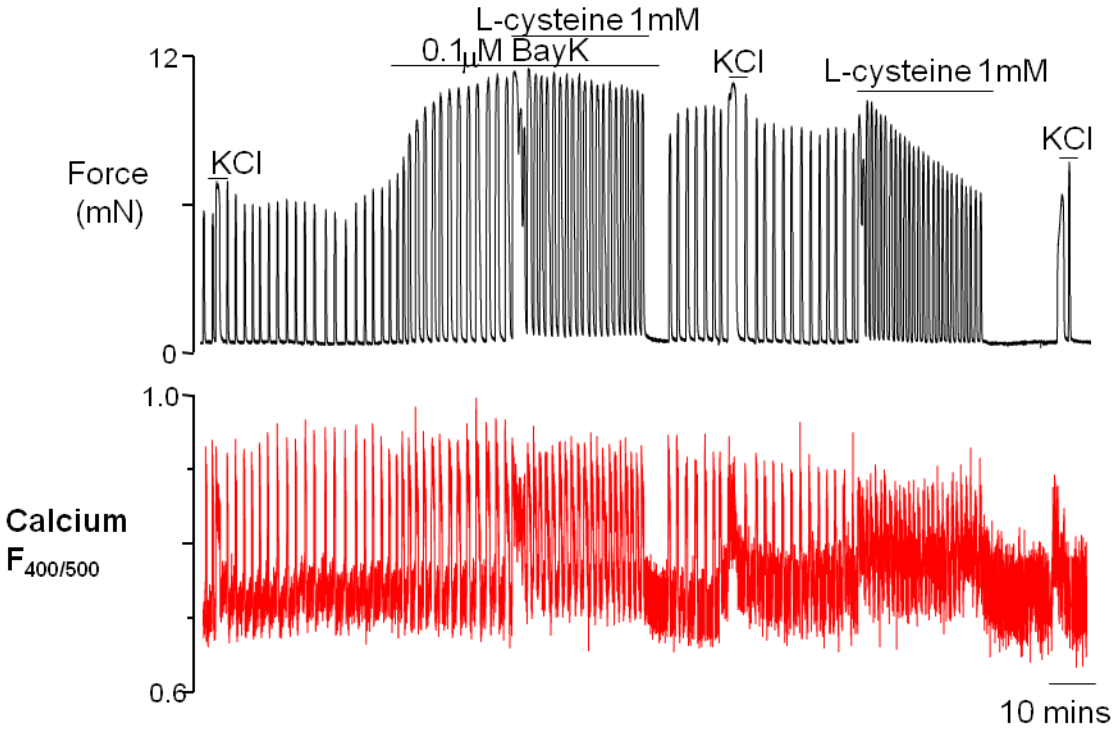


**Figure 5.8: Effect of L-cysteine on myometrial contractility in the presence of BayK 8644**

A representative trace showing the reduced effects of L-cysteine on the contractility of term pregnant rat myometrium in the presence of BayK 8644. BayK 8644 L-type calcium channel opener. The decrease in contractions is abolished in the response to L-cysteine with BayK 8644 compared to the response to L-cysteine alone. In indo1AM loaded stripes whilst being excited at 340nM by a xenon lamp. The emitted light was measured by photomultiplier tubes at 400nM and 500nM. The ratio of the two fluoresces gives a measurement of the underlying calcium transients. All experiments were carried out a 37°C and pH7.4

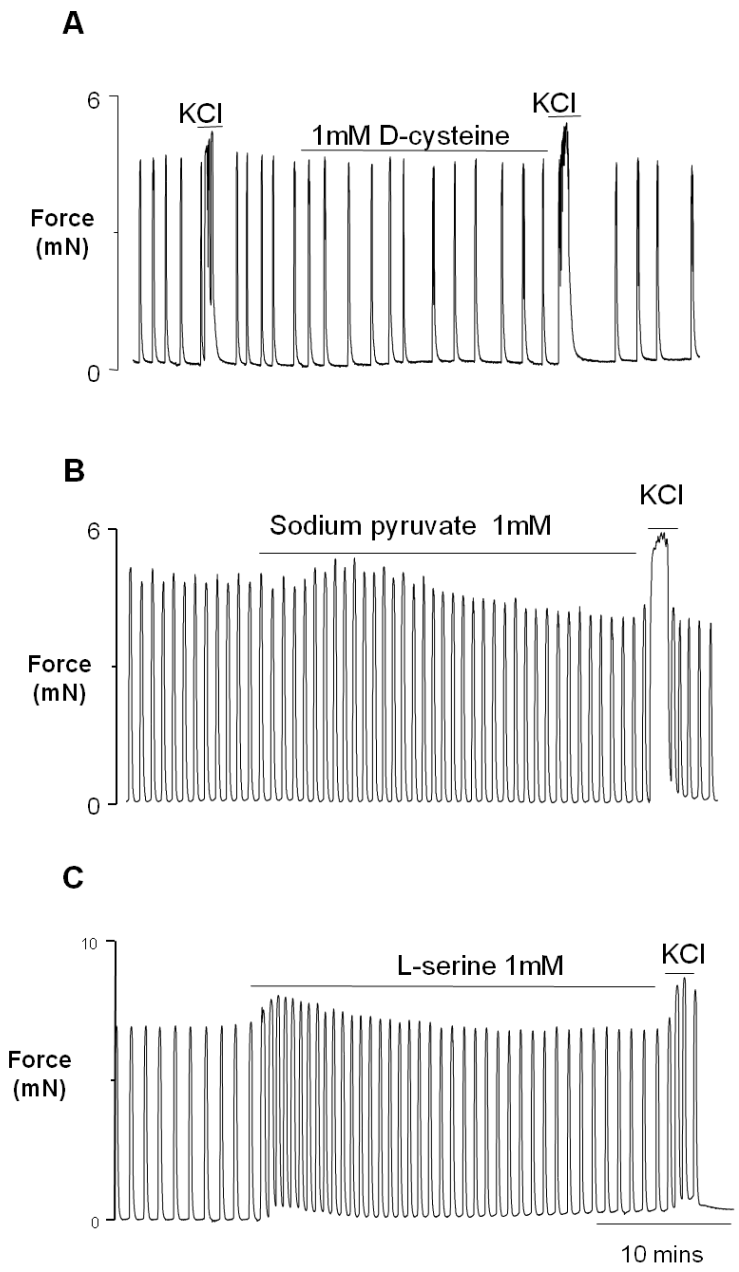


**Figure 5.9: Effect of D-cysteine, sodium pyruvate and L-cysteine on term pregnant rat myometrium**

A representative trace showing A) no effect of 1mM D-cysteine on spontaneous contractility of term pregnant rat myometrium and the stimulation upon initial perfusion in B) 1mM sodium pyruvate and C) 1mM L-serine. All experiments were carried out a 37°C and pH7.4.

### 5.4.6 Monitoring intracellular pH of term pregnant myometrial contractions upon perfusion with L-cysteine.

To investigate if the initial stimulation of frequency upon incubation with L-cysteine was due to an intracellular alkalinisation (Heaton 1992, Heaton 1993) the pH sensitive indicator carboxy SNARF-1 was used. In 6 myometrial strips from different term pregnant rats pH was measured simultaneously with spontaneous contractile activity and L-cysteine added. The initial changes in frequency and amplitude of contractions upon application of L-cysteine coincides with the period when pH decreased i.e. no alkalinisation occurred. (**Figure 5.10A**). Addition of pH 6.9 PSS illustrates the effects of external acidification (**figure 5.10B**) using Carboxy SNARF-1, supporting that upon incubation in L-cysteine an acidification roughly half the size is seen.

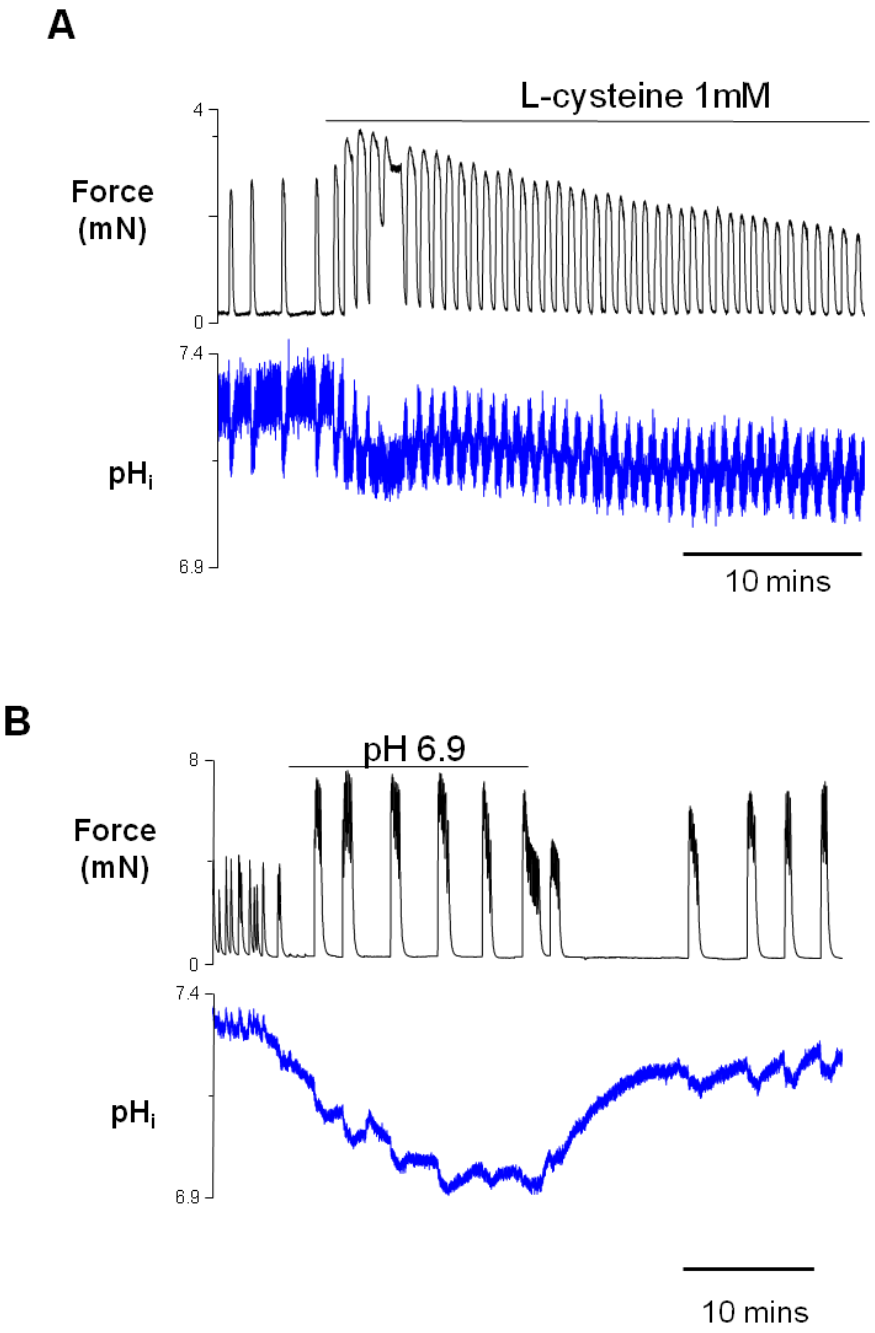


**Figure 5.10 The effects of L-cysteine and external pH 6.9 on intracellular pH**

Simultaneous recording of force and $pH_i$ in term pregnant rat myometrium loaded with the pH sensitive indicator carboxy-SNARF. The tissue was excited at 530nm and a ratio of the emission signals at 590 and 640. Traces show a small decrease in $pH_i$ is associated with each phasic contraction. A) Shows an intracellular decrease in pH upon application of L-cysteine (n=6). B) Illustrates decreasing the pH of the perfusate from pH 7.4 to pH 6.9 increased force of contractions.

### 5.4.7 Do inhibitors of both $H_2S$ production enzymes abolish the response of L-cysteine?

Both CSE and CBS enzymes produce $H_2S$ through use of substrate L-cysteine. In order to confirm that the effects of 1mM L-cysteine were through producing $H_2S$, CBS and CSE inhibitors were administered 20 mins before addition of L-cysteine to term pregnant rat myometrium. Within this section I describe the use of CSE inhibitor, PAG (0.1mM) and CBS inhibitors, AOAA (0.1mM) and HA (1mM). PAG and AOAA have been used in human myometrium at these concentrations (You et al 2011).

An original trace showing L-cysteine's effects in the presence of PAG can be seen in **Figure 5.11**. As shown in **Figure 5.12A**, CSE inhibitor PAG did not block the initial stimulation caused by L-cysteine. Significant elevations in amplitude, duration and AUC remain ($p=0.03$, $p=0.03$, $p=0.009$, respectively, $n=4$). Frequency is elevated but not significantly, with increased n numbers this may change. However, illustrated in **figure 5.12B**, the decrease in amplitude of the contractions in the final ten minute period of L-cysteine exposure is partially blocked at 88±13% rather than 67±6% in L-cysteine alone. Amplitude, duration and AUC remain significantly elevated.
CBS inhibitors HA ($n=5$) and AOAA ($n=4$) were tested in the presence of L-cysteine and original traces can be found in **figure 5.13**. HA totally abolished contractions as well as the effects of L-cysteine. AOAA abolished the L-cysteine-induced decrease in spontaneous contraction amplitude found within the last ten minute of L-cysteine application. This is shown in the mean data within **figure 5.14**. All other parameters of contractility were not significantly changed in response to L-cysteine in the presence of AOAA at the beginning or end period of L-cysteine application, although overall resultant affects still remain on AUC.

Overall both inhibitors block or partially block the decrease in contractility, most likely a result of $H_2S$ production by the enzymes through L-cysteine. Although the effects of the acidification found upon application of L-cysteine remain, with an increase in the frequency and AUC of contraction demonstrated in **figures 5.11-5.14**.

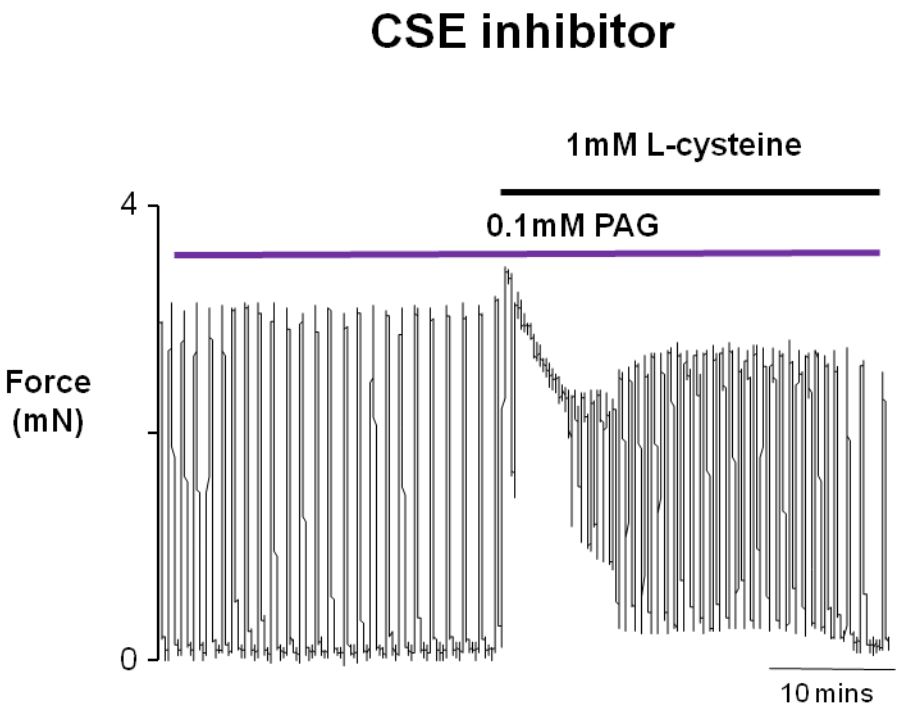


**Figure 5.11 The effect of L-cysteine in the presence of CSE inhibitor**

CSE inhibitor PAG (0.1mM) was applied to term pregnant rat myometrial strips 20 minutes prior and throughout the 30 minute superfusion of L-cysteine (1mM). A representative trace is illustrated above (n=4). All solutions were superfused at 37$^{o}$C, pH7.4.

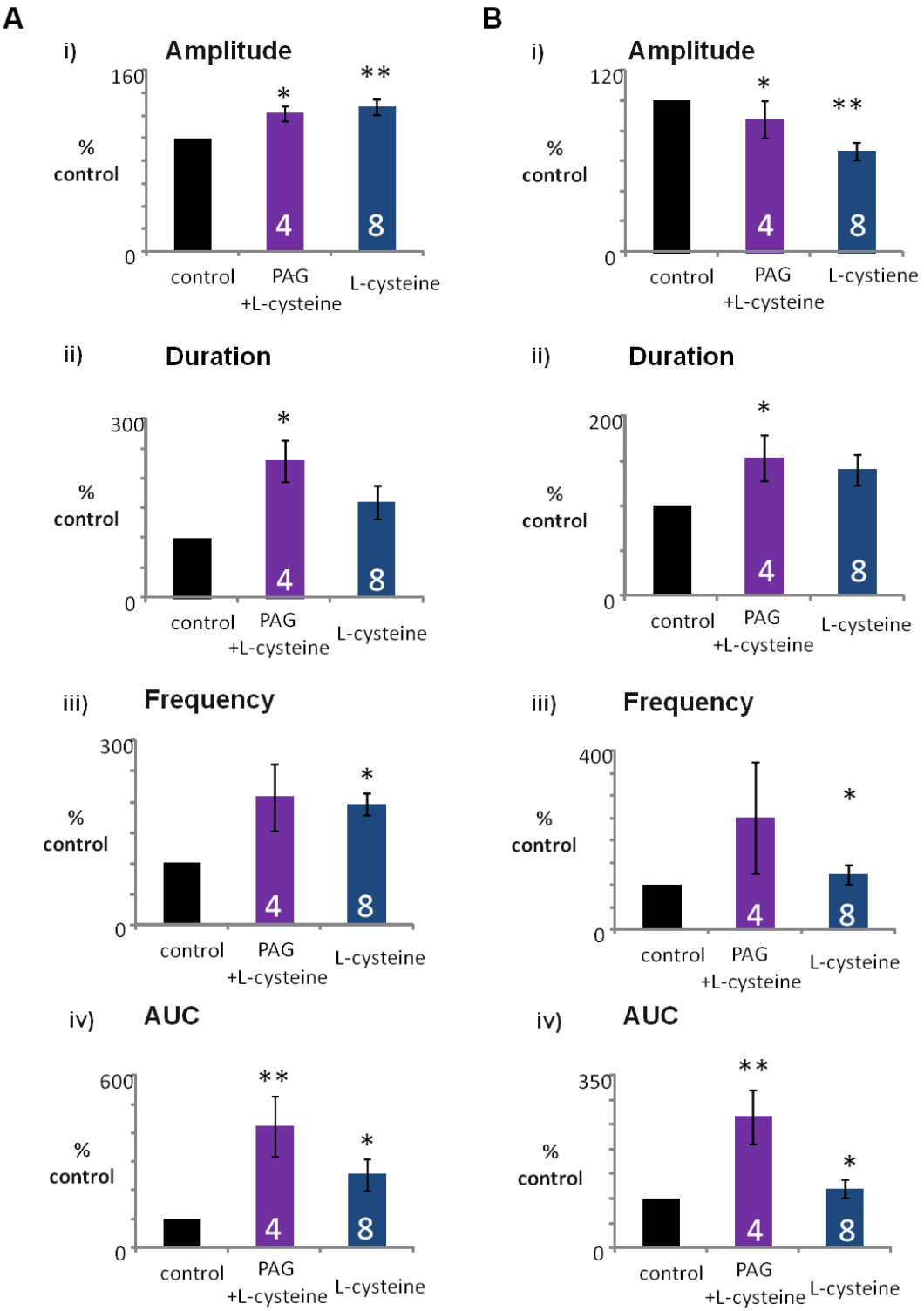


**Figure 5.12: Mean data showing the responses of L-cysteine with and without CSE inhibitor.**

Mean data± s.e.m, denoted by error bars, showing the first 10 minutes (A) and the last 10 minutes (B) of applications of L-cysteine (1mM) with and without CSE inhibitor PAG (0.1mM). The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is $p<0.05$. ** is $p<0.01$, using student t-tests.

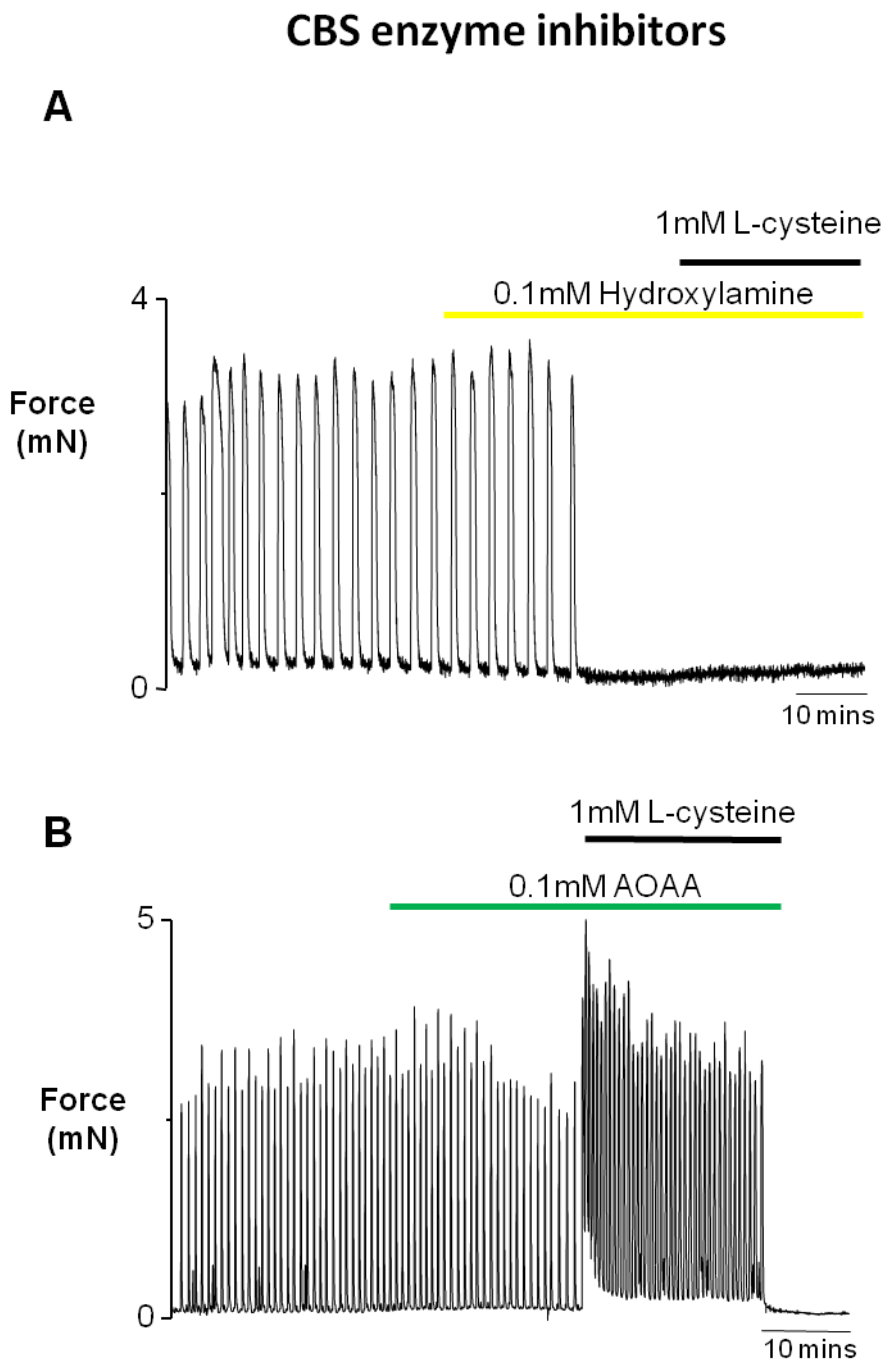


**Figure 5.13 The effect of L-cysteine in the presence of CBS inhibitors**

CBS inhibitors A) HA(1mM) and B) A0AA (0.1mM) were applied to term pregnant rat myometrial strips 20 minutes prior and throughout the 30 minute superfusion of L-cysteine (1mM). A representative trace is illustrated above (n=5, n=4 respectively). All solutions were superfused at 37$^{o}$C, pH7.4.

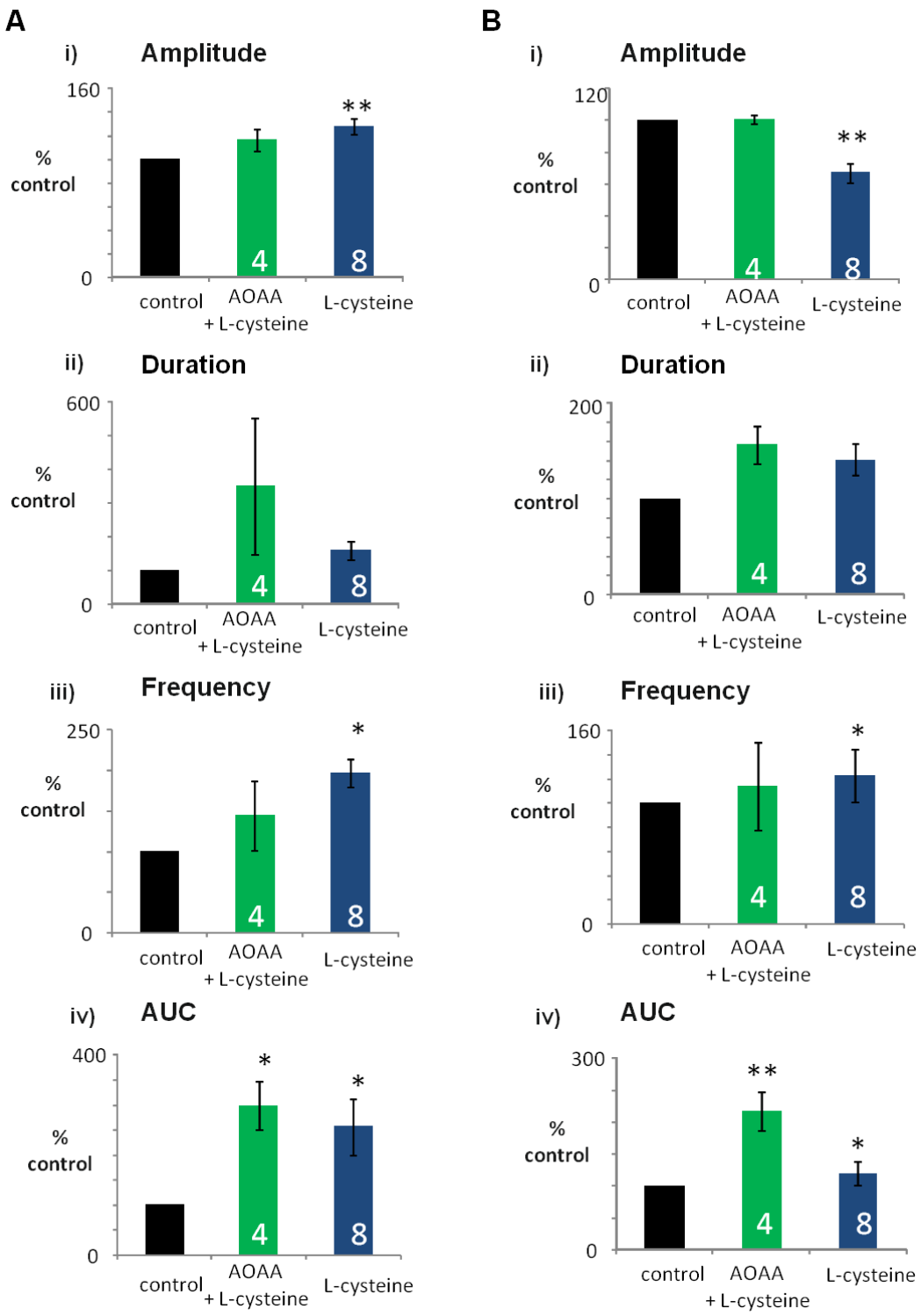


**Figure 5.14: Mean data showing the responses of L-cysteine with and without CBS inhibitor A0AA.**

Mean data± s.e.m, denoted by error bars, showing the first 10 minutes (A) and the last 10 minutes (B) of applications of L-cysteine (1mM) with and without CBS inhibitor AOAA (0.1mM). The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is $p<0.05$. ** is $p<0.01$, using student t-tests.

### 5.4.8 The dose dependent effect of L-cysteine on term pregnant human myometrium

The dose response of L-cysteine was examined in the term pregnant human myometrium. To see if similar effects were found. To accommodate the slower rate of contractions the full 30 minute exposure to L-cysteine was assessed. Human myometrium was exposed to increasing concentrations of L-cysteine from 1nM to 1mM.

A trend dose-dependent decrease in the amplitude of force of contractions was found. At the highest concentration (1mM) this decrease in force almost reached significance at 57±8% compared to control (100%) (p=0.060, n=6). A typical dose response force trace can be seen in **figure 5.15** and the mean data for amplitude, duration, frequency and AUC of contractions in response to L-cysteine are presented in graphical form in **figure 5.16.** All other parameters showed an increase in contraction (**5.15 ii-iv**) in response to increasing doses of L-cysteine, only the elevated frequency reached significance, from 1nm and 1µm to 1mM (p=0.033, p=0.025 respectively). Individual Student t-tests between control and 1mM L-cysteine contractions showed a significant decrease in amplitude (p=0.001) and significant increases in duration, frequency and AUC (p=0.023, p=0.017, p= 0.040, respectively). Student t-tests between control and 0.1mM L-cysteine showed a significant decrease in amplitude as well as a significant increase in frequency (p=0.03, p=0.019).

A dose response curve for the effect of L-cysteine on the force of term pregnant human myometrial contractions showing the logEC50 is illustrated in **Figure 5.17**.

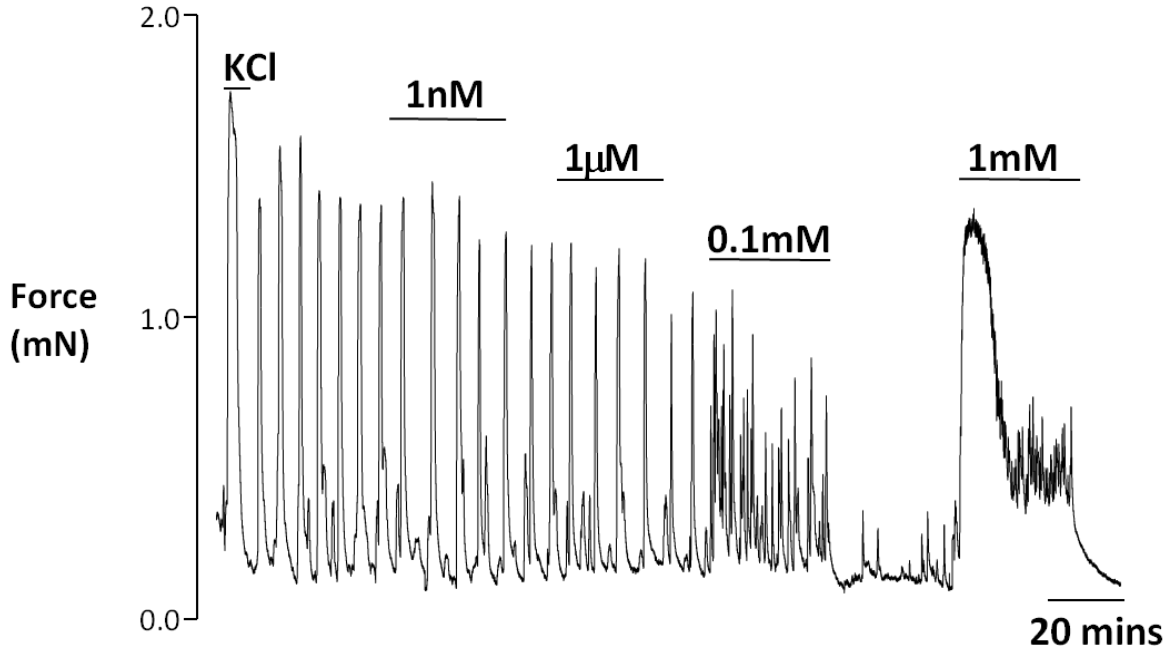


**Figure 5.15: Dose dependency of L-cysteine in term pregnant Human myometrium.**

Isometric recordings of spontaneously contracting term pregnant human myometrial strips superfused with increasing doses of L-cysteine from 1nm to 1mM at 37$^{o}$C, pH7.4.

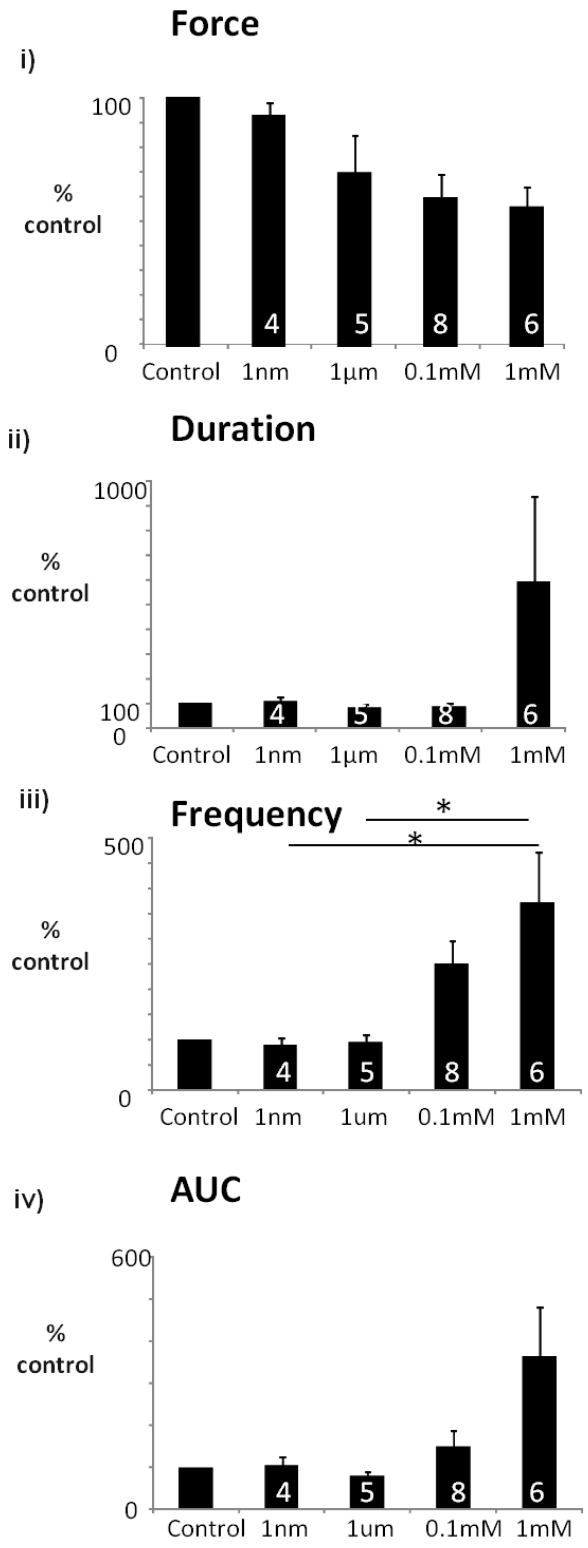


**Figure 5.16: Mean data of dose dependency of L-cysteine in term pregnant human myometrium.**

Mean data± s.e.m, denoted by error bars, showing the mean data in the parameters of contractility i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is p<0.05. ** is p<0.01, ***p<0.001 using Anova with Bonferroni *post hoc* tests.

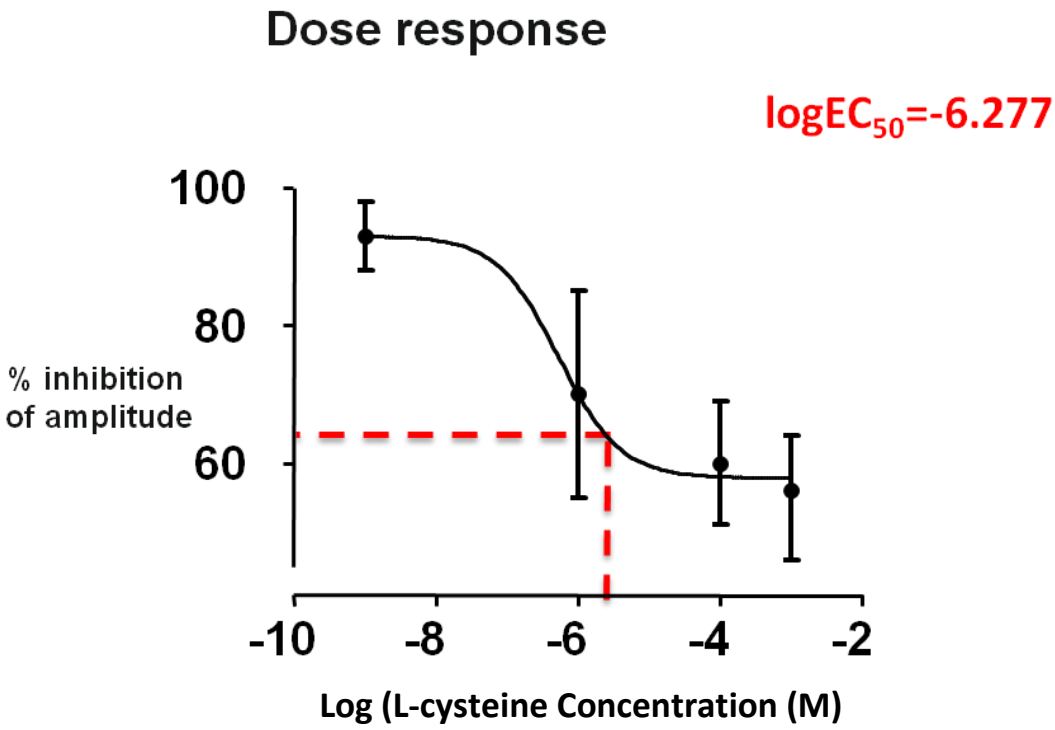


**Figure 5.17 LOG dose response curve for term pregnant human myometrium**

Log dose response curve for the effect of L-cysteine on term human myometrial contractility, showing the sigmoidal relationship. The log EC50 value of -6.277 depicts the concentration required to reduce myometrial contractility by 50% compared to minimum and maximum values.

### 5.4.9 The effect of 1mM L-cysteine on non-pregnant and Term human myometrium

Looking at the statistical effect of 1mM L-cysteine on non-pregnant human myometrium (n=5) alone showed a significant decrease in amplitude of contractions at 90±2% (p=0.015) and significant increases in frequency at 364±100% (p=0.009) and AUC at 268±65% (p=0.048) when compared to control period (100%).

Differences between the responses found to 1mM L-cysteine in non-pregnant and term pregnant human myometrial contractions were then examined. Representative traces of a non-pregnant (**5.18A**) and term pregnant human (**5.18A**) 1mM L-cysteine response is illustrated in **figure 5.18**. The mean data are demonstrated in **figure 5.19 i-iv**. As in rat myometrium l-cysteine responses, the only significant difference in response was to the decline in contractility as within the term human pregnant myometrium the contractions under L-cysteine application were significantly more reduced at 56±8% (p=0.008). Therefore in conclusion the changes in contractile activity on application of L-cysteine within both groups are not significantly different apart from a residing significant decrease in amplitude when compared to non-pregnant.

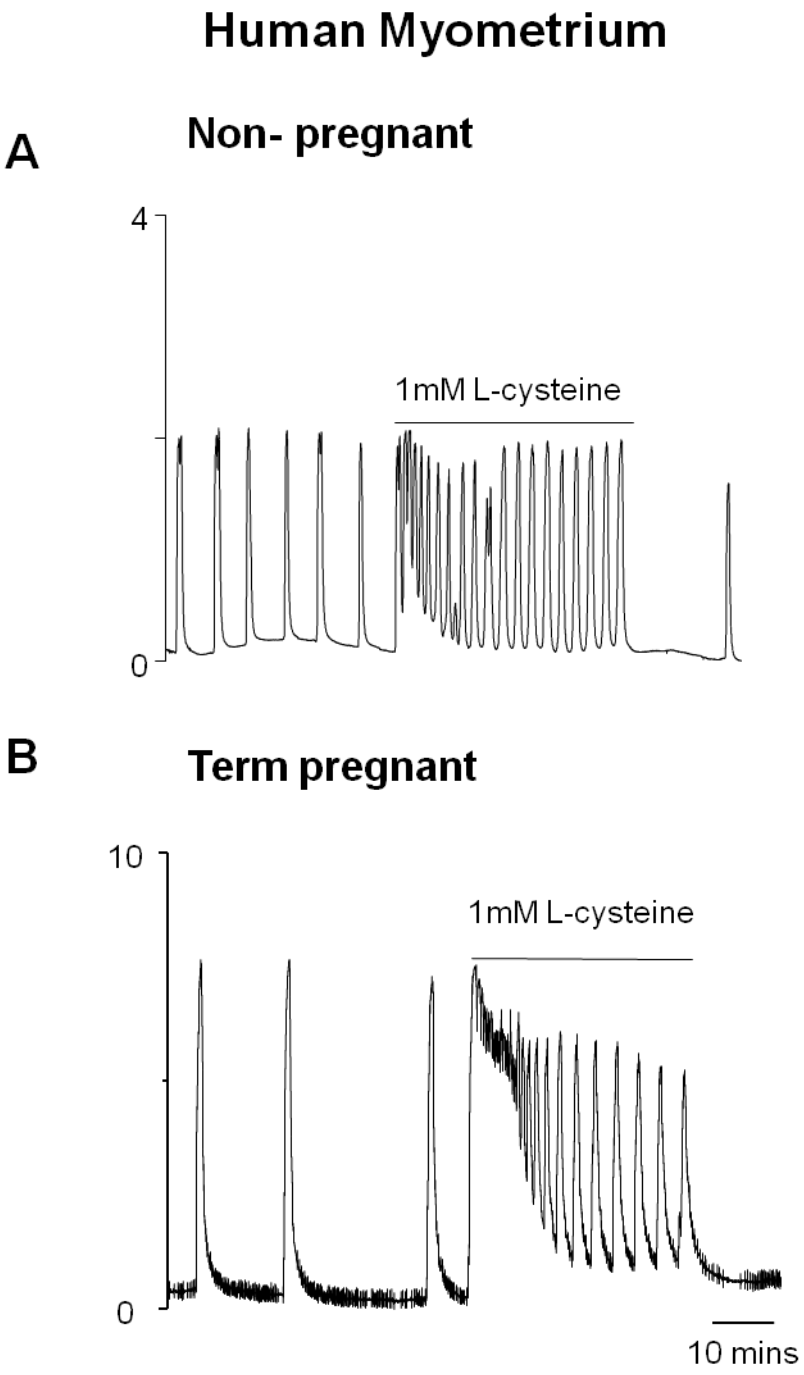


**Figure 5.18: Effect of 1mM L-cysteine in non-pregnant and term pregnant myometrium.**

Isometric recordings of spontaneously contracting (A) non-pregnant and (B) term pregnant human myometrial strips superfused with 1mM L-cysteine at 37$^{o}$C, pH7.4.

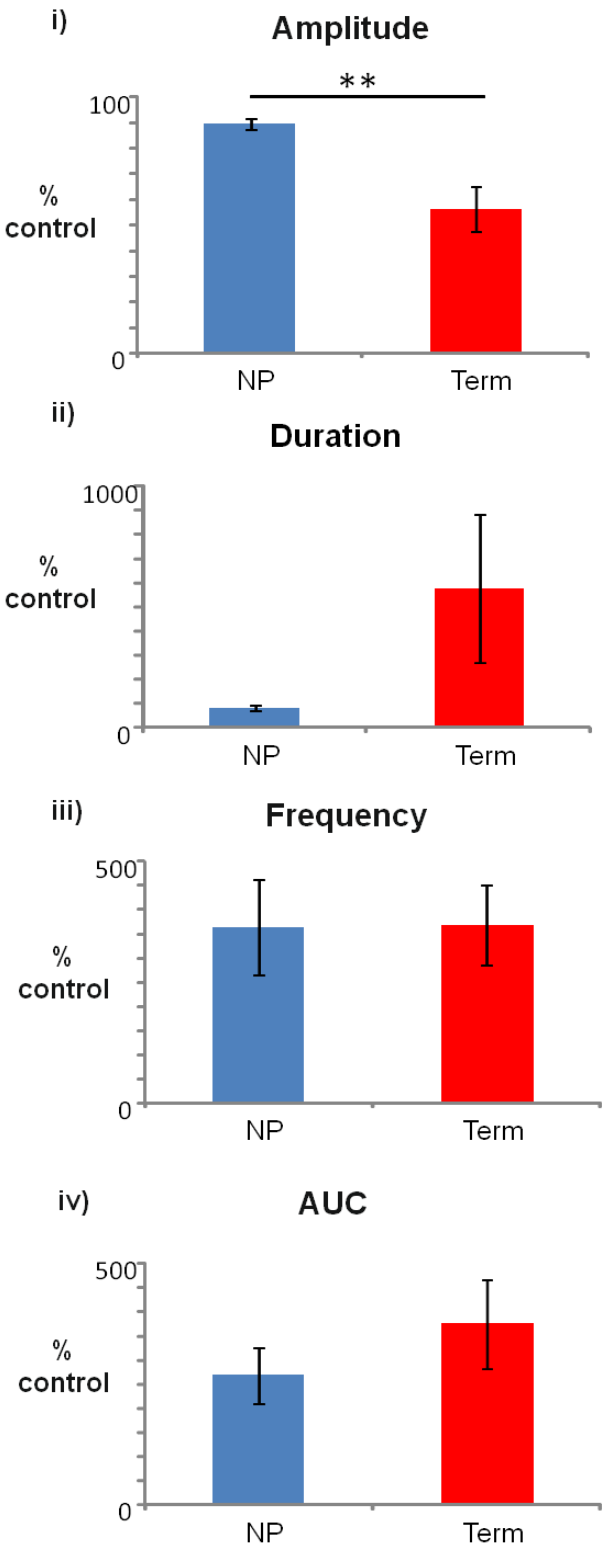


**Figure 5.19: Comparison of non-pregnant and term pregnant L-cysteine responses.**

Mean data± s.e.m, denoted by error bars, showing non-pregnant (NP) and term pregnant differences in final ten minute perfusion with L-cysteine. The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. * is $p<0.05$. ** is $p<0.01$, ***$p<0.001$ using student ttests.

### 5.4.10 Does garlic affect the myometrium?

Here preliminary experiments have been performed superfusing different concentrations of garlic powder, most likely a mixture of garlic derived organic polysulfides (Benavides et al 2007**)**, within PSS for 20 minute periods.

Initial experiments show a similar response to L-cysteine at 0.714mg/ml concentration of garlic (**Figure 5.20A**). The different concentration responses can be seen within **figure 5.20 A-C**. Upon increasing doses of garlic powder there is a trend of a dose-dependent increase in duration, frequency and AUC with not much change in amplitude for both the initial and end 10 minute period responses of garlic (**Figure 5.21 and 5.22**). The most prominent effects were found at 0.714mg/ml and greater concentrations of garlic powder. At 0.714mg/ml the initial ten minute period of garlic application illustrated a small initial increase in amplitude of contraction at 103±19% with increases in duration, 117±3%, frequency, 152±14% and AUC, 169±9% (**Figure 5.21 i-iv**, n=4). At 0.714mg/ml the last ten minutes of garlic application showed amplitude decreased to 96±25% with increases in duration, 125±11%, frequency, 163±15% and AUC, 172±13% (**Figure 5.22 i-iv** , n=4). Only underlying AUC reached significance for both initial and final exposures to 0.714 mg/ml garlic (5.21iv and 5.22ivp=0.030, p=0.007 respectively). All other doses were not assessed for significance due to low n-numbers of 1 or 2.

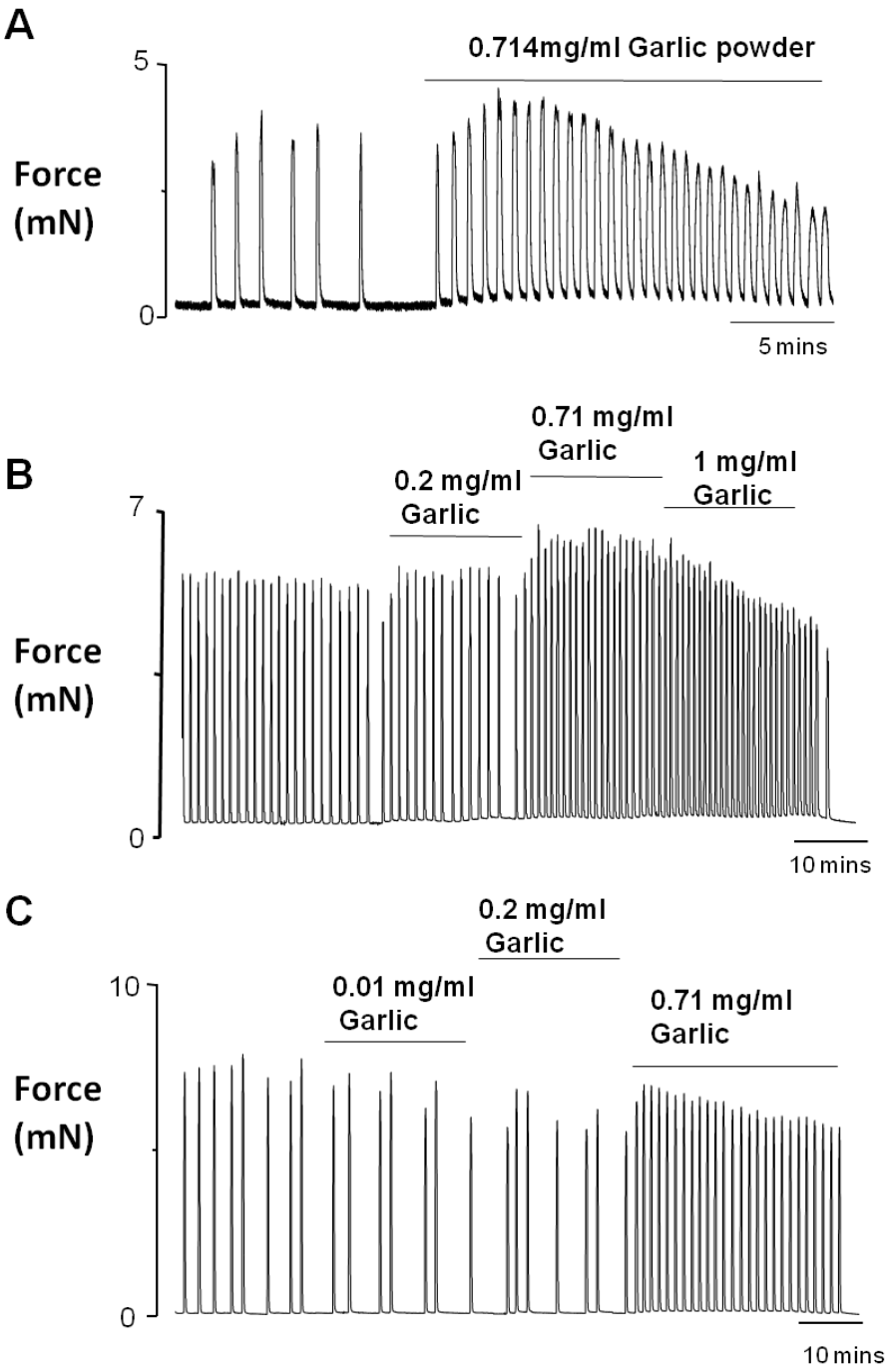


**Figure 5.20: Effect of increasing doses of garlic powder on term pregnant rat myometrium.**

Isometric recordings of spontaneously contracting term pregnant human myometrial strips superfused with 0.01-1mg/ml garlic powder. A) shows the effect of 0.714mg/ml garlic B) shows the effect of 0.2, 0.714 and 1mg/ml garlic C) shows the effect of 0.01, 0.2 and 0.714mg/ml garlic on contractility.

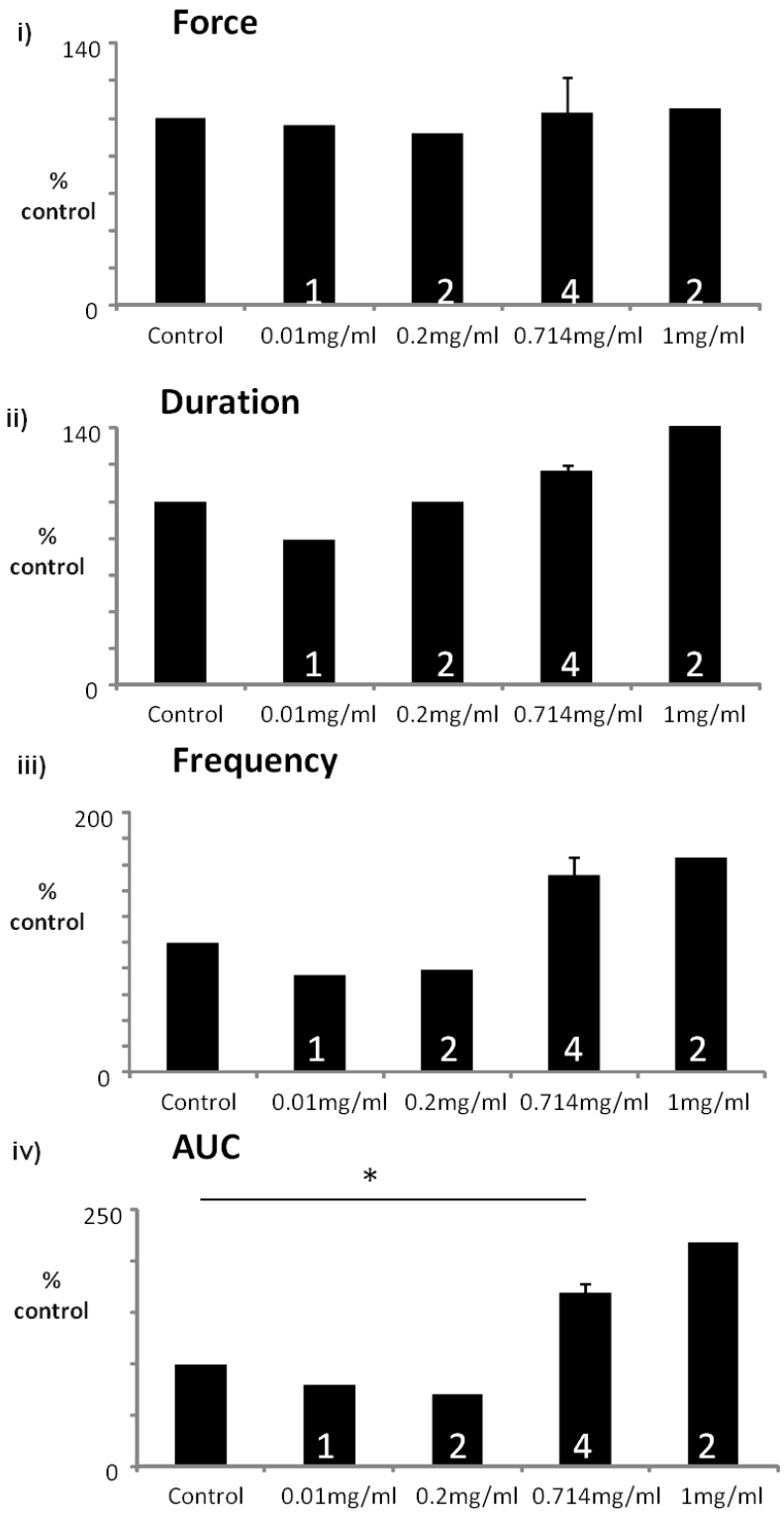


**Figure 5.21: Mean data for the initial effects of increasing concentrations of Garlic on term pregnant rat myometrium.**

Mean data± s.e.m, denoted by error bars, showing the changes in contractility within the first 10 minutes of applications of Garlic. The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. Only 0.714mg/ml garlic was tested for significance using student t-tests * is $p<0.05$.

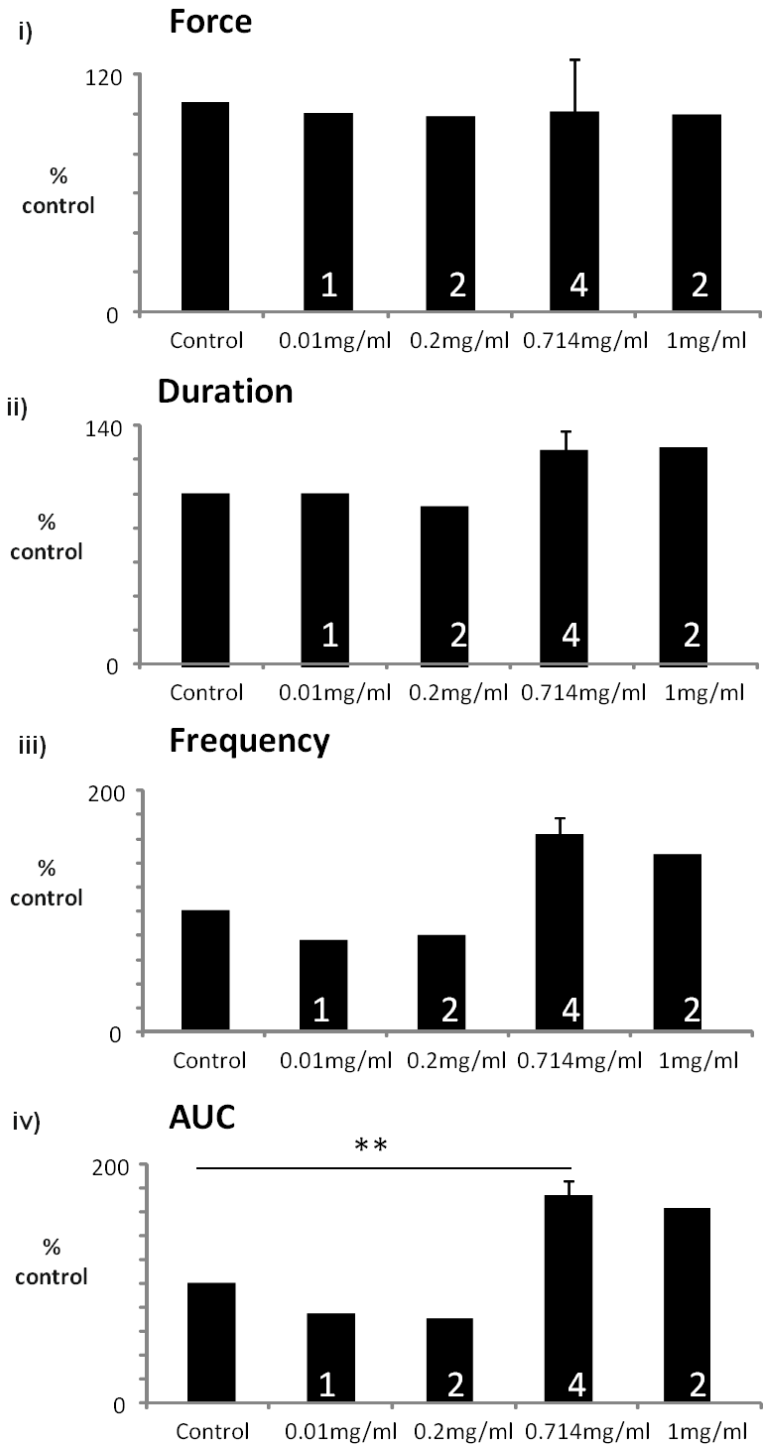


**Figure 5.22: Mean data for the final effects of increasing concentrations of Garlic on term pregnant rat myometrium.**

Mean data± s.e.m, denoted by error bars, showing the changes in contractility within the last 10 minutes of applications of Garlic. The mean data for i) Amplitude, ii) Duration iii) Frequency, iv) AUC in response to L-cysteine. Values within bars indicate n-numbers. Only 0.714mg/ml garlic was tested for significance using student t-tests ** is $p<0.01$.

### 5.4.11 Expression of the CBS and CSE in the Myometrium of Non-pregnant, 10 day, 14 day, 18 day and 22 day rat myometrium – Immunohistochemistry

The expression of the CBS and CSE, $H_2S$ production enzymes were examined by light microscope of DAB staining which is equivalent to CBS and CSE expression. Positive controls for CBS (Brain) and CSE (Aorta) as well as negative controls for non-pregnant, 10 day, 14 day, 18 day and 22 day rat myometrium are seen in **figure 5.23**, illustrating DAB staining in the positive controls and no staining in the negative controls. This shows that the staining is specific to either the CBS or CSE antibody depending on which antibody is used. A representative image of NP, 10, 14, 18 and 22 day rat myometrium is shown in **figure 5.24** (n=3 each tissue type). Staining was found in the myometrium of all samples as well as the circular muscle.

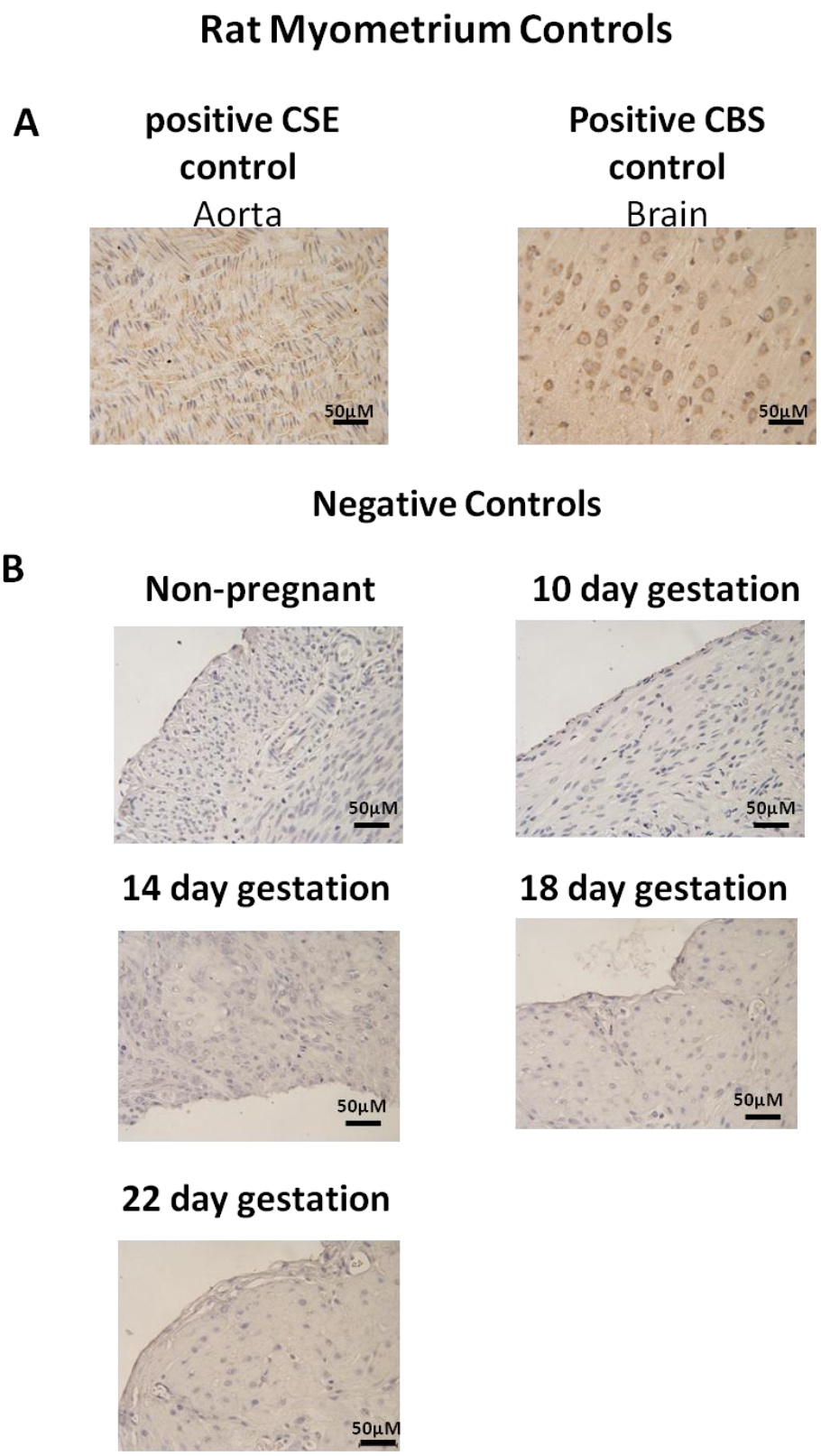


**Figure 5.23 Positive and negative controls**

Positive staining for CBS and CSE in brain and aorta respectively was observed (A). No staining was found using BSA as negative control under the same conditions in non-pregnant, 10, 14, 18 and 22 day rat myometrium (B).

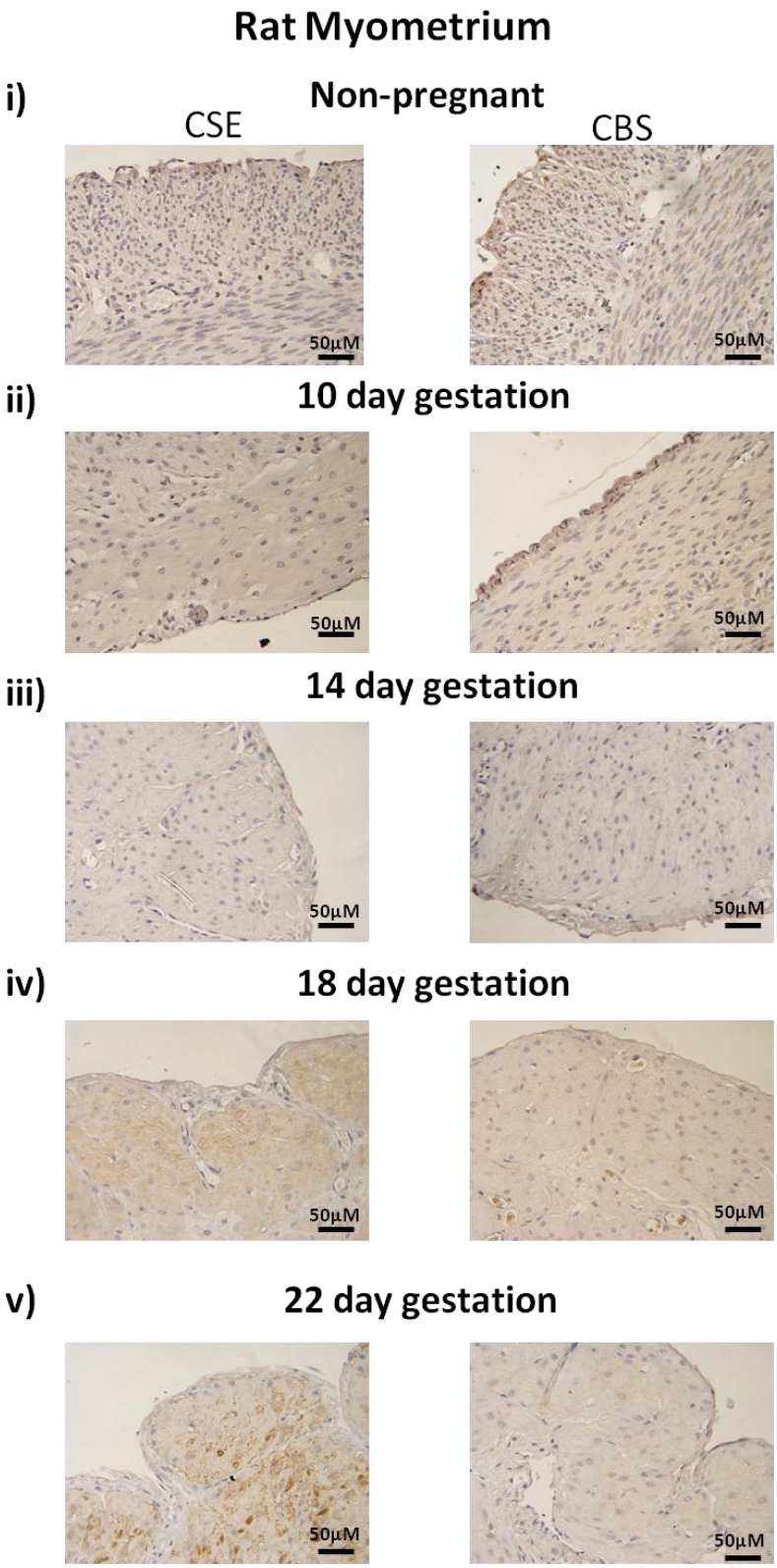


**Figure 5.24 Expression of $H_2S$ producing enzymes CBS and CSE in rat myometrium.**

Expression of CBS and CSE can be see using DAB staining in i) non-pregnant, ii)10 day, iii)14 day, iv)18 day and v)22 day rat myometrium.

### 5.4.12 Expression of the CBS and CSE in the myometrium of non-pregnant, and term pregnant human myometrium- Immunohistochemistry

The expression of the CBS and CSE, $H_2S$ production enzymes were examined by light microscope of DAB staining which is equivalent to CBS and CSE expression. Positive control beta actin as well as negative controls for non-pregnant and term pregnant human myometrium are demonstrated in **Figure 5.25C&D and Figure 5.26C&D, respectively**, illustrating DAB staining in the positive controls and no staining in the negative controls. This shows that the staining is specific to either the CBS or CSE antibody depending on which antibody is used. A representative image of non-pregnant and term pregnant human myometrium stained with CBS (n=6) and CSE (n=6) can be found in **Figure 5.25A&B and 5.26A&B**, respectively. Staining for both enzymes was present in both types of myometrium showing $H_2S$ could be produced in the myometrium.

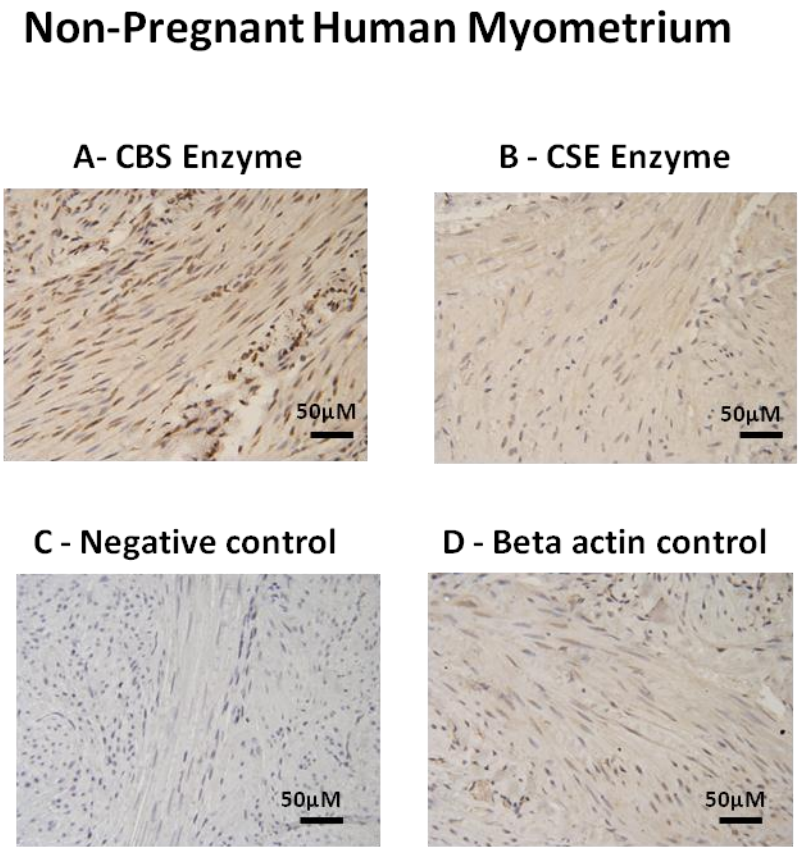


**Figure 5.25: Expression of CBS and CSE in non-pregnant myometrium as shown by immunohistochemistry.**

A) A representative image showing the expression of CBS in the myometrium of a non-pregnant women, as shown by the brown DAB staining n=6

B) A representative image showing the expression of CSE in the myometrium of a non-pregnant women, as shown by the brown DAB staining n=6

C) Positive control & D) Negative control

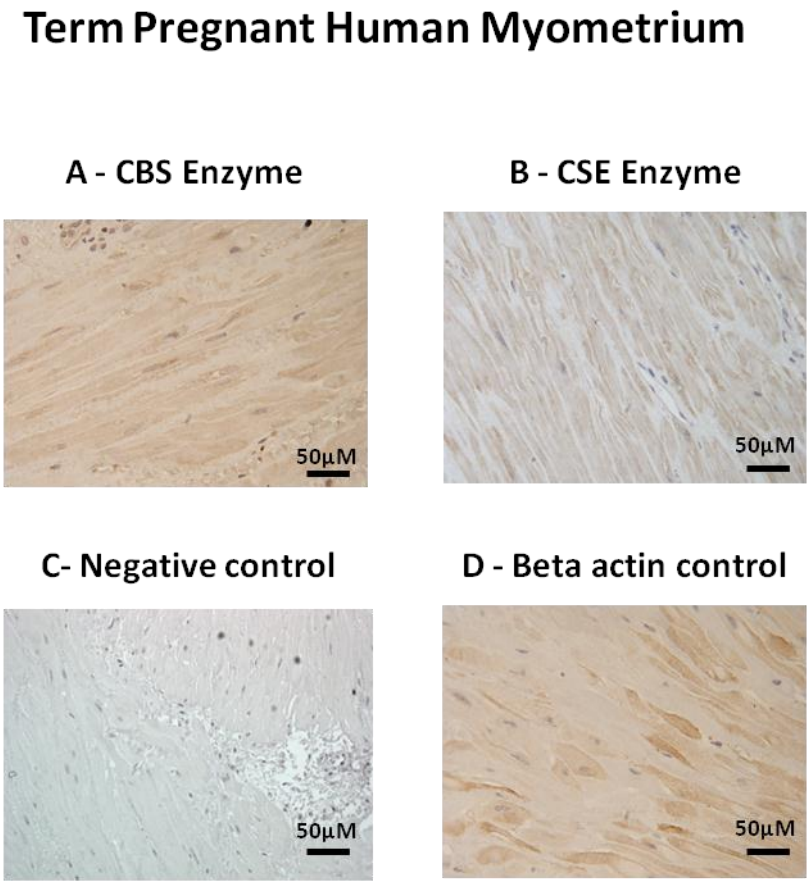


**Figure 5.26: Expression of CBS and CSE in term pregnant myometrium as shown by immunohistochemistry.**

A) A representative image showing the expression of CBS in the myometrium of a term pregnant women, as shown by the brown DAB staining n=6

B) A representative image showing the expression of CSE in the myometrium of a term pregnant women, as shown by the brown DAB staining n=6

C) Positive control & D) Negative control

### 5.4.13 Expression of the $H_2S$ producing enzymes CBS and CSE in non-pregnant and term pregnant myometrium – Western Blotting

To investigate whether the differences in the effect of $H_2S$ on human myometrial contractility is related to more than modifications to ion channels, the expression of $H_2S$ production enzymes CBS and CSE were investigated, this was also to help understand the physiological relevance of $H_2S$ in the myometrium.

The quantification of CBS (**Figure 5.27**) and CSE (**Figure 5.28**) was performed in non-pregnant and term pregnant myometrium. Western blotting was carried out from 6 non-pregnant and 6 term pregnant women and the expression of each enzyme measured. A representative image of 2 term pregnant and 4 non-pregnant repeats of western blots for CBS is shown in **figure 5.27**. The expression of CBS was measured as a percentage of the actin loading control. As can be seen in **figure 5.27** there was a significant reduction in expression of the CBS enzyme in the myometrium from term pregnant women (10.1%) compared to non-pregnant (100%) $p=<0.05$. A representative image of 3 repeats of western blots in non-pregnant and term pregnant myometrium for CSE is shown in **figure 5.28**. The expression of CSE was measured as a percentage of the actin loading control. As can be seen in **figure 5.28** there was also a significant reduction in expression of the CSE enzyme in the myometrium from term pregnant women (51.0%) compared to non-pregnant (100%) $p=<0.05$.

Overall both the CSE and CBS $H_2S$ producing enzymes are present in the non-pregnant and term pregnant human myometrium, allowing $H_2S$ production to occur, although both are down regulated in the term pregnant myometrium.

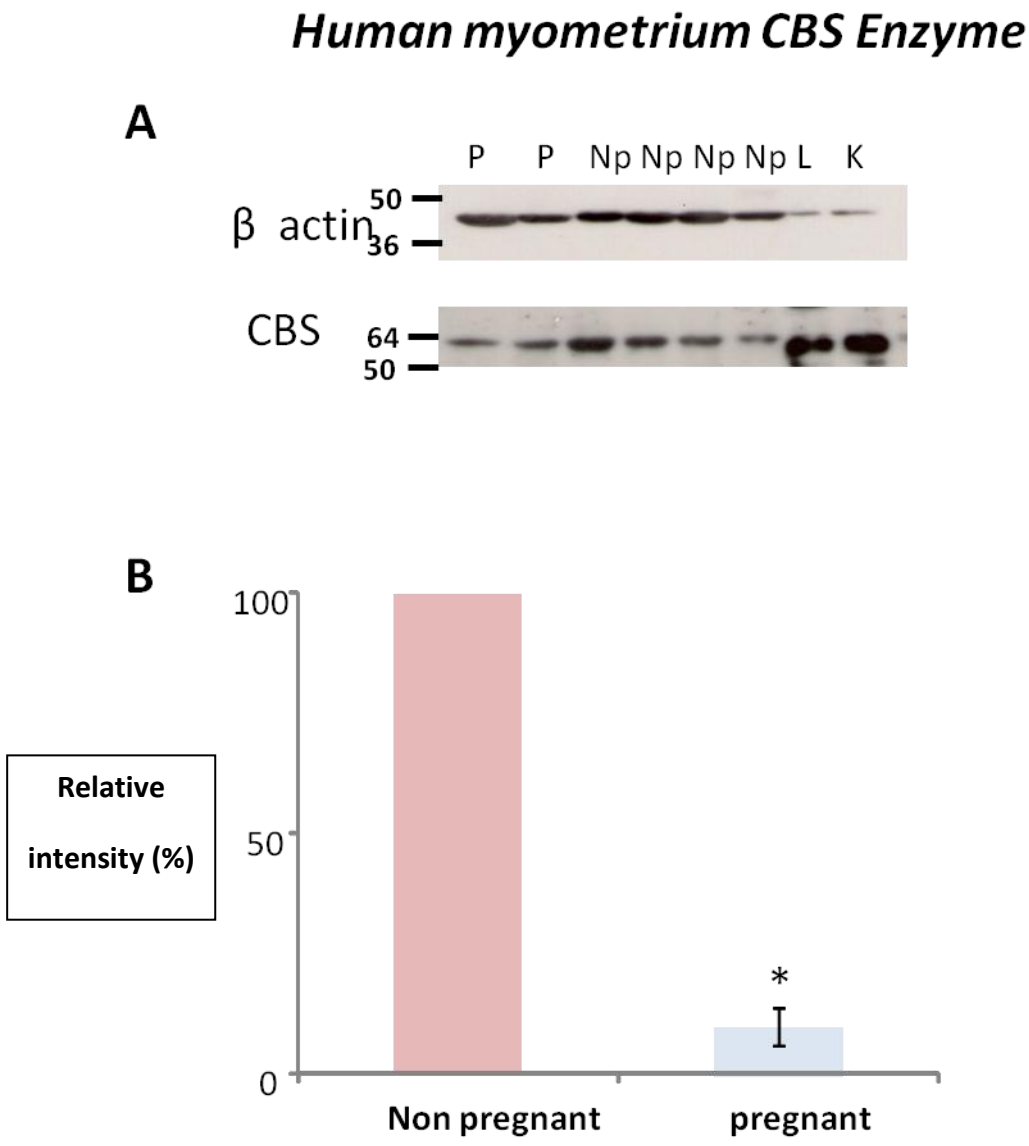


**Figure 5.27. The expression of CBS $H_2S$ production enzyme in non-pregnant and term pregnant myometrium – Western Blotting**

A) A representative image showing the results of western blot for CBS in human non-pregnant and term pregnant myometrial protein extracts. L is for liver and K is for kidney.

B) Bar chart showing the significant reduction in CBS expression in the myometrium of term pregnant women.

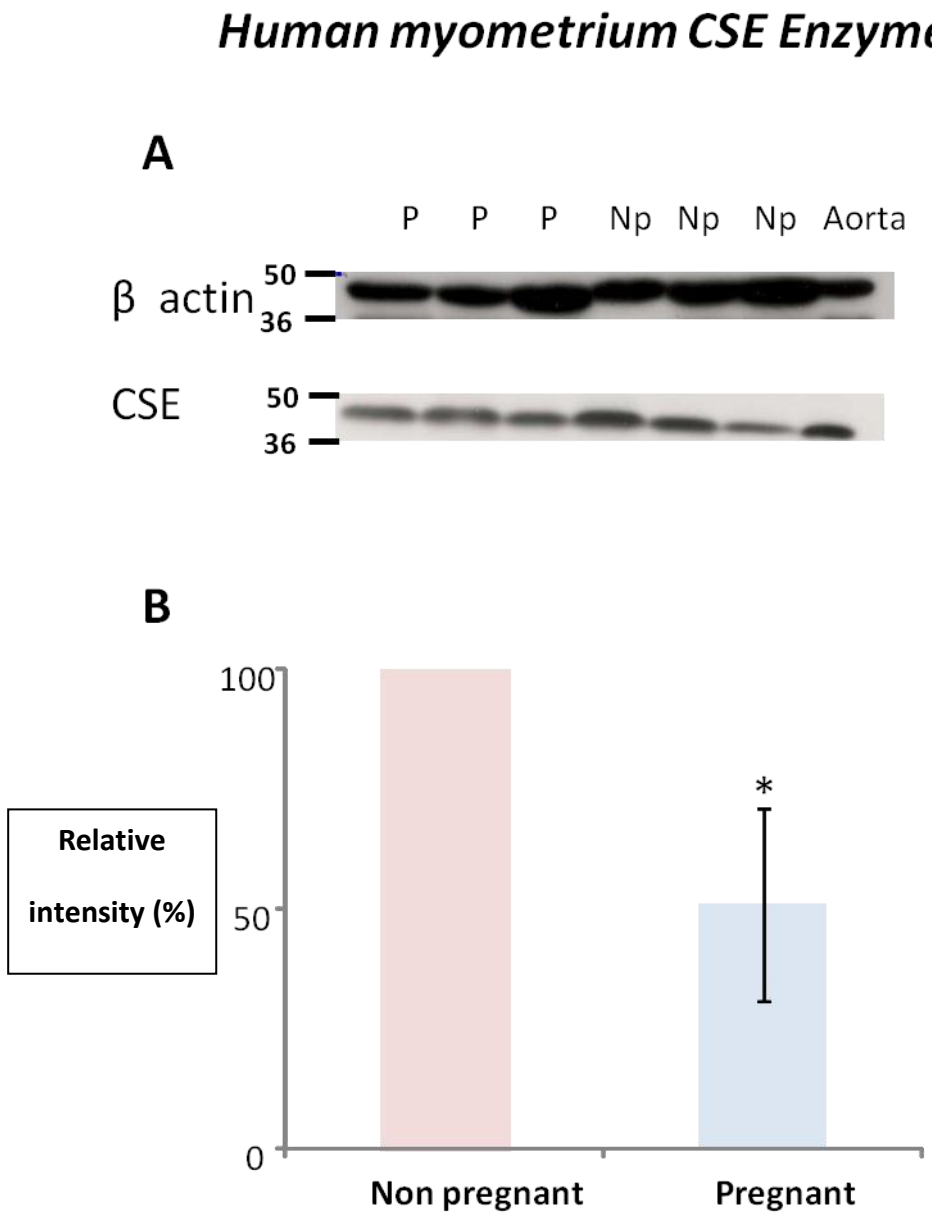


**Figure 5.28. The expression of CSE $H_2S$ production enzyme in non-pregnant and term pregnant myometrium – Western Blotting**

A) A representative image showing the results of western blot for CSE in human non-pregnant and term pregnant myometrial protein extracts.

B) Bar chart showing the significant reduction in CSE expression in the myometrium of term pregnant women.

### 5.4.14 Expression of the $H_2S$ detoxifying enzyme – Western Blotting

Contrary to what I thought, there was a down regulation of both $H_2S$ producing enzymes at term. The next question was whether there was an upregulation in $H_2S$ breakdown in non-pregnant women, which help to reduce the effect of $H_2S$ producing compounds on contractility? TST has been implicated in $H_2S$ breakdown. The quantification of TST was performed in non-pregnant and term pregnant myometrium. Western blotting was carried out on tissue from 5 non-pregnant and 5 term pregnant women and the expression of the TST isoform of Rhodanese measured. A representative image of 5 repeats of Western blots is shown in **figure 5.29A** for its presence in non-pregnant human myometrium and **figure 5.29B** for its presence in the term pregnant human myometrium. No detectable expression of this enzyme was present in the human myometrium of either non-pregnant or term pregnant women, however its presence was strong in the rat liver and kidney positive controls. The presence in the non-pregnant, 14 day, 18 day, and 22 day rat myometrium also showed no presence of TST (**Figure 5.29C**).

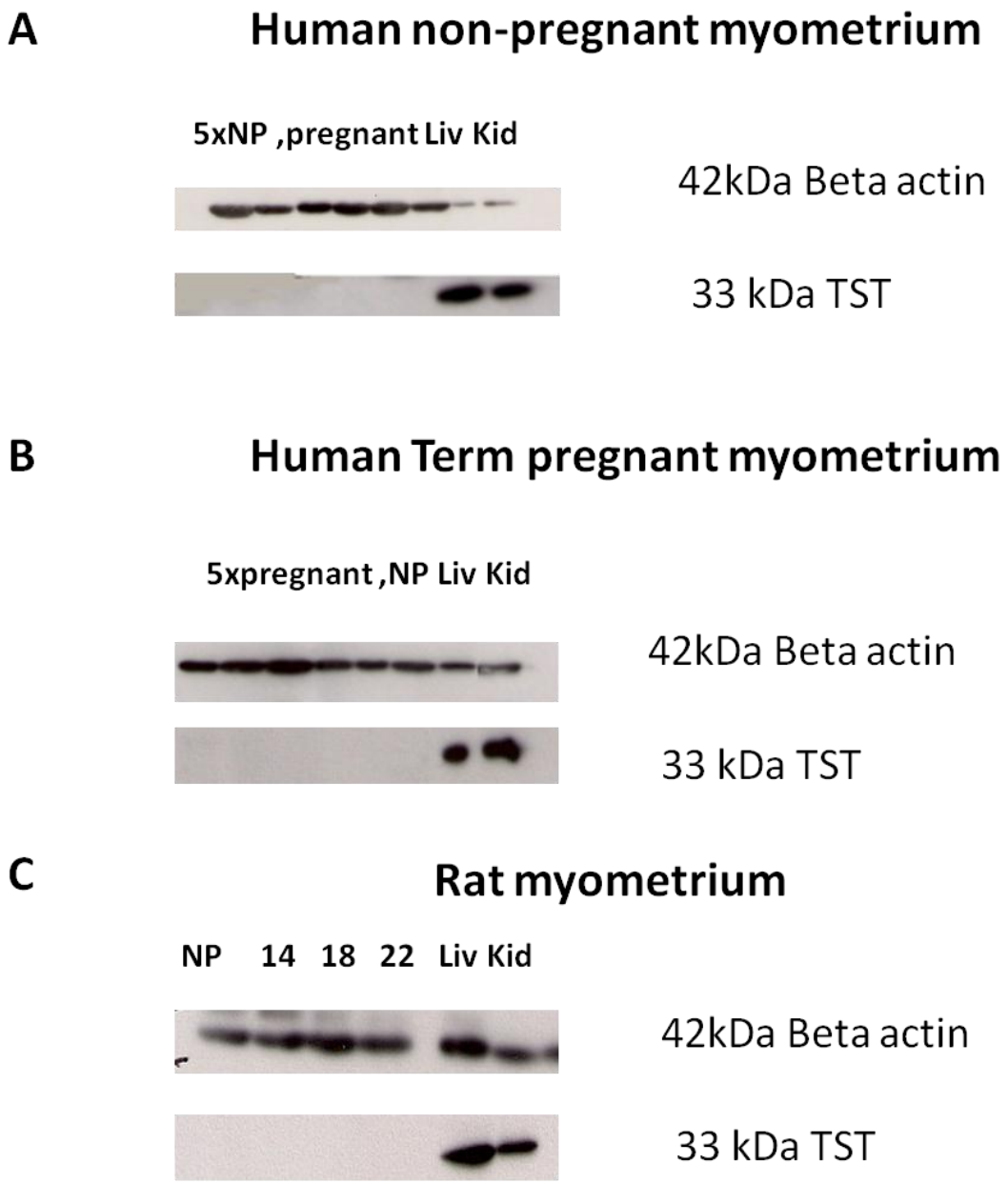


**Figure 5.29. The expression of TST, $H_2S$ detoxifying enzyme in non-pregnant, term pregnant human myometrium and rat myometrium – Western Blotting**

A representative image showing the results of western blot for TST in A) human non-pregnant, B) Human term pregnant and C) rat myometrial protein extracts.

## 5.5 Discussion

This is the first study to investigate the effect of L-cysteine within the term pregnant rat and non-pregnant myometrium as well as to simultaneously measure force-calcium and underlying intracellular pH changes in response to L-cysteine. This chapter is also the first time the response to garlic has been looked at in the myometrium. The presence of $H_2S$ producing enzymes in non-pregnant myometrium as well as in the myometrium of different stage rat gestations have never been examined. Neither has the $H_2S$ detoxifying enzyme isoform TST been monitored in the myometrium.

### 5.5.1 The effect of L-cysteine on myometrial contractility

This study has shown that L-cysteine, a precursor of $H_2S$ produces uterine relaxation in term pregnant rat and human myometrium although effects were not as pronounced as those of GYY4137 or NaHS. The decrease in contractility appeared to be dose dependent and was more pronounced in the human myometrium. At the highest concentration of L-cysteine 1mM there was a significant decrease in the force amplitude of myometrial contractions relative to control period. Similar relaxant effects have been reported in vascular smooth muscle (Hosoki 1997, Teague 2002, Zhao 2009, Ohia 2010, d'Emmanuele di Villa Bianca 2009). A single study within the rat myometrium has presented similar effects in 19 day gestation rats (sidhu 2001). The data inside this chapter was performed on 22 day gestation pregnant rat (term) myometrium, along with this relaxation an increase in frequency and overall AUC was found. This response was established also in the term pregnant human myometrium. The only other study within human myometrium by You et al used cumulative administration of L-cysteine ($10^{-7}$–$10^{-2}$ mol/L) and found a dose-dependent decrease in the amplitude of spontaneous contractions in non labouring and labouring myometrial strips. In addition to L-cysteine at high concentration ($10^{-3}$ mol/L), increased frequency of spontaneous contractions and sometimes a tonic contraction were also found (You 2011), in support of the results within the rat and human myometrial data demonstrated in this chapter. Other research on neonatal rat bladders illustrated this facilitation of contractions was also produced in the

presence of L-cysteine. Its effects were reduced by the use of L-type calcium channel blocking agent nifedipine and a calcium activated $K^+$ channel opener (Buyu knacar,2010).

In the non-pregnant uterus it was found that the application of L-cysteine showed similar excitation without the pronounced decrease in force amplitude, implying once again that the non-pregnant tissue seems more resilient to the diminished force caused by $H_2S$. This maybe similar to the effect found when using NO donors, where NO production was found to be lower in non-pregnant myometrium possibly through some involvement of oestrogens (Kakui 2004). Also within NO research, as with $H_2S$, CBS and CSE produces $H_2S$ from L-cysteine, so arginase metabolizes L-arginine. NO production depends on the availability of L-arginine to NOS. It was found L-arginine decreased during $7^{th}$-$21^{st}$ day of gestation. However, the enzyme activity became significantly higher at term gestation (22 day) than that in the non-pregnant myometrium (Hirata 2006). Both factors may play a part in the reason for the difference in responses found to L-cysteine in non-pregnant and term pregnant myometrium.

### 5.5.2 The mechanism of L-cysteine's effects in the myometrium

One potential mechanism of action is that $H_2S$ is produced from L-cysteine endogenously in uterine tissues, which relaxes the smooth muscle. This is a biochemical pathway documented (Abe 1996, stipanuk 1982) and involves two main enzymes, CSE and CBS. These enzymes have been shown to be present in rat myometrium by a functional study (Sidhu 2001) and human myometrium (Patel 2009) and quantified in non-labouring and labouring human myometrium (You 2011) although within the non-pregnant versus the term pregnant myometrium, each enzyme's presence has not been quantified, as discussed below. Intracellular L-cysteine concentrations are reported to be 30-200μM (Griffith 1987) with tissue levels as 10-100μM (cooper 1983). This illustrates that concentrations used for dose responses are within an adequate range.

L-cysteine reduced contractions via decreased Ca transient amplitude, suggesting that it could affect L-type Ca channels, possibly by sulfhydration as is proposed to happen when $K_{ATP}$ channels are affected within vascular smooth muscle (Tang 2010) and L-type calcium channels in cardiomyocytes (Zhang 2012). BayK 8644 data also shows a reduction in the

response to L-cysteine, pointing towards L-type calcium channels being targeted. No differences in the response to L-cysteine were found in high $K^+$ suggesting membrane potential is affected. Intracellular calcium leading to contraction is controlled by a multitude of mechanisms beyond the classic entry through voltage operated calcium channels, calmodulin binding pathway. Alterations too many of the ion channels in the myometrium can have a profound effect on contractility.

Researchers working on vascular smooth muscle have shown $K_{ATP}$ channels are directly involved in the mechanism of $H_2S$ by increasing their open probability, most likely through S-sulfhydration of specific cysteine residues of the $K_{ATP}$ channel, promoting outward $K^+$ flow and hyperpolarization, to cause a decrease in contractility (Zhao 2001, Wang 2012). This conclusion is largely based on the ability of glibenclamide, a $K_{ATP}$ channel antagonist, to block the vasorelaxant effects of $H_2S$ (Zhao 2001, Cheng 2004), in addition to electrophysiological experiments providing direct evidence that exogenous $H_2S$ increases $K_{ATP}$ currents, blocked by glibenclamide in isolated rat aortic and mesenteric SMCs (Cheng 2004, Zhao 2001). $K_{ATP}$ channels have been suggested as one of the targets of $H_2S$ that lead to reduced myometrial contractility using glibenclamide (Hu 2011). In contrast, other studies showed that the $K_{ATP}$ channels are not involved in $H_2S$ relaxation in smooth muscle tissues including vascular, bronchial, and gastrointestinal smooth muscle (Kubo 2007, Teague 2002, Lee 2007, Kiss 2008). Our data with glibenclamide in **Chapter 4** would support a main role for these channels in the mechanism of $H_2S$ effects in the myometrium. This decrease in rat myometrial contractility could be inhibited by both CSE and CBS inhibitors, suggesting that endogenous $H_2S$ generated by CBS and CSE can modulate the contractility of human myometrium during pregnancy as found in non-labouring and labouring human myometrium (You 2011).

Hydrogen ions are known to interfere with uterine smooth muscle contraction and both extracellular and intracellular pH changes have been found to effect contractility (Parrat 1995, Taggart 1997). All solutions were pH readjusted to 7.4, so a change in extracellular pH could not explain the relaxant effect of L-cysteine in the myometrium. D-Cysteine was found to have no effect on contractility, which suggests that the relaxation was not produced by a simple chemical effect. Although

application of by products of $H_2S$ production through use of L-cysteine by CSE and CBS, L-serine and sodium pyruvate, caused initial excitation with significant increase in force amplitude upon application of sodium pyruvate. This led to the testing of Intracellular pH upon L-cysteine exposure. A decrease in intracellular pH was illustrated. The initial changes in frequency and amplitude of contractions upon application of L-cysteine coincided with the period when pH was seen to change. This rapid increase in $[H^+]_i$ may lead to this initial stimulation by displacing $Ca^{2+}$ from intracellular binding sites as protons and $Ca^{2+}$ are known to compete for intracellular binding sites (Wray, 1998). In contrast decreased intracellular pH using NaHS within rat aortic smooth muscle was related to the relaxation monitored (Lee 2007). This is the first study that has shown the effects of cysteine are seen even though there is an intracellular acidification and that an alkalinisation cannot explain the effects of cysteine.

It is possible that NaHS, GYY4137 and L-cysteine have quite different mechanisms of action and therefore different potencies. L-cysteine could act as an agonist at amino acid receptors, as this is usually excitory, this could be also a target to produce the stimulation upon incubation of myometrial tissue in L-cysteine. This is something that could be further investigated. L-cysteine is thought to achieve its effect on the cell through the help of an excitory amino acid transporter subtype and possibly by a zwitter ion amino acid transporter subtype (EAAT3)(Zerangue, 1996) and possibly by a zwitterionic amino acid transporter (ASCT1)(Palacin 1998). Both of these transporters are widely distributed in tissues. Once inside the cell as $H_2S$ could be released enzymatically from L-cysteine and therefore L-cysteine could act as a direct intracellular donor $H_2S$ or HS-. Normally L-cysteine is transported into cells as cystine, which then splits into two molecules of cysteine, but it is possible that L-cysteine could be transported directly when applied extracellularly (Sidhu 2001).

### 5.5.3 Effects of garlic

$H_2S$ is produced naturally by garlic as well as from L-cysteine within human tissue. Here I found that the myometrial contractility changes of garlic were comparable to L-cysteine with initial stimulation in contractility, which then results in decreased

amplitude of contractions (something needed to be further investigated, shown in **figure 5.20 A**). Further work on Garlic power needs to be performed to investigate its effects. Although garlic oil produced through crushing garlic would be better to use as Allicin (diallyl thiosulfinate), the main organosulfur compound, is produced from the amino acid alliin by action of the enzyme alliinase when garlic is crushed. Allicin, unstable in aqueous solution, rapidly decomposes mainly to diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and ajoene (Amagase 2006). It would also be of interest to look at active garlic derived polysulfide compounds, which are converted to $H_2S$. This could help find a naturally occurring $H_2S$ compound to reduce contractility allowing the monitoring of different polysulfides active in garlic. Within vascular smooth muscle different polysulfides have been looked at and $H_2S$ production amounts monitored, DATS and DADS produce the greatest amount of $H_2S$ and cause the greatest relaxation in aorta (Benavides 2007), good candidates to further research garlic's use on the uterus.

Preliminary investigations show 0.714mg/ml and above concentrations of garlic show similar responses to L-cysteine with increased contractility although no significant changes in amplitude of contraction was found with low n numbers. The frequency of myometrial contractions is directly related to changes in membrane potential and the frequency of burst or the increased frequency of contraction may also be attributed to faster repolarisation or recovery of the membrane to resting potential. Further investigation is needed to examine these effects and mechanism found by the use of L-cysteine and garlic. Electrophysiological techniques such as voltage clamp could be used to measure the membrane potential of the smooth muscle cells in the presence and absence of L-cysteine and garlic to examine their effect on membrane potential. Overall in this chapter we find differences and similarities in myometrial contractility changes in response to L-cysteine when compared to the response found through use of $H_2S$ producers NaHS and GYY4137. It would also be of interest to monitor $H_2S$ production using a polarographic electrode (Olson 2012), to compare the $H_2S$ produced by NaHS, GYY4137, L-cysteine and garlic and to assess whether the decrease in contractility was directly related to $H_2S$ production or an unidentified sulfide moiety.

### 5.5.4 Regulation of $H_2S$ production and breakdown in the myometrium.

In the myometrium, Patel et al found the presence of both $H_2S$ producing enzymes in rat non-pregnant and 19 day gestation uterus as well as human term pregnant myometrium. You et al quantified the presence of both enzymes in term non labouring and labouring human myometrium at the protein and mRNA level as well as looking for their expression in sections, finding both present and during labour $H_2S$ enzymes are down-regulated. No work has been undertaken to look at the expression of both enzymes within different stage gestation rat myometrium or looked at the presence in non-pregnant human versus term pregnant myometrium.

This chapter showed the expression of both the CSE and CBS enzyme in non-pregnant, 14 day, 18 day and term pregnant rat myometrium as well as non-pregnant and term pregnant human myometrium. This showed that the myometrial tissues assessed are able to produce $H_2S$ endogenously. Their presence was further defined through quantification of CBS and CSE in non-pregnant and term pregnant human myometrium via Western blotting. It was found that the presence of both enzymes was significantly decreased in the term pregnant myometrium, perhaps in preparation for the onset of labour as the enzymes further decline upon the onset of labour (You et al), allowing the production of $H_2S$ to no longer keep the powerful contractions at bay to cause labour.

As I have found that $H_2S$ producers have more of an effect at term the question was, could the breakdown of $H_2S$ be higher at term when compared to non-pregnant human myometrium as clearly there are the tools to have a greater production of $H_2S$ in the non-pregnant myometrium although we see the effect at term. TST is the isoform of rhodanese implicated in detoxifying $H_2S$ in human colon (Ramasamy 2006), however its presence has not been investigated in the uterus. I have shown clearly that in human non-pregnant and term pregnant human myometrium in addition to non-pregnant, 14 day, 18 day, term rat myometrium that it was not detectable and is not likely to be involved in $H_2S$ breakdown in the myometrium. This is not to say that the increased effects seen upon application of NaHS, GYY4137 and L-cysteine throughout gestation are not due to $H_2S$ removal rates as other enzymes may exist. Recently, mice lacking ethylmalonic encephalopathy 1 (Ethe1) exhibit

elevated sulphide levels, suggesting that Ethe1 is the sulphur dioxygenase involved in $H_2S$ metabolism (Linden 2012). Also could this affect be due to the up regulation of a SH- channel at term? Recent work identified a channel permeable to HS− anions in the bacterium *Clostridium difficile*, although there is a long way to go and much research to be performed to elucidate the mechanism of $H_2S$ although perhaps such channels/ receptors are present in mammalian tissues (Czyzewski 2012).

### 5.5.5 Conclusions

L-cysteine causes an initial dose dependent increase in frequency and a decrease in amplitude of myometrial contractility in both term pregnant rat and human myometrium. The effect on frequency could not be explained by intracellular alkalinisation, as pH falls when L-cysteine is applied. The effects of L-cysteine are not as prominent as those upon incubation in GYY4137 or NaHS. The mechanism of the initial enhanced contraction frequency remains to be elucidated. The mechanism of L-cysteine inhibitory action on myometrial contractions is suggested to be due to corresponding decreases in the calcium transients, probably due to $H_2S$ production; CSE and CBS inhibition with PAG and AOAA prevented this decrease in contraction. In the human term pregnant myometrium the dose response of L-cysteine was more apparent than in rats with a log $EC_{50}$ value approximately doubled, similar to the differences in NaHS and GYY4137 responses in non-pregnant and term pregnant tissues, reduced inhibitory effects of L-cysteine were found when applied to non-pregnant myometrium. Preliminary investigations show 0.714mg/ml and above concentrations of garlic show similar responses to L-cysteine with increased contractility although no significant changes in amplitude of contraction was found due to low n numbers.

This chapter has also highlighted that both $H_2S$ enzymes are present within all the myometrial tissues tested within this thesis and that there is a down regulation of both enzymes at term. Also no presence of the $H_2S$ detoxifying isoform of rhodanese, TST, was found in rat or human myometrium.

### 5.5.6 Limitations of study

- Low n numbers for sodium pyruvate, L-serine, Garlic powder work. More n numbers are required

- Results using CBS and CSE inhibitors are limited due to their poor selectivity.

- Garlic oil is more active and contains a greater content of polysulfides to produce H2S due to crushing garlic rather than drying in the case of garlic powder. Future work should use garlic oil.

# Chapter 6- Final Discussion

The aims of the work described within this thesis were firstly to characterise the non-pregnant human myometrial tissue samples to see how contractility changes with age, menopausal status or with the condition endometriosis, and then to examine $H_2S$ and its effects on myometrial contractility of human and rat tissue, using three $H_2S$ producing compounds, NaHS, GYY4137 and L-cysteine. In addition to investigating the mechanism by which $H_2S$ reduces contractility I also investigated any differences in response to $H_2S$ over gestation and thus its potential involvement in quiescence. The presence of $H_2S$ producing enzymes CBS and CSE were also explored to confirm that $H_2S$ could be produced within the myometrial tissues studied. My overall aim is to gain a better understanding of the role of $H_2S$ in the myometrium and to investigate its potential as a preterm preventative. I consider I have addressed and achieved many of my specific aims and that the work presented here has built on previous studies and highlighted the physiological relevance of $H_2S$ in the myometrium and its effects throughout gestation as well as in human myometrium.

### 6.1 Characterisation of non-pregnant myometrium

Myometrial contractility from pre-, and post-menopausal women as well as from women with endometriosis were compared. The effect of advancing age on the non-pregnant myometrium was also addressed. The data demonstrated clear effects of age and menopausal state upon myometrial contractility. It shows that aging significantly decreases uterine contractility as does progressing menopausal state, which is not surprising as there is a strong relationship between the two. Endometriosis also shows an increase in frequency of contractions and a non-significant but consistent decrease in the other parameters of contractility. Consistent with an earlier *in vitro* study which had found spontaneous activity in post-menopausal women, and earlier *in vivo* studies showing more frequent contractions within myometrium with endometriosis (Domali 2001, Bulletti 2002), these changes in the myometrial contractions of women with endometriosis could have a causal role in the genesis and continuation of the disease.

The reasons behind this impaired contractility may be related to alterations in calcium signalling in the post menopausal women and women with endometriosis. Reduced calcium transients are indicative of less calcium entry through L-type voltage calcium channels or perhaps alterations in the expression of L-type calcium channels. This is supported in the high K data within **chapter 3**, where High $K^+$ contractions were reduced in the post menopausal women as well as a trend to decrease in women with endometriosis. Reduced muscle content could also be contributing to the reduced contractility, especially with advancing age. Further work needs to be performed to investigate this.

Overall, contractions of the non-pregnant human myometrium were stable allowing for investigation of the effects of $H_2S$ compared with the term pregnant human myometrium. Therefore pre-menopausal women were used as the non-pregnant myometrium in chapters **4 and 5**.

### 6.2 The effect of $H_2S$ producers NaHS and GYY4137 on myometrial contractility

The effects in rat and human myometrium were studied and I found that: i) GYY4137 causes a concentration-dependent reduction in contractility of myometrium, ii) The ability of $H_2S$ to inhibit contraction is not constant but rather is greatest close to term before disappearing during labour iii) GYY4137 and NaHS significantly reduced contractility in pregnant but not non-pregnant human myometrium, iv) $H_2S$ significantly reduced tonic force produced by high-K depolarization and oxytocin-stimulated contractions, and v) GYY4137 reduced the intracellular Ca transients underlying contractions and inhibition of $K_{ATP}$ channels prevented the effects of GYY4137. A recent study supported the reduced effects of $H_2S$ upon labour in the human labouring myometrium in response to L-cysteine (You 2011). Other research also found NaHS reduced contractility of human term labouring myometrium and that the effects found were abolished in the presence of glibenclamide supporting the data found in this thesis at term (Hu 2011). No significant effects on contractions in the non-pregnant tissue to addition of either $H_2S$ source were found, but clear effects were seen by mid-gestation. The inhibitory effect on contraction then further increased until term. These data suggested that the relaxant effects of $H_2S$ were increased as pregnancy

advances. The most striking effect however was the abrupt transition upon labour; $H_2S$ was without effect as seen by the data for both compounds. This suggests that $H_2S$ contributes to uterine quiescence in late pregnancy and that the myometrium can rapidly change its responses to $H_2S$, perhaps through changes in $H_2S$ removal rates or channel susceptibility to sulfhydration.

Studies were limited by the lack of a compound to better mimic the endogenous release of $H_2S$ in cells. The commonly used NaHS or $Na_2S$ release $H_2S$ instantaneously in aqueous solutions, producing very large and transient increases in its concentration. GYY4137's potential as a slow-releasing $H_2S$ compound with effects on vascular smooth muscle *in vitro* and *in vivo* were shown, its time scale of $H_2S$ production measured and its lack of toxicity to aortic cells shown (Li 2008). Subsequent work has supported low toxicity (Yu 2010) and also indicated anti-cancer properties (Lee 2011), anti inflammatory activity (Li 2009), and anti apoptotic (Lavu 2011) activity of GYY4137. Thus GYY4137 or subsequent compounds, (Predmore 2012) may well be suitable for a variety of patho-physiological conditions, including tocolysis in threatened preterm labour, i.e. to stop the onset of labour, although further work, including studies on labouring samples, are needed to develop this suggestion.

Recently, $H_2S$ releasing drugs are an active area of research as these molecules have such benefits as suppressing breast cancer cell support for osteoclastogenesis and prevent osteolysis (Frantzias 2011), prevention of smooth muscle cell proliferation in diseases such as atherosclerosis and vascular restenosis (Baskar 2008) to anti inflammatory and anti cancer properties (Kodela 2012, Chattopadhyay 2012). Other drugs investigated include naturally occurring $H_2S$ producing agents such as DATS produced by garlic, which readily vasodilate rat aortas (Benavides 2007) and Sulforaphane, the isothiocyanate compound from broccoli, which has shown neuroprotective and anti inflammatory actions (Jackson 2007, Shan 2010, Zhu 2008). I speculate there will be further research and more slow releasing $H_2S$ molecules to come to help benefit therapeutically and give insight into the physiological roles of $H_2S$. Interestingly, sulfide sensitive dyes similar to the well known ion-sensitive dyes such as the calcium reporter Indo-1 are currently being developed as to date they lack sensitivity, these molecules would resolve many of

the key questions regarding $H_2S$ signalling mechanisms (Lippert 2011, Qian 2011, Olson 2012), this further demonstrates $H_2S$ and its releasing compounds are an active area of research.

My data and that of previous studies suggest $H_2S$ could contribute to uterine quiescence and that increasing its level in myometrium could be an attractive target for therapeutics to inhibit the onset of labour. Increased understanding of the mechanisms for transition to labour should also follow from obtaining a better understanding of $H_2S$ in the myometrium.

### 6.3 The effect of $H_2S$ producing enzyme substrate, L-cysteine

L-cysteine at 1 mM, caused an initial increase in the frequency of uterine contractions, but a decrease in amplitude of myometrial contractility in both term pregnant rat and human myometrium. The effect on frequency could not be explained by intracellular alkalinisation, as pH falls when L-cysteine is applied. The effects of L-cysteine are not as prominent as those upon incubation in GYY4137 or NaHS. The mechanism of the initial enhanced contraction frequency remains to be elucidated. The mechanism of L-cysteine inhibitory action on myometrial contractions is suggested to be due to corresponding decreases in the calcium transients, probably due to $H_2S$ production; CSE and CBS inhibition with PAG and AOAA prevented this decrease in contraction. In the human term pregnant myometrium the dose response of L-cysteine was more apparent than in rats with a log $EC_{50}$ value approximately doubled. Similar to the differences in NaHS and GYY4137 responses in non-pregnant and term pregnant tissues, reduced inhibitory effects of L-cysteine were found when applied to non-pregnant myometrium.

### 6.4 Mechanism of $H_2S$ in the myometrium

There are many suggestions for the mechanism by which $H_2S$ exerts its effects and it is likely that there will be many targets (Li 2011). The main mechanism appears to be due to $H_2S$ modifying cysteine residues in many proteins through S-sulfhydration (Mustafa 2011) i.e. cysteine's covalent modification by which -SH groups on cysteine residues of a protein are converted to –S-SH, via addition of sulphur from $H_2S$

(Mustafa 2009). The data in this thesis using DTT, a reductant sulfhydryl modifier and Diamide, an oxidant sulfhydryl modifier supported this showing that firstly free sulfhydryl groups have no effect on contractility and that disulphide bonds cause a similar effect to NaHS and GYY4137. Although it would be interesting to see whether using DTT could reverse GYY4137 and NaHS effects on force and calcium as in Zhang et al 2012 where it could markedly reverse the $H_2S$ donor-induced inhibition of $I_{Ca,\ L}$ (L-type calcium current) in cardiomyocytes. Illustrating that if $H_2S$ targets the crucial free-sulfhydryl groups on the L-type $Ca^{2+}$ channel and inhibits the L-type calcium current, the inter-chain disulfide bond linkages would be rapidly reduced by DTT, and therefore the inhibition would be reversed (Zhang 2012).

The most documented effect of $H_2S$ is on $K_{ATP}$ channels within smooth muscle. In vascular smooth muscle cells $H_2S$ stimulated single-channel activity of $K_{ATP}$ channels by directly increasing their opening probability (Wang 2012). Recent work has made progress in identifying which residues in the channel are affected by $H_2S$, with Cys 6 and 26 on the extracellular N terminal of the SUR1 subunit of the channel being identified (Jiang 2010). $K_{ATP}$ channels have been suggested as one of the targets of $H_2S$ that lead to reduced myometrial contractility (Hu 2011). The data with glibenclamide would support a role for these channels in the mechanism of $H_2S$ in the myometrium, as GYY4137 had no significant effect on myometrial contractility when $K_{ATP}$ channels were blocked with glibenclamide.

The data within this thesis also suggested that L-type $Ca^{2+}$ entry was reduced by $H_2S$ sources. Reduction of $Ca^{2+}$ by $H_2S$ has previously been demonstrated in non-contractile arterial segments (Tian 2012) but had yet to be shown in the myometrium. My simultaneous measurements of intracellular $Ca^{2+}$ and contractions show a $H_2S$-dependent reduction in intracellular $Ca^{2+}$ accompanies the decrease in amplitude of the phasic contractions. Also when BayK 8644, a calcium channel opener is used in the presence of L-cysteine to produce $H_2S$, reduces the reduction in contractility also pointing towards L-type calcium channels being targeted. L-type $Ca^{2+}$ channels have also been shown to contribute to $H_2S$ effects, possibly by sulfhydration as proposed to happen when $K_{ATP}$ channels are affected within vascular smooth muscle (Tang 2010) and L-type calcium channels in cardiomyocytes (Zhang 2012). There is now

mounting evidence that the L-type $Ca^{2+}$ channels themselves are targets of $H_2S$. Sun et al, (Sun 2008) in cardiac myocytes were the first to show that $H_2S$ can inhibit L-type $Ca^{2+}$ channels. Recently others have shown inhibition of these channels by NaHS also occurs in vascular smooth muscle (Tian 2012, Al-Magableh 2011) and Zhang et al (Zhang 2012) have gone on to show that this is dependent upon the protein sulfhydryl state of the channel.

The above gives insight into the mechanism of action of $H_2S$ but does not explain the reasons for susceptibility changes over gestation. My suggestions include (i) increased vulnerability to sulfhydration of L-type calcium channels as L-type calcium channel subunits increase toward term (Collins 2000); (ii) reduction in kir6.1 and 6.2 $K_{ATP}$ subunits once myometrium is labouring, as before labour $H_2S$ exerts its effects on these subunits (Xu 2011); (iii) up regulation of the $H_2S$ breakdown enzymes with gestation, or (iv) changes in uterine environment with labour, such as hypoxia an pH changes, (Quenby 2004, Wray 1992) may result in faster breakdown of $H_2S$, but this remains controversial, (Olson 2012, Doeller 2005). The lack of specific inhibitors of these enzymes and the difficulty of accurately measuring $H_2S$ in tissues, hinders further study of these last two points.

### 6.5 Expression of $H_2S$ production enzymes in the myometrium

Within this thesis $H_2S$ producing enzymes CBS and CSE were expressed in non-pregnant, 10 day, 14 day, 18 day, and term pregnant rat myometrium as well as in non-pregnant and term pregnant myometrium. This showed that $H_2S$ can be produced endogenously within the tissues assessed. Both enzymes have already been shown to be expressed in term non-labouring and labouring human myometrium in addition to 19 day gestation rat myometrium (Patel 2009, You 2011).

Here we have also shown through quantification of the CBS and CSE enzymes that there is a down regulation of both enzymes within the term pregnant human myometrial tissue when compared to the non-pregnant human myometrium. This could be in preparation for the onset of labour as the enzymes are further declined upon the onset of labour (You et al), allowing the production of $H_2S$ to no longer keep the powerful contractions at bay to cause labour. In addition $H_2S$ production is

also found to be lower in labouring compared to non-labouring human myometrium (You 2011).

### 6.6 Expression of $H_2S$ detoxifying enzyme TST

TST is the isoform of rhodanese implicated in detoxifying $H_2S$ in human colon (Ramfrez 2004), however its presence has not been investigated in the uterus. I have shown clearly using Western blotting that in human non-pregnant and term pregnant human myometrium in addition to non-pregnant, 14 day, 18 day, term rat myometrium that it is not detectable, this is supported by TST being present in positive controls. A Western blot using increasing quantities of myometrial protein up to 100µg was also performed further demonstrating no detectable presence of the enzyme TST (not shown). This makes it highly likely that TST is not involved in $H_2S$ breakdown in the myometrium although this is not to say other $H_2S$ breakdown enzymes such as sulfur dioxygenase enzymes may be present.

I found in this thesis that there was greater inhibition of contractility at term in the presence of $H_2S$ producers, but that $H_2S$ producing enzymes are up regulated in the non-pregnant myometrium compared to the term myometrium. Thus the non-pregnant uterus has the potential to produce $H_2S$, but it does not affect contractility, at least under my experimental conditions. The question was, could the resistance against $H_2S$ effects be due to an up-regulation in $H_2S$ removal rates? However as my data on TST were negative, it was not possible to further test this suggestion. Recently, mice lacking ethylmalonic encephalopathy 1 (Ethe1) exhibit elevated sulphide levels, suggesting that Ethe1 is the sulphur dioxygenase involved in $H_2S$ metabolism (Linden 2012), this enzymes presence in the myometrium could be investigated.

### 6.7 Future work

There are several subsequent studies that could be pursued following my work:

The finding that post menopausal and old age cause reduced myometrial contractility could be further explored by examining the potential mechanisms that could be affected. Through monitoring changes in underlying calcium transients

using simultaneous force and calcium measurements using calcium sensitive indicator Indo-1. It would also be of interest to monitor changes in the expression of L-type calcium channel to see if there is a down regulation in the post menopausal and older age group. A reduction in L-type calcium channel expression has been shown to be linked with the decreased force produced in the diabetic myometrium (Al-Qahtani 2011). In the laboratory reduced muscle content in diabetic women was also found, a potential factor that could ultimately contribute to the poor contractility found in **chapter 3**, especially with advancing age. Also working on further endometriosis samples would define the contractility better especially increasing the n numbers in the 'no medication' group. I could also test the hypothesis that ageing myometrium can respond to hormonal treatment and retains the ability to increase its strength. It would be interesting to look at the effect of different medications the non-pregnant women were on and compare contractility differences also once n numbers are increased. Likewise a comparion could then be made between Progestagen treated non- pregnant myometrium and pregnant myometrium to see possible similarities.

It would also be worth monitoring the changes in $H_2S$ production over gestation and looking at the difference in $H_2S$ production from the $H_2S$ producers NaHS, GYY4137 and L-cysteine used within this thesis. Using either analysis of $H_2S$ evolution into headspace gas and subsequent gas chromatography which has been used to measure both tissue and plasma $H_2S$ in the nanomolar range (Furne 2008, Levitt 2011) or the well-known method of measuring thiols by derivatization with excess monobromobimane (MBB) and subsequent measurement of the stable sulfide-diamine product with reverse phase high pressure liquid chromatography (HPLC) coupled with fluorescence detection, recently used to measure plasma $H_2S$ levels at the nanomolar range (Shen 2011, Wintner 2010). A good review of the $H_2S$ monitoring techniques was published by Olson 2012 concluding that there appears to be a lack of sensitivity to measure endogenous $H_2S$ at submicromolar levels and in real-time. This will give insight into $H_2S$ and its production throughout pregnancy and maybe confirming its involvement in quiescence. It would also be important to look at the effect of NaHS and GYY4137 on human labouring tissue to see whether the

effect seen in the rat can be related to the human. As well as investigating the effects of both $H_2S$ producers invivo and on the fetus using the rat model to help understand more clearly their side effects and potential use in pregnancy.

L-type calcium channels have been implicated in the mechanism of $H_2S$ within this thesis. In non-contracting (butanedione monoxime treated) cerebral artery, Tian et al, used fluo-4 and showed decreases in $Ca^{2+}$ levels as NaHS was increased from 0.1 to 1 mM, and suggested that NaHS relaxes these vessels by reducing L-type $Ca^{2+}$ current. A recent study using cardio myocytes suggested, $H_2S$ inhibits L-type [$Ca^{2+}$] channels through sulfhydration, as NaHS decreased the functional free sulfhydryl groups in the channels (Zhang 2012). To further demonstrate the effect of $H_2S$ on L-type calcium channels in the myometrium, I could monitor the function free sulfhydryl groups in the myometrial tissue protein extract of a control incubated and $H_2S$-producer incubated tissue then using a biotin switch assay as used in Zhang 2012 monitor any difference in functional free Sulfhydryl.

$K_{ATP}$ also play a role in the reduction in contractility of term myometrial tissue shown through use of glibenclamide a $K_{ATP}$ blocker. To confirm if $H_2S$ affects membrane potential through $K_{ATP}$ channels, patch clamp studies could be used to see how much the membrane hyperpolarises after exposure to $H_2S$ producers as examined in vascular smooth muscle (Zhao 2001).

$H_2S$ producing enzymes CBS and CSE were expressed in non-pregnant, 14 day, 18 day and 22 day (term) rat myometrium. This could be further investigated through protein quantification via use of Western blotting to see whether there are any changes in the production over gestation implicating $H_2S$ in the mechanism of quiescence.

TST the $H_2S$ detoxifying isoform of the enzyme rhodanese. This is not present within the uterus. Other breakdown mechanisms maybe involved such as ethylmalonic encephalopathy 1 (Ethe1) which is a sulphur dioxygenase enzyme involved in $H_2S$ catabolism as mice lacking Ethe1 exhibit elevated sulphide levels (Linden 2012).

Naturally producing $H_2S$ compounds may be of use therapeutically, these include garlic derived poly sulphides already implicated to reduce cardiovascular risk. Human red blood cells convert garlic-derived organic polysulfides into $H_2S$ (Benavides 2007).

Diallyl disulfide (DADS), and Diallyl trisulfide (DATS) are the highest producing $H_2S$, garlic-dervied polysulfides producing approximately 35 and 100 µM $H_2S$, respectively on the addition of 100 µM of each polysulfide (Benavides 2007). There effect on the myometrium could be of interest because if a reduction in contractility is found they could help to reduce the onset of labour within threatened pre-term births. The use of garlic oil which produces these polysulfides when crushed would a better compound to perform experiments with as garlic powder is dried rather than crushed and does not retain the activity of the polysulfides.

The investigation of intact tissue RNAi technique, reversible permeablisation to deliver SiRNA (Morgan 1982, Lesh 1995) was investigated as a potential useful technique for use in the uterus over the period of studying for my PhD. Preliminary experiments using Indo-1 pentapotassium salt (cell-impermeable calcium sensitive indicator) were performed (**Figure 6.1**).

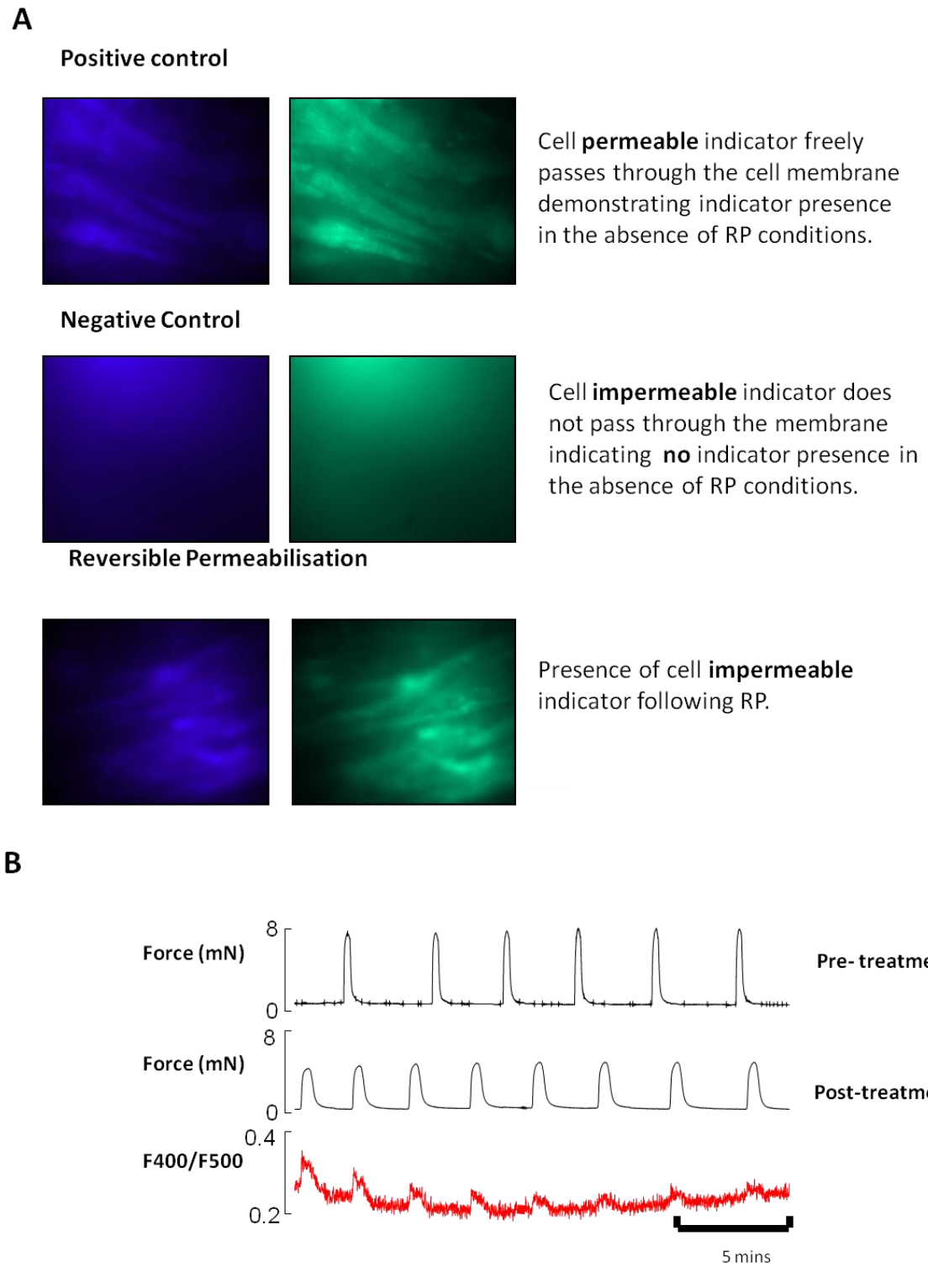


**Figure 6.1: Reversible permeabilisation of 22 day gestation rat myometrial strips.** Figure A demonstrates the presence of cell impermeable Indo-1 inside the myocytes showing successful permeabilisation. The scale bar (white) in the right hand corner of the picture corresponds to 50µm. Figure B shows the contractility before and after treatment and the indicator emission ratio confirming some cells were successfully reversibly permeabilised.

Under the reversible permeabilisation technique, signalling will only be present if the cell impermeable Indo-1 manages to enter the cell and is cleaved by esters enabling it to bind to calcium and fluoresce. One problem with examining calcium signalling of reversible permeabilised strips is that the period of time the incubations occur over allows Indo-1 to enter and escape from the permeabilised membrane. Hence the indo fluorescent signalling may not be very strong. Considering this, pregnant tissue was dissected thinly and tested for contractility. Once 20 minutes of contractions occurred, the strips were incubated firstly in a solution containing EGTA, ATP and $MgCl_2$ to permeabilise the tissue and then increasing concentration of $MgCl_2$ was used to increase the permeability of the cell membranes. Following this, cell-impermeable calcium sensitive indicator, Indo-1 was introduced to the tissue and after this the holes in the membrane were closed using increasing concentrations of calcium in the presence of NaCl. This shows antagonism exists between Mg and Ca ions and alterations of the surface layer of cells which regularly accompanies changes of irritability, initiates changes in permeability (Gellhorn 1930). The contractility of the strip after treatment was tested as well as the presence of cell impermeable Indo-1 which is illustrated in figure 6.1. This figure shows that under the conditions of the reverse permeabilisation, contractility still occurred. The contractions post treatment were more regular and smaller in amplitude (Figure 6.1A). Changes in indicator emission, which indicates the change in intracellular calcium, is shown in figure 6.1B suggesting that some smooth muscle cells were successfully loaded.

This technique could possibly be used to knockdown ethe-1 a potential $H_2S$ breakdown enzyme, if present within the uterus, to monitor whether the same responses to $H_2S$- producers are found throughout gestation and implicate a role for this enzyme in the breakdown of $H_2S$ in the myometrium.

## 6.8 Final Conclusions

The key findings demonstrated in this thesis are:

- Changes in contractility of the non-pregnant myometrium can be attributed to menopausal state, age or condition
- A progressive decline in contractility over gestation in response to $H_2S$ producers GYY4137 and NaHS up until labour onset, an effect rapidly reversed as labour starts. A decrease was also found from non-pregnant to term human myometrium.
- $H_2S$ effects involved both $K_{ATP}$ channels and L-type Ca channels.
- GYY4137 and L-cysteine reduced the intracellular Ca transients that underlie spontaneous contractions.
- $H_2S$ producing enzymes have been shown to be down regulated at term perhaps in preparation for labour onset.

Therefore my data and that of previous studies suggest $H_2S$ could contribute to uterine quiescence and that increasing its level in myometrium could be an attractive target for therapeutics to inhibit the onset of labour in threatened pre-term pregnancies perhaps via use as a gel or pessary.

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# Appendix 1 List of publications and conference presentations

## Publications

Arrowsmith, S., H. Robinson, et al. (2012). "What do we know about what happens to myometrial function as women age?" J Muscle Res Cell Motil **33**(3-4): 209-217.

Robinson H and Wray S. (2012). "A new slow releasing, $H_2S$ generating compound, GYY4137 relaxes spontaneous and oxytocin-stimulated contractions of human and rat pregnant myometrium", PLoS ONE 7(9): e46278. doi:10.1371/journal.pone.0046278.

## Conference Poster presentations

Investigating the effect of Hydrogen Sulfide on myometrial contractility **– SGI Miami March 2011**

A new slow releasing, $H_2S$ generating compound, GYY4137 relaxes human and rat pregnant myometrium- **SGI San Diego March 2012**

## Conference Oral presentation

A new slow releasing, $H_2S$ generating compound, GYY4137 relaxes human and rat pregnant myometrium- **PHYSOC Edinburgh July 2012**

This text box is where the unabridged thesis included the following third part copyrighted material:

Appendix 2: Ethical approvals. REC References 09/H1005/55, 10/H1002/49, and 11/H1005/4

Appendix 3: Myometrial tissue bank application

Appendix 4: Consent forms