An Investigation into the Effects of Extracellular Acidification on Mouse Uterine Contraction and Possible Mechanisms of Action

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Published papers

1. **Almohanna AM** & Wray S., 2018. Hypoxic conditioning in blood vessels and smooth muscle tissues: effects on function, mechanisms, and unknowns. Am J Physiol Heart Circ Physiol. Review. 315(4):H756-H770

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National and international conference presentations

- **Almohanna AM**, Noble K, Wray S. The effects of extracellular acidification on uterine contractions. Early Career Physiologists Symposium. Dublin, Ireland. July 28th 2016 (Poster presentation)

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- **Almohanna AM** & Wray S. The effects of extracellular acidification on uterine contractions. Early Career Symposium. Liverpool, UK. September 04th 2017 (Poster presentation)

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- I was awarded the Excellence in Research award from the Saudi Ambassador. (I was one of fifteen students selected who are studying for a PhD in the UK and the only student from the University of Liverpool to receive the award). Embassy of Saudi Arabia, London, UK. September 21th 2017

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List of Abbreviations

AM	Acetoxymethyl
ANOVA	Analysis of Variance
ASICs	Acid-Sensing Ion Channels
ATP	Adenosine Triphosphate
ATPγS	ATP-gamma-S
AUC	Area under the curve
AVP	arginine vasopressin
BCECF	2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein
BK_{Ca}	Large Conductance Calcium Activated Potassium Channel
Ca^{2+}	Calcium ion
Ca-CaM	Calcium Calmodulin Complex
$CaCl_2$	Calcium Chloride
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
CAPSO	N-Cyclohexyl-2-hydroxyl-3-AminoPropaneSulfOnic acid
CCE	Capacitative Calcium Entry
Cl^-	Chloride ion
CaCC	Calcium activated chloride channel
CO_2	Carbon dioxide
DAG	Diacylglycerol
DEA	Diethylamine
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide

EMC	Electro-Mechanical Coupling
EtOH	Ethanol
GPCR	G-Protein Coupled Receptor
GTP	Guanine Triphosphate
H&E	Hematoxylin and Eosin staining
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHC	Immunohistochemistry
IP_3	Inositol 1, 4, 5-trisphosphate
IP_3R	Inositol 1, 4, 5-trisphosphate receptor
K^+	Potassium ion
K_{ATP}	ATP sensitive potassium channel
K_{Ca}	Calcium activated potassium channel
KCl	Potassium chloride
Kv	Voltage dependent potassium channel
L-VGCC	L-type voltage-gated calcium channel
Mg	Milligrams
Mg^{2+}	Magnesium ion
$MgSO_47H_2O$	Magnesium Sulfate
MES	2-N-MorpholinoEthaneSulfonic acid
Mins	Minutes (time)
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
mM	Milli-Molar
mN	Milli-Newton
mV	Millivolts
n	Number of samples from different animals

Na^+	Sodium ion
NaCl	Sodium Chloride
NaBut	Sodium Butyrate
NCX	Na^+/Ca^{2+} exchanger
NFA	Niflumic Acid
NH_4C	Ammonium Chloride
NIOK	Non-inactivating outward K^+ Channels
nM	Nanomolar
NaOH	Sodium Hydroxide
O_2	Oxygen
OT	Oxytocin
OTR	Oxytocin Receptor
P1	Adenosine Receptors
P2	ATP receptors
PCr	Phosphocreatine
PG	Prostaglandin
PGE2	Prostaglandins E2
PGF2α	Prostaglandins F2α
pH_o	Extracellular pH
pH_i	Intracellular pH
Pi	Inorganic phosphate
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PIP2	Phosphatidylinositol 4, 5 Biphosphate
PLA2	Phospholipase A2
PLC	Phospholipase C
PMCA	Plasma Membrane Ca^{2+}-ATPase

PMT	Photo Multiplier Tube
ROCC	Receptor operated calcium channel
RyR	Ryanodine receptor
SEM	Standard Error of the Mean
SERCA	Sarcoplasmic Reticulum Ca^{2+}-ATPase
SNARF	Seminaphtharhodafluor
SOCC	Store-operated calcium channel
SR	Sarcoplasmic reticulum
TASK	TWIK related acid-sensing K^+ channels
TEA	Tetraethylammonium
TMA	Trimethyl-amine
TREK	TWIK related potassium channels
TWIK	Two-pore domain weak inwardly rectifying K^+ channels
VGCC	Voltage-gated calcium channel
WB	Western Blot
$[Ca^{2+}]_i$	Concentration of the intracellular calcium
µL	Microlitre

Abstract

An Investigation into the Effects of Extracellular Acidification on Mouse Uterine Contraction and Possible Mechanisms of Action

By: Asmaa Almohanna

Introduction: Adequate uterine contraction is crucial to safe delivery of the foetus. Acidity can play an important role in affecting myometrial contraction but the effects, especially concerning external acidification, have not been consistent. External acidification is produced as a result of ischemia develops due to uterine blood vessels occlusion by strong uterine contractions. This study was designed to determine the effects of acidic pH_o on myometrium, any gestational differences and possible mechanisms underlie that effect. ***Methods:*** Uterine strips dissected freshly from C57BL/6J mice were used either on day 14 or 19 of gestation or as 8 weeks non-pregnant. Changes in pH_o to 6.9 (or 6.5) and 7.9 were made for 10 minutes, by adding HCl or NaOH, respectively to physiological saline. Intracellular acidification and alkalinization at a constant external pH (7.4) was made by the addition of the Na butyrate and ammonium chloride, respectively (40 mM). Uterine strips were dissected and mounted in organ baths, bubbled with HEPES-buffered, oxygenated physiological saline solution for isometric force recording. Carboxy SNARF—1 AM was used to measure intracellular pH (pH_i). ***Results and Discussion:*** Both extra- and intracellular acidification significantly increased uterine contractility in pregnant, but not in non-pregnant, uteri while extra- and intracellular alkalinization had the opposite effect, i.e. both decreased uterine contractility. No gestational differences were found between term and mid-term stages of pregnancy but clearly, pregnancy enhanced uterine response to acidification. Pregnant uterus is known to be more sensitive to the changes in pH than the non-pregnant in different species. My work looking for mechanisms underlying the stimulatory effect of extracellular acidification on pregnant uterus revealed interesting findings. Simultaneous measurement of force and pH_i, showed that pH_i started to decrease after one minute after the acidic pH_o start its action. Corresponding changes of pH_i may explain partially the effect of acidic pH_o on pregnant uterus. Unpublished work done to measure intracellular calcium levels $[Ca^{2+}]_i$ in response to decreasing pH_o revealed that the increase in force produced by pH_o 6.9 was preceded by an increase in $[Ca^{2+}]_i$. Therefore, I carried out the following experimental work to check to source of this increase in Ca^{2+}. Using nifedipine as an inhibitor of the L-VGCCs revealed that L-type calcium entry is needed for the acidic pH_o in order to stimulate the uterus. Also, extracellular acidification requires depolarization for its mechanism of action. Moreover, oxytocin abolished the stimulatory effect of pH_o 6.9 mainly by limiting the drop in pH_i in response to acidic pH_o and, even, increasing intracellular pH. Calcium-activated chloride channels and P_{2x7} were not involved in the stimulatory effect of extracellular acidification. Ion channels sensitive to external acid and conducting inward current (ASICs) have been reported. My novel data gained from western blot and IHC suggesting ASICs are expressed in pregnant myometrium from mouse, and thus may contribute to increases in force. Although, blocking the ASICs didn't affect the stimulatory effect of acidic pH_o on pregnant uterus, role of ASICs in uterine contraction should be the focus of other work. In conclusion, extracellular acidification increases uterine contraction mainly due to the increase in L-type calcium entry and partially because of the corresponding changes in pH_i. This effect might be mitigated by the physiological presence of oxytocin. This research hold a lot of promise for further development and the findings will be rewarding for this project area.

Chapter One

General Introduction

Chapter-1

General Introduction

Structure of Introduction to the Thesis

In this introductory chapter, I will outline the knowledge and literature that forms the basis for my studies into the effects of acidic external pH in the uterus, and the possible mechanisms that underlie these effects. To do this, I will firstly and briefly describe the anatomy of the uterus, and the cells responsible for contractions, the myocytes of the myometrium. I will then discuss excitation-contraction coupling (or electro-mechanical coupling), including membrane potentials and ion channels present in the myometrial cells. Next, I will give a review of the literature about the work has been done on the effects of external pH alteration on smooth muscle contractility. Finally, I will explain the aims of my work in this thesis.

1.1 The Uterus

1.1.1 Anatomy of the Human Uterus

The uterus has a major role in mammalian reproduction (Mona e Pinto et al., 2014, Sosa-Stanley and Peterson, 2019). Its shape is like an inverted pear with a thick muscular wall located in the pelvis, anterior to the rectum and posterior to the urinary bladder. The essential function of the uterus is to host and nourish a fertilized egg until the offspring is ready for labour. The uterus consists of four regions: fundus, body, isthmus, and cervix. **The fundus** is the upper curved area where it attaches to the fallopian tubes. **The body** is the main part of the uterus which starts below the fallopian tubes and extends downward. **The isthmus** is the lower narrow region which connects the uterine body to the cervix. The lowest section of the uterus is **the cervix** which extends downward from the isthmus and opens into the vagina (Figure 1.1).

Histologically, the uterus is composed of three layers named, the endometrium, myometrium, and perimetrium. The innermost layer is **the endometrium** which is composed of basal cells and responds to reproductive hormones. The middle layer is **the myometrium** which consists of smooth muscle cells, and is the largest part of the

uterus. The outer thin layer is **the perimetrium**, also known as the serosal coat, which composed of epithelial cells (Sosa-Stanley and Peterson, 2019).

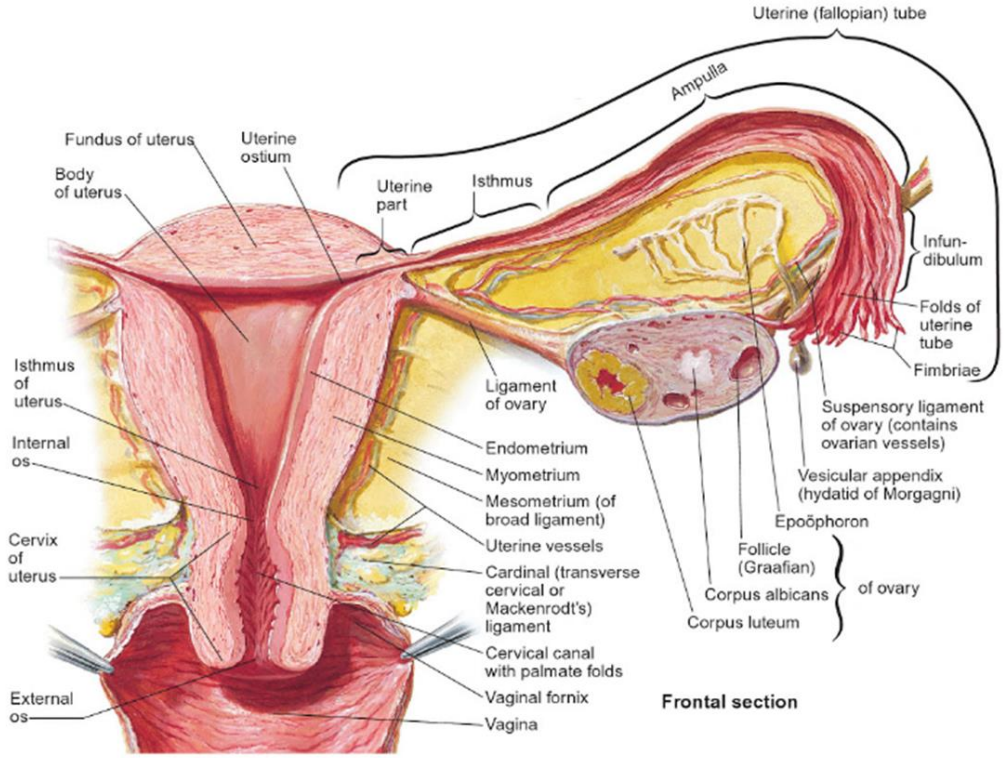

Figure 1.1 Gross anatomy of the human uterus

Adapted from (Treuting et al., 2012).

1.1.2 Mouse uterus

The mouse uterus is composed of two lateral horns joining into a single body, at the cervical end. The inner surface is the inner epithelial-lined mucosa which forms **the endometrium**. The middle part is the muscular layer of **the myometrium** which consists of two muscular layers, the inner circular and outer longitudinal layers and the outer serosal layer, **the perimetrium** (Treuting et al., 2012) (Figure 1.2).

In a mouse, the uterus is called *Duplex Uterus*, a Y-shaped with two horns, extending up into the abdomin and this allows it to support multiple foetuses.

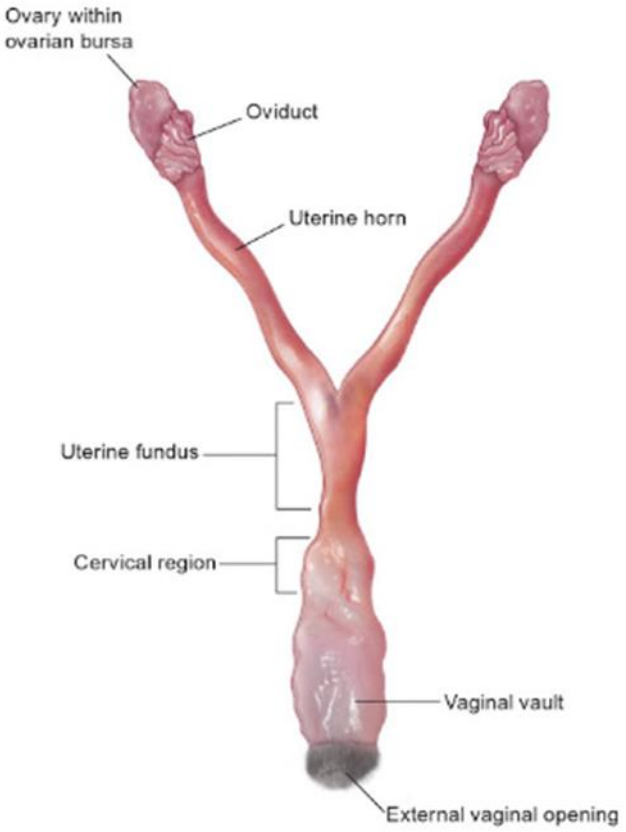

Figure 1.2 Gross anatomy of the mouse uterus

Adapted from (Treuting et al., 2012)

1.1.3 Uterine blood supply

The main blood supply for the uterus came from the uterine arteries, which arise from the anterior branch of the internal iliac artery. As the blood supply enters the myometrium, it branches into the arcuate arteries, which branch into the radial arteries. As they enter the level of the endometrium, they branch into the basal and spiral arteries (Sosa-Stanley and Peterson, 2019). There are two uterine arteries (right and left). The uterine artery courses from lateral to the medial direction through the lowest part of the broad ligament or the cardinal ligament (Chaudhry and Chaudhry, 2019) (Figure 1.3).

At the uterine isthmus level, the uterine artery is divided into ascending and descending branches. The ascending artery runs a tortuous course superiorly along the lateral side of the uterus. The ascending branch of the uterine artery anastomoses to the ovarian artery which is a direct branch of the abdominal aorta. The ovarian artery supplies blood to the ovary, uterus, and fallopian tube. The descending branch supplies blood to cervix and vagina (Osol and Mandala, 2009).

The arcuate arteries supply blood to the myometrium. The arcuate arteries penetrate throughout the circumference of the myometrium and supply blood to anterior and posterior walls of the uterus. The arcuate artery terminates in the spiral artery, which supplies the endometrium, decidua, and placenta during pregnancy. The regular contraction and relaxation of these blood vessels supports endometrial stability and controls it blood supply (Akerlund, 1994).

The spiral arteries give blood supply to the endometrium, more specifically, the functional zone which sheds during menstruation. The basal arteries supply the blood to the endometrium, more specifically, the basal zone. The basal zone starts the regeneration of the denuded endometrium after menstruation (Hwuang et al., 2019).

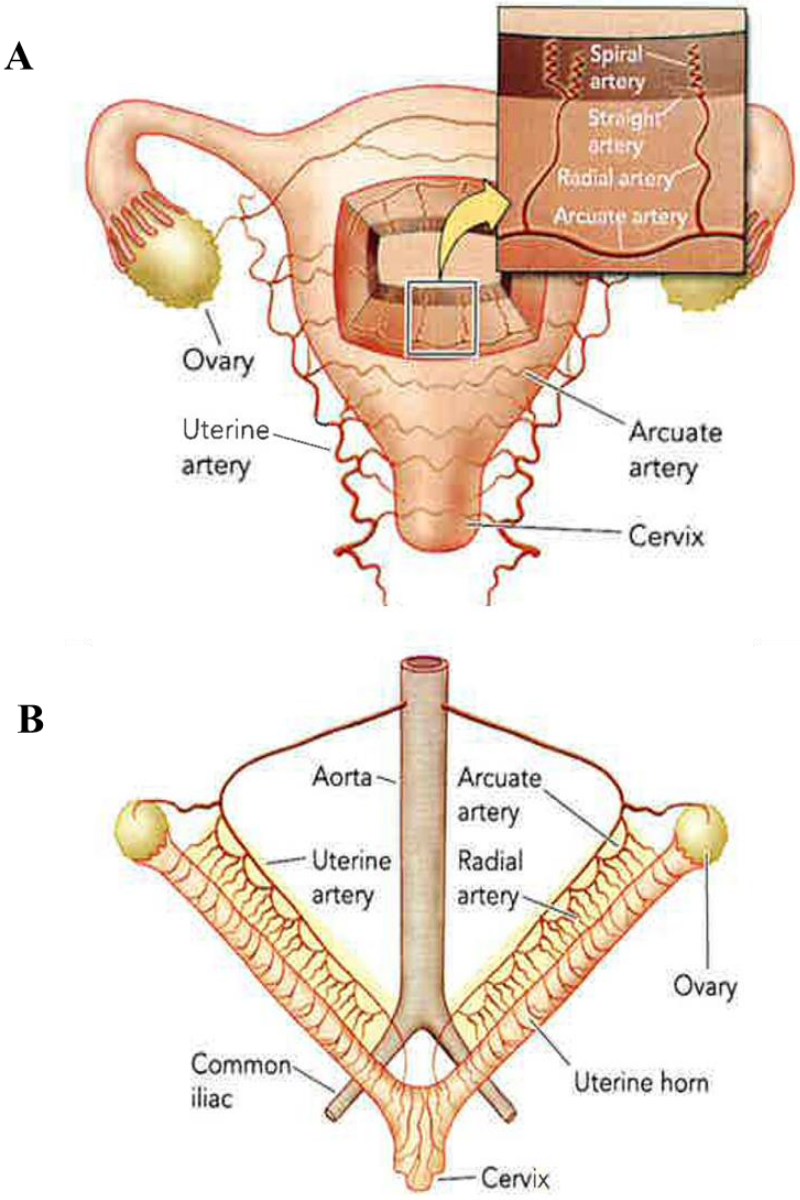

Figure 1.3 Blood supply of the (A) human and (B) mouse uteri

Adapted from (Osol and Mandala, 2009)

1.2 Myometrium

1.2.1 Structure of the myometrium

The myometrium is the muscular layer of the uterus which consists of smooth muscle cells located in the middle layer of the uterine wall between the endometrium and the perimetrium. It significantly expands during pregnancy. It is a hormonally sensitive structure that undergoes hyperplasia and hypertrophy during pregnancy (Chaudhry and Chaudhry, 2019). The myometrium is composed of two layers of smooth muscle. The muscle fibres of the inner circular layers are arranged concentrically around the long axis of the uterus, and those of the outer longitudinal layer are arranged in parallel fashion (Treuting et al., 2012). The myometrium is myogenic which means that it is spontaneously active smooth muscle, producing regular contractions without neural or hormonal stimulation (Wray, 1993). For contraction to occur, there should be interaction between myosin and actin myofilaments. Myometrial contractions are stimulated by temporary elevation in the intracellular calcium concentration. Uterine contraction is initiated and controlled by myometrial action potentials (Wray, 1993).

In mouse uterus, the myometrium consists of two layers; the outer longitudinal and the inner circular smooth muscle layers. They are clearly separated by a highly vascular loose layer of connective tissue, the stratum vasculosum (Treuting et al., 2012). While in the human myometrium, such distinction between longitudinal and circular layers does not exist (Young and Hession, 1999, Treuting et al., 2012).

1.2.2 Cellular structure of the myometrium

Myometrium is the largest component of the uterus and myometrial cells occupy around 90% of the total cellular content of the uterus (Dawson and Wray, 1985). Myometrial cells are typical spindle-shaped smooth muscle cells arranged in bundles and enclosed in a connective tissue matrix (Yu and Lopez Bernal, 1998). This matrix is composed of elastin, collagen, glycoproteins and proteoglycans, and it helps transmitting the contractile force throughout the uterus (Yu and Lopez Bernal, 1998). Myometrial cells contain actin thin filaments and myosin thick filaments which are the main contractile proteins and distributed throughout the cell (Word, 1995). Myofilaments, intermediate filaments, dense bodies and dense plaques occupy about 80-90% of the myometrial cell volume with the remaining 10-20% space consists of the

nucleus and other organelles like mitochondria, sarcoplasmic reticulum (SR), Golgi apparatus, etc. (Broderick, 1990, Kao and Carsten, 1997, Jain et al., 2000) (Figure 1.4).

Gap junctions are essential for cell to cell communication in the myometrium. They play a vital role in passing calcium ions, and hence depolarization between cells and synchronizing activity (Garfield et al., 1977, Huizinga et al., 1992). Expression of connexin 43, a key protein of gap junctions, was found to be enhanced toward term and with the onset of labour (Chow and Lye, 1994).

In cardiomyocytes, gap junctions are responsive to the changes in intracellular pH (pH_i). Mild decrease in cytoplasmic pH enhances the permeability of the gap junctions and hence passively passing protons to the adjacent cell to regulate pH_i. On the other hand, with more severe intracellular acidification, the junctional permeability is inhibited, allowing for more advanced and energy-consuming methods of pH regulation (Chow and Lye, 1994, Swietach et al., 2007, Zaniboni et al., 2003). Such relation between protons and the permeability of the gap junction in the myometrium is not yet identified.

Uterine caveolae (small caves) are small invaginations present in the plasma membrane of myometrial cells (Smith et al., 2005, Taggart et al., 2000) with a 2.4% of the cellular volume of the myometrium in Wistar rat (Popescu et al., 2006). They are a special type of lipid raft increasing the surface area by ≈70% and contain the protein caveolin which is responsible for maintaining the caveolae, and giving them their distinctive omega shape (Taggart et al., 2000, Noble et al., 2006). Their expression in relation to labour is controversial (Ciray et al., 1995, Turi et al., 2001) but they were downregulated by oestrogen (Wang et al., 2005). They may be involved in excitation-contraction coupling because of their proximity to sarcoplasmic reticulum and their involvement in PKC regulation (Taggart et al., 2000, Shmygol and Wray, 2004, Turi et al., 2001). Moreover, the large conductance, Ca^{2+}-sensitive K^+ channels (BK) were found to be localized close to caveolae (Brainard et al., 2005).

The sarcoplasmic reticulum (SR) is the intracellular store of calcium ions in the myometrium and has a volume of ≈7% in the cell (Somlyo et al., 1985, Wray and Shmygol, 2007, Wray and Burdyga, 2010). Its role in modulating uterine contractility has only recently been understood (Noble et al., 2014). Calcium ions actively enter the SR against their gradient using the SR Ca^{2+} -ATPase (SERCA 2&3) (Tribe et al., 2000, Khan et al., 1993). While they are released from the SR through inositol 1, 4, 5

triphosphate (IP_3) binding to its receptors (IP_3-induced Ca^{2+} release-IICR). Although the myometrial SR expresses ryanodine receptors, they have been shown by molecular and pharmacological studies to be non-functional (Wray et al., 2001, Mironneau et al., 2002, Wray and Shmygol, 2007, Dabertrand et al., 2007, Matsuki et al., 2017). Calcium release from SR is required in agonist-induced, but not spontaneous, uterine contraction and that is achieved through IICR. The role of the SR in myometrium took much investigation to elucidate. It was first noted, unexpectedly that a full SR inhibited spontaneous contraction, while inhibiting SR re-filling and thereby keeping SR Ca^{2+} content low stimulated contractility (Wray et al., 2001, Shmygol and Wray, 2005). Further work showed that this could not be explained by SR Ca^{2+} release stimulating Ca^{2+}-activated K^+ channels, as occurs in blood vessels, and contributes to their relaxation. Finally it was found that the agonist-induced depletion of SR Ca^{2+} stimulated store-operated calcium entry (SOCE) and depolarization (Noble et al., 2014). Thus emptying the SR is associated with increased excitability and contraction increase.

Mitochondria are present in the myometrial cells as they are in all other mammalian cells apart from red blood cells. Their main functions are energy production, by phosphorylating the ADP into ATP through respiration, and metabolic regulation. In addition, mitochondria have a large capacity, but low affinity, to store Ca^{2+} (Somlyo et al., 1985). A role for mitochondria Ca^{2+} signalling has been proposed during oxytocin stimulation (Gravina et al., 2011). Other studies have indicated that myometrial mitochondria may fall in number or perform less well as a woman ages or develops preeclampsia, but does not change with diabetes (Patel et al., 2017, Gam et al., 2018, Vishnyakova et al., 2019).

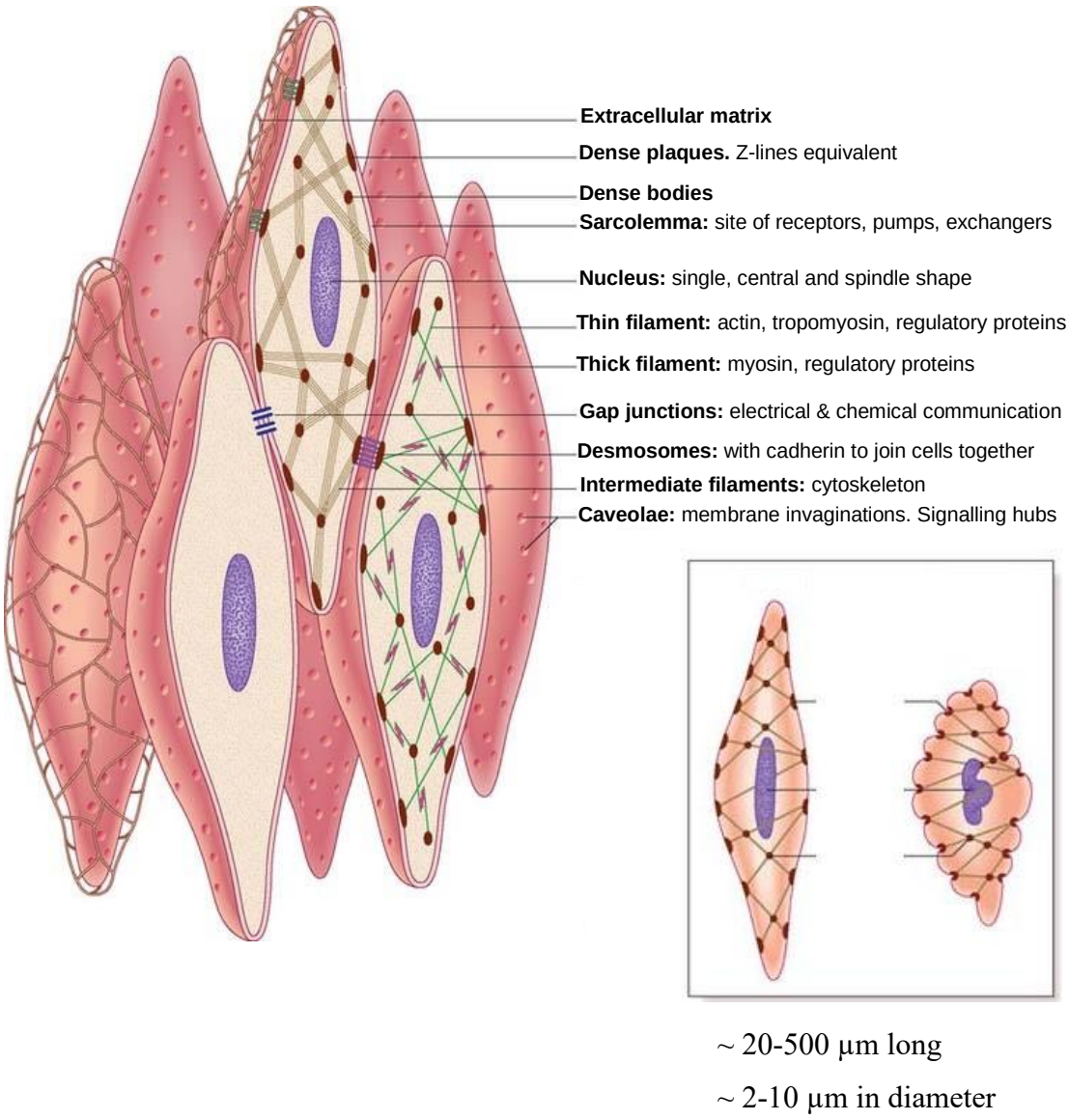

Figure 1.4 Three-dimensional illustration of smooth muscle cells and their cellular components.

Adapted from https://clinicalgate.com/smooth-muscle-and-the-cardiovascular-and-lymphatic-systems/ (2015)

1.3 Myometrial Cell Excitability

It is suggested that the pregnant uterus remain quiescent throughout pregnancy and when approaching labour, a cascade of hormonal and mechanical events initiate the contraction to deliver the foetus (Challis et al., 2005). As mentioned already, the myometrium is spontaneously active, i.e. it contracts without the need for hormonal or neuronal stimuli (Wray, 1993). Uterine contractions are phasic in nature with cycles of strong and slow contractions and relaxation periods in between. The main key initiating the action potential is Ca^{2+} entry while relaxation is initiated by inactivation of the Ca^{2+} channels and activation of K^{+} efflux (Wray, 1993, Matthew et al., 2004).

1.3.1 Electrical activity and membrane potential

Action potentials in the myometrium are spontaneous electrical activity that is composed of cycles of depolarization and repolarization (Kao and Carsten, 1997). Uterine contraction depends on the changes of membrane potential, a transient increase in intracellular calcium, and the presence of a contractile myofilaments and a conducting system between uterine myocytes (Garfield et al., 1977). The excitability of uterine myocyte is mainly affected by the ions movement across the cell membrane which modifies its potential (Wray, 1993, Wray et al., 2015).

The resting membrane potential occurs when there is minimal movement of ions across the plasma membrane. It has been recorded, and estimated to be between -35 and -80 mV in the uterine smooth muscles (Sanborn, 2000, Aguilar and Mitchell, 2010). In the pregnant mouse longitudinal myocyte, the resting membrane potential was measured to be -55 mV at mid-gestation and, depolarized to -46 mV at term, suggesting that the potassium channels density may decline in the late pregnancy in rat myometrium (Matharoo-Ball et al., 2003).

The resting membrane potential gradually becomes less negative towards term and this will bring it closer to the threshold for firing action potentials. Moreover, the activity of the uterus becomes synchronized and coordinated, and this regularity is secondary to the increase in gap junctions between the adjacent cells, that facilitate the rapid transmission of electrical activity, hence producing coordinated and regular contractions (Khan et al., 2001).

1.3.2 Electro-mechanical coupling

Electro-mechanical coupling (EMC) in the uterus is the events sequence between the start of action potential and beginning of muscle contraction. This could happen through two mechanisms which are pharmacomechanical or electrochemical coupling (Somlyo and Somlyo, 1994b).

During electrochemical coupling, the increase in intracellular Ca^{2+} level is due to plasma membrane depolarization. Cell membrane depolarization from -55 mV to -40 mV opens the L-type voltage-gated calcium channel, causing influx of Ca^{2+} into the cell and therefore binding of calcium to calmodulin (abbreviation for calcium-modulated protein, CaM). The Calcium-CaM complex stimulates myosin light chain kinase (MLCK) (Shmigol et al., 1998). Consequently, the serine 19 on the regulatory light chain of myosin (MLC20) will be phosphorylated, permitting acto-myosin crossbridge interaction and cycling, hydrolysis of Mg-ATP, and eventually contraction occurs (Word, 1995, Taggart et al., 1997a) (Figure 1.5).

In pharmacomechanical coupling, the rise in Ca^{2+} level is caused by binding of receptor-agonist such as neurotransmitter, hormones or drugs on plasma membrane. Consequently, the small monomeric G-proteins bind to GTP and stimulate phospholipase C (PLC). Therefore, phosphatidylinositol biphosphate (PIP_2) is cleaved and produces Inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 binds to its receptor on sarcoplasmic reticulum (SR) and thus increasing Ca^{2+} concentration (Somlyo and Somlyo, 1994b).

Studies showed that phosphorylation of serine 19 on the light chains by calcium-CaM dependent enzyme (MLCK) is the main determining factor of smooth muscle contraction in both human and rat myometrium (Word, 1995, Longbottom et al., 2000). Figure1.6 shows mechanisms of spontaneous uterine contraction.

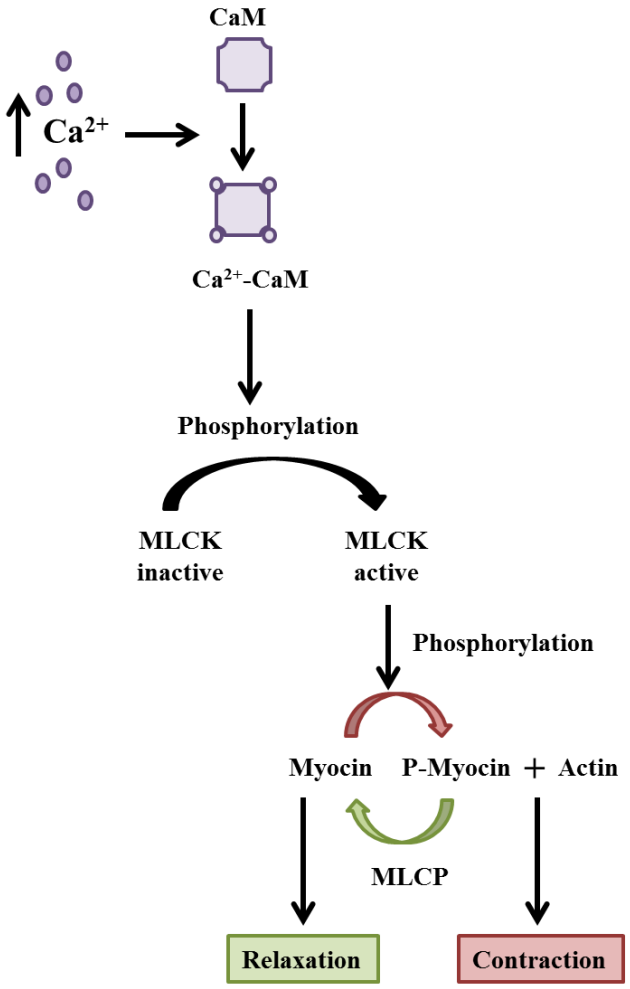

Figure 1.5 Scheme of contraction in a uterine myocyte showing the electro-mechanical coupling (EMC)

The increase in $[Ca^{2+}]_i$ causes 4 Ca^{2+} ions to bind to calmodulin producing CaM (Ca-calmodulin complex). CaM then activates the enzyme myosin light-chain kinase (MLCK). MLCK then phosphorylates myosin into active P-myosin and allows actin binding. Thus contraction will occur with ATP hydrolysis. Relaxation then occurs by dephosphorylating the p-myosin by phosphatases (MLCP).

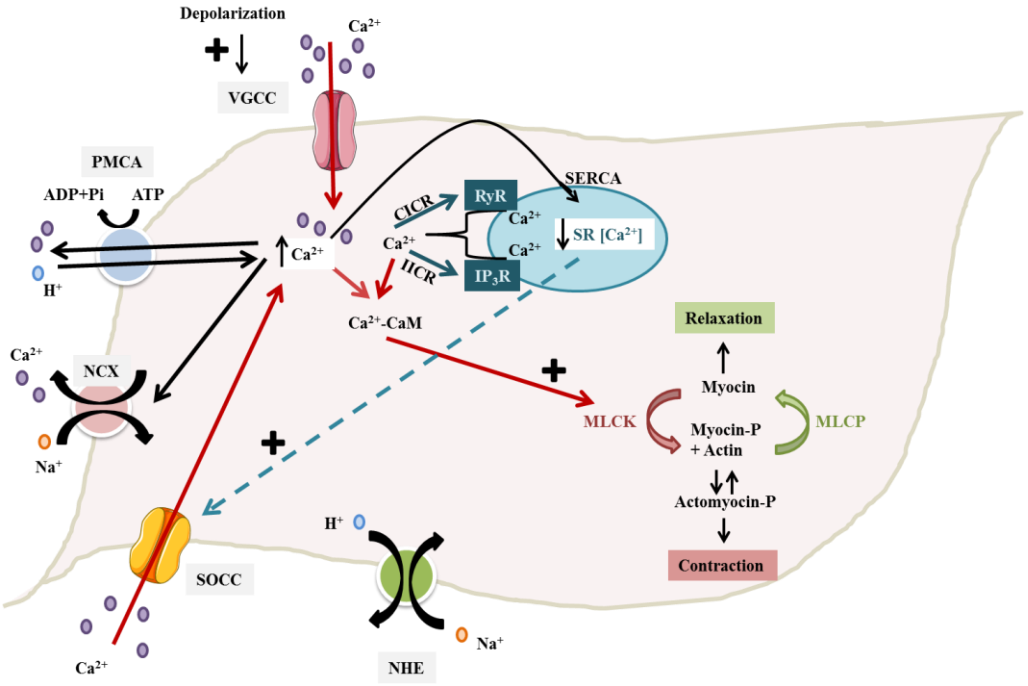

Figure 1.6 Schematic diagram showing mechanisms of spontaneous uterine contraction

Depolarization of the membrane opens the L-type voltage-gated calcium channels (L-VGCCs) leading to influx of calcium ions into the cytoplasm and that leads to the release of the Ca^{2+} from sarcoplasmic reticulum (SR) mainly through inositol trisphosphate receptor (IP_3R). Drop of the [Ca^{2+}] in the SR stimulates more influx of Ca^{2+} through store-operated calcium channels (SOCCs). Calcium ions (Ca^{2+}) connect to calmodulin proteins (CaM) to produce Ca^{2+}-CaM complexes which activate myosin light chain kinase (MLCK). MLCK phosphorylates myocin and join actin to start cross-bridging and contraction. Myosin light chain phosphatase (MLCP) then dephosphorylates myocin-p-actin to induce relaxation. Relaxation is associated with Ca^{2+} extrusion outside the cell 70% through plasma membrane Ca-ATPase (PMCA) and 30% through Na/Ca exchanger (NCX). Some of the Ca^{2+} will be actively entering SR through sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

1.4 Ion Channels in the Myometrium

1.4.1 Calcium channels

There are two major subtypes of calcium channels including L-type and T-type. "L'' stands for long-lasting, or slow, and "T'' stands for transient-lasting, or fast, and both types of calcium channels, referred to as voltage gated calcium channels, have been reported in human uterine smooth muscles (Blanks et al., 2007).

1.4.1.1 L-type calcium channels

The main channel for calcium entry pathway in myometrium is the L-type calcium channel and nifedipine which is a specific blocker, inhibits uterine contractions (Wray et al., 2001). L-type channels were found to change massively during pregnancy in guinea pig and human myometrium (Collins et al., 2000, Longo et al., 2003). L-type channels are gated by membrane potential; they open as a result of depolarization, to around -40 mV, causing calcium influx and initiates myometrial contractions (Wray et al., 2003). L-type channels are then inactivated by Ca^{2+} itself and their half-inhibition was also noticed at ≈45mV (Sanborn, 2000, Wray et al., 2003). Unlike nifedipine, Bay K 8644 is a dihydropyridine used as a Ca^{2+} channels agonist. Bay K8644 was found to increase phasic uterine contraction and its mechanism of action was suggested to be through activation of P_i-PLC pathway and CICR and by increasing Ca^{2+} influx and action potential (Chien et al., 1996, Bechem and Schramm, 1987).

1.4.1.2 T-type calcium channels

Studies showed that T-type calcium channels are expressed in the myometrium of pregnant human, rat and mouse but their expression is gestation-independent (Blanks et al., 2007, Lee et al., 2009). T-type current is present in only 50% of the myometrial smooth muscle cells unlike L-type channels which has 100% expression (Blanks et al., 2007) and they are unlikely to be activated during normal activity as smooth muscle cells have resting membrane potentials within the range for steady-state inactivation of the T-type channel (Wray et al., 2015).

1.4.2 Potassium channels

Membrane repolarization is the result of potassium efflux from myometrial cells and this efflux is the main ionic current to maintain the resting membrane potential. In myometrial cells, changes in the activity or expression of potassium channels can lead to inadequate repolarization causing aberrant uterine activity (Khan et al., 2001). There are several types of potassium channels that have been recognized in the myometrium. The well-studied types include voltage-gated K^+ channels (Kv), Calcium-activated potassium channels (Kca), ATP-sensitive potassium channels (K_{ATP}) and two-pore domain potassium channels (K2P) (Brainard et al., 2007).

1.4.2.1 Voltage-gated potassium channels (Kv)

Voltage-gated K^+ channels (Kv) are abundantly expressed in uterine smooth muscle and they play a key role in maintaining resting membrane potential (Brainard et al., 2007, Parkington et al., 2014). Depolarization of the cell membrane opens L-type Ca^{2+} channels causing Ca^{2+} influx and hence activation of the Kv channels inducing an efflux of K^+ which repolarizes the membrane to a resting membrane potential and maintain the uterus quiescence (Brainard et al., 2007, Wray et al., 2015). Inhibition of Kv channels using tetraethylammonium (TEA) or 4-aminopyridine (4-AP) markedly enhances uterine contractions (Wray et al., 2015). Among many subtypes of Kv channels, few have been described in relation to the myometrium, Kv4 (Smith et al., 2007), Kv7(Wang et al., 1998) and Kv11(Knock et al., 1999, Knock et al., 2001).

1.4.2.2 Calcium-Activated Potassium Channels (K_{Ca})

There are three types of K_{Ca} that have been recognized in the myometrium; big conductance (BK_{Ca}), intermediate-conductance (IK_{Ca}), and small-conductance (SK_{Ca}) potassium channels (Khan et al., 2001). Among these types, **BK_{Ca} channels** (also known as Maxi-K) are well studied and most dominant K_{Ca} channels encountered in pregnant and non-pregnant myometrium (Khan et al., 2001, Wray et al., 2015). They are large conductance, voltage-gated and calcium-sensitive potassium channels. During normal gestation, intracellular Ca^{2+} is elevated and the BK_{Ca} channels will be activated, and thus help to maintain uterine quiescence throughout gestation through inhibition of the membrane excitability (Brainard et al., 2007, Wakle-Prabagaran et al., 2016, Brainard et al., 2005). Their expression was found to decrease in human myometrium during labour (Gao et al., 2009) however, blocking them pharmacologically did not

affect uterine contractility (Aaronson et al., 2006, Noble et al., 2010). On the other hand, **SK_{Ca} channels** are calcium-sensitive but voltage-insensitive potassium channels. They were found expressed in the myometrium and similar to the BK, their pharmacological inhibition resulted in little effect on contractility and Ca^{2+} signalling (Noble et al., 2010, Rosenbaum et al., 2012). Little is known about the role of **IK_{Ca} channels** in myometrium.

1.4.2.3 ATP-Sensitive Potassium Channels (K_{ATP})

Expression of K_{ATP} was found in pregnant and non-pregnant human myometrium and they are one of other seven subfamilies of inwardly rectifying K^+ channels (K_{ir}) (Curley et al., 2002, Xu et al., 2011). Despite a relatively low open state probability, the K_{ATP} in uterine smooth muscle maintains basal membrane potential (Hong et al., 2016). Kafali *et al* found that K_{ATP} channels might mediate the relaxation phase of spontaneous contractions in late pregnant rats (Kafali et al., 2002). Sawada *et al* found that over-expression of K_{ATP} subunits inhibits oxytocin-induced contractions in pregnant rats (Sawada et al., 2005).

1.4.2.4 Two-pore-domain potassium channels (K2P)

Two-pore potassium channels consist of 5 families and 15 subtypes. Specifically, two main types were found expressed in animal and human myometrium: TWIK-related acid-sensitive K^+ channels (TASK1, 2, 4 and 5) and TWIK-related K^+ channels (TREK1) (Bai et al., 2005, Buxton et al., 2011, Monaghan et al., 2011, Hong et al., 2013, Kyeong et al., 2016, Yin et al., 2018, Buxton et al., 2010). Extracellular acidification was found to activate TREK-1 and inhibit TASK-1 (Bai et al., 2005, Buxton et al., 2011, Monaghan et al., 2011). TREK-1 was activated by arachidonic acid and mechanical stretch during gestation while hypoxia was found to inhibit both TASK1 and TREK1 (Buxton et al., 2011). Hormones also modulate K2P as the expression of TREK1 channels was found significantly decreased in ovariectomized mice (Monaghan et al., 2011).

It was hypothesized that they may participate in setting a resting membrane potential and maintaining uterine quiescence (Brainard et al., 2007). Activation of TREK1 was correlated to hyperpolarization and hence decreased uterine contraction while their blockade increased uterine excitability (Buxton et al., 2011, Monaghan et al., 2011).

Moreover, their expression was found to increase during pregnancy and drop quickly near term (Buxton et al., 2010, Monaghan et al., 2011).

Less work was done to investigate biophysical and functional effects of TASK channels in relation to pregnancy and parturition. TASK1, 4 and 5 were found expressed in pregnant human myometrium (Bai et al., 2005) while TASK2 was found expressed in both pregnant and non-pregnant mouse myometrium (Hong et al., 2013, Kyeong et al., 2016). Latest authors found that inhibiting TASK2 results in relaxation of the mouse uterus. Despite the great interest lately in investigating the effect of K2P on uterine activity, more work is needed to define their role in pregnancy and labour.

1.4.3 Chloride channels

Chloride channels are responsible for Cl ion movement across the cell membrane. It should be noted that in smooth muscles the [Cl^-] is higher than in many other mammalian cells (Chipperfield and Harper, 2000, Saravanaperumal et al., 2018). The accumulation of Cl^- is energetically demanding, but the membrane in smooth muscle has a very low permeability to Cl^-. Thus in smooth muscles cells, if chloride conductance is increased by opening of channels, chloride ions will leave the cells. This in turn will tend to depolarize the cells, and increase excitability. Chloride movement is also linked to the transport of other anions for pH and volume regulation, for example Cl^-/HCO_3^- exchange, and Na^+, for example, Na-K-2Cl cotransport, which has been associated with hypertension (Chipperfield and Harper, 2000).

Studies showed that Cl^- channels are present in smooth muscle cells including myometrium. It was believed that chloride channels have no role in uterine electrophysiology (Young, 2007) but recent studies have showed otherwise (Bernstein et al., 2014, Dodds et al., 2015, Mijuskovic et al., 2015, Danielsson et al., 2018). There are two types of Cl^- channels identified; calcium activated chloride channels (Cl_{Ca}) and volume regulated chloride channels (Cl_{VR}) (Jones et al., 2004, Bernstein et al., 2014).

1.4.3.1 Calcium-Activated Chloride Channels (Cl_{Ca})

Calcium-activated chloride channels are stimulated by calcium entry to the cells. Activation of Cl_{Ca} in the myometrium leads to chloride efflux that stimulates membrane depolarization and can initiates contraction (Jones et al., 2004, Dodds et al., 2015). Studies showed that the potent Cl_{Ca} channel blocker, niflumic acid, can inhibit the spontaneous and oxytocin-induced contractions in pregnant rat and non-pregnant mouse

myometrium (P Ganesan Adaikan, 2005, Bernstein et al., 2014, Dodds et al., 2015). Using L-type agonist (Bay K 8644) was found to potentiate Ca-activated chloride current in the pregnant rat myometrium (Jones et al., 2004, Song et al., 2009)

1.4.3.2 Volume-Regulated Chloride Channels (Cl_{VR})

When water enters the cell under normal condition, it is joined by the entry of amino acid and organic osmolytes hence swelling of the cell can occur. Consequently, regulatory volume decrease processes are generated. These include the activation of Cl^- and K^+ channels causing efflux of these electrolytes and water (Zhou et al., 2005). Studies showed that the main mechanism by which cell size return to normal is through Cl^- efflux via Cl_{VR} channels (Eggermont et al., 2001).

1.4.4 Sodium channels

Sodium channels play a major role in physiology. They facilitate depolarization and mediate transmission of electrical impulses through muscles. Sodium channels move extensively in the course of gating and ion translocation i.e. they are not static. They bind several toxins and anaesthetics (Marban et al., 1998). During gestation, the averaged density of fast sodium channels is increased because of an increase of cells which have fast sodium channels (Inoue and Sperelakis, 1991, Sanborn, 2000). It was found in rat that fast Na^+ channels participate in myometrial excitability (Inoue and Sperelakis, 1991, Seda et al., 2007).

1.4.5 Acid-Sensing Ion Channels (ASICs)

Acid-Sensing Ion Channels are voltage-insensitive, amiloride-sensitive channels activated by extracellular protons producing fast, but transient, inward current (Waldmann et al., 1995, Waldmann et al., 1997b, Horisberger, 1998, Waldmann et al., 1999). ASICs are permeable to different cations, including Na^+, Ca^{2+} and H^+. These channels have been most identified with the nervous system and a role in pain sensation. In smooth muscle, studies have found them to be expressed in cerebral (Chung et al., 2010, Chung et al., 2011) and pulmonary arteries (Jernigan et al., 2009) and detrusor muscle (Kobayashi et al., 2009, Corrow et al., 2010). Their expression or functional importance in myometrium is not yet documented. Given that they are gated by extracellular protons, and therefore may be activated when external pH becomes acidic, I considered it important to determine if they are present in myometrium, and undertake preliminary studies to investigate if they are functionally important. More details are given in Chapter 6.

1.5 Calcium Signalling in the Uterine Smooth Muscle Cells

As mentioned above, uterine contraction depends primarily on the increase of $[Ca^{2+}]_i$, which occurs mainly by L-type calcium entry, which is gated by depolarization (Wray, 1993, Somlyo and Somlyo, 1994a, Wray et al., 2015). The relation between Ca^{2+} signalling and uterine contractility is synchronized by action potentials (Lammers et al., 2008, Burdyga et al., 2009). Intracellular calcium concentration $[Ca^{2+}]_i$ is markedly low when compared to its concentration extracellularly (100nM and 2mM, respectively). Increasing $[Ca^{2+}]_i$ to 0.6-1μM is needed to induce uterine contraction (Horowitz et al., 1996). Calcium may increase within the myometrial cells via voltage-independent calcium entry through either store-operated calcium channels (SOCCs) and/or receptor-operated calcium channels (ROCCs) and/or calcium release from SR (Ichida et al., 1984, McFadzean and Gibson, 2002). Increases of free calcium ions inside the cells will activate efflux mechanisms into the extracellular space through plasma membrane Ca^{2+}-ATPase (PMCA) and/or sodium-calcium exchanger (NCX). Calcium lost from the SR will be taken up by the SR Ca^{2+}-ATPase (SERCA).

1.5.1 Calcium influx

Voltage-gated L-type calcium entry is the main key for the myometrium to contract (Wray, 1993). Blocking this port with specific L-type calcium channel blocker, e.g. nifedipine, inhibits both spontaneous uterine contraction as well as agonist-induced contraction in human and animal uteri (Downing et al., 1988, Taggart et al., 1996, Parkington et al., 1999). Other voltage-independent, calcium permeable channels were also identified in the uterus. They are namely ROCCs and SOCCs (Albert and Large, 2003, Bolton and Imaizumi, 1996).

Receptor-operated calcium channels (ROCCs) are membrane-bound channels which allow calcium to enter the cell in response to the binding agonists. Very little is known about their expression or biophysical characteristics in myometrium. However, there are two types of ROCCs in smooth muscle cells, ATP-activated and acetylcholine-activated ROCCs (McFadzean and Gibson, 2002). They are also permeable to Na^+ and K^+ in addition to their permeability to the Ca^{2+} (Wray, 1993).

Capacitative calcium entry through store-operated calcium channels (SOCCs) is an understudied area in the myometrium and smooth muscle generally (McFadzean and Gibson, 2002). They are ion channels which allow calcium influx through cell

membrane as a result of depletion of calcium stores from the SR (Putney and Ribeiro, 2000).

1.5.2 Calcium efflux

Calcium extrusion is an essential step for the relaxation of the myometrium. At the end of each contraction, cytosolic calcium will be pumped in the SR (through SERCA) and/or pumped outside the cell against its gradient (using PMCA and NCX). The PMCA is located in the cell membrane and actively sends the Ca^{2+} outside the cell in exchange of protons by ATP hydrolysis. The sodium-calcium exchanger (NCX) exchanges one calcium ion for 3 sodium ions. Both PMCA and NCX are present in the myometrium (Burdyga et al., 1994, Kosterin et al., 1994, Guerini, 1998). The SERCA, as mentioned earlier, re-sequester the Ca^{2+} into the SR in exchange for protons by ATP hydrolysis. In addition, calcium uptake by mitochondria was proposed in the uterine smooth muscle cells but its involvement was found to be insignificant (Smith, 1996, Shmigol et al., 1999) unlike its role in some other smooth muscle cells (Drummond and Fay, 1996, Kamishima et al., 2000).

1.5.3 Calcium sensitization

Calcium sensitization can be defined as a pathway that causes smooth muscle contraction without affecting intracellular Ca^{2+} concentration, i.e. in a Ca^{2+}-independent manner (Somlyo and Somlyo, 1994b, Somlyo, 1997). Calcium sensitization in smooth muscles is mainly mediated by RhoA/ROK pathway (Taggart et al., 1999). The expression of the mRNA of both RhoA and RhoA-associated kinase (ROK) proteins was detected in human pregnant myometrium (Moran et al., 2002), pregnant and non-pregnant myometrium of the rat (Kim et al., 2003) and the mouse (Oh et al., 2003). The RhoA/ROK pathway acts by the activation of RhoA that activates ROK, which in turn, phosphorylates myocin light chain phosphatase (MLCP) inhibiting its activity which enhances the contraction. The specific inhibitor of the ROK (Y-27632) decreased the agonist-induced Ca^{2+} sensitization of myometrium contractility (Oh et al., 2003, Woodcock et al., 2004). Using Y-27632 caused minor decrease in spontaneous contraction which indicates that this pathway is not essential for uterine spontaneous activity (Kupittayanant et al., 2001).

1.6 Modulation of Force

The uterus is a myogenic organ contracting spontaneously without the need for hormonal or neuronal stimuli. However, properties of the contractions can be controlled by neurotransmitters and hormones. I will briefly discuss them below with most focus on the effect of oxytocin and purinergic signalling, as these were used in part of my study into the effects of acidic pH.

Neuronal stimulation is not needed to initiate myometrial contraction (Wray, 1993). However, the uterus is innervated by sympathetic, parasympathetic and sensory nerves. Innervation of the myometrium is completely lost during pregnancy due to hormonal changes and/or mechanical stretching with complete restoration after delivery, which may rule out the neuronal effect during parturition (Monica Brauer and Smith, 2015).

During pregnancy, the uterus responds willingly to the growing embryo and it stretches allowing more room and the resulting **mechanical stretch** *per se* affects contractility (Rouse et al., 1993, Li et al., 2009, Yin et al., 2018). It is accompanied by activation and/or up-regulation of some channels and receptors in the myometrial cell membrane (Csapo, 1977, Manabe et al., 1983, Loudon et al., 2004, Terzidou et al., 2005, Tichenor et al., 2005, Buxton et al., 2010, Monaghan et al., 2011, Yin et al., 2018). In addition, mechanical stretching may affect $[Ca^{2+}]_i$ (Himpens et al., 1988, Himpens and Somlyo, 1988, Davis et al., 1992).

Sex hormones, namely oestrogen, progesterone and androgen are strong modulators for uterine contraction. Their receptors are expressed in the myometrium and their expression might change during labour (Arrowsmith et al., 2010, Makieva et al., 2014, Renthal et al., 2015). It is thought that **progesterone** is the key in maintaining the uterus quiescent throughout pregnancy via different molecular cascades (Mesiano, 2004, Blanks and Brosens, 2012, Wu and DeMayo, 2017). *In vitro*, progesterone inhibited spontaneous and oxytocin-induced contractions may be by preventing Ca^{2+} entry and SR release (Arrowsmith et al., 2010, Arrowsmith et al., 2016). It may also prevent binding of oxytocin to its receptors (Arrowsmith et al., 2010, Arrowsmith et al., 2016). Studies on the effect of progesterone on K^+ current are inconsistent (Knock et al., 2001, Anderson et al., 2009). Progesterone withdrawal was linked to the initiation of parturition in animals. However, this withdrawal could not be found in human as the progesterone levels remained high during labour (Mitchell and Taggart, 2009). **Oestrogen**, on the other hand, becomes active during parturition (Renthal et al., 2015,

Menon et al., 2016, Sivarajasingam et al., 2016). Its concentration and/or receptor activity increased near term and hence increased uterine contractility (Renthal et al., 2015). There is a growing interest in studying the effect of **androgen** on myometrial activity. So far, androgen was found to have a significant relaxing effect on the myometrium which may make it a possible target for tocolysis, i.e. inhibit uterine contraction to prevent preterm labor (Makieva et al., 2014).

Prostaglandins (PGs) are naturally available and physiologically active prostanoids, lipid compounds, in all living tissues including the uterus. They are produced enzymatically from the fatty acid, arachidonic acid. Their production increased dramatically during labour in response to the influence of mechanical stretch, oxytocin, platelet activating factor, endothelin and nitric oxide (Patel and Challis, 2001). Structurally different PGs have different effect on myometrial contraction. For example, $PGF_{2\alpha}$ and $PGE_{2,}$ increases $[Ca^{2+}]_i$ which enhance the contractility while PGI_2 relaxes the uterus by activating the adenylate cyclase (Hertelendy and Zakar, 2004).

Oxytocin is a nanopeptide hormone produced in the hypothalamus and secreted by the posterior pituitary gland. Its biochemical structure is similar to the arginine vasopressin (AVP) which makes AVP binds to the oxytocin receptors and acts as an agonist (Ph, 2000). Oxytocin is a potent uterine stimulus and it is widely used to augment labour (Blanks and Thornton, 2003, Osilla and Sharma, 2019).

Oxytocin receptors (OTRs) belong to G protein-coupled receptor family (GPCR) and they are upregulated and increased towards labour. However, OTRs may desensitise following continuous stimulation like other GPCRs. Progesterone was found to relax the uterus by binding to OTRs and inhibiting their activation (Kimura and Saji, 1995, Gimpl and Fahrenholz, 2001). Inhibition of OTRs, for example with atosiban, has been used clinically to prevent preterm labour (Kim and Shim, 2006).

The mechanisms by which oxytocin stimulates uterine contractility have been extensively studied. Generally, oxytocin increases intracellular Ca^{2+} concentration by enhancing Ca^{2+} entry from the extracellular space either through voltage-gated L-type channels or ROCCs or both. Also, it increases Ca^{2+} release from SR and inhibits its extrusion by the PMCA (Arrowsmith and Wray, 2014). Due to these additional mechanisms, oxytocin stimulation can be independent from the extracellular Ca^{2+} (Monga et al., 1999). Oxytocin was found also to activate RhoA proteins which then

activate RhoA associated kinase (ROK). ROK then inhibits the dephosphorylation of myosin which prevents relaxation of the uterine myocytes (Somlyo et al., 1999).

Purinergic receptors

There are two types of purinergic receptors; P1 receptors which are known as "adenosine purinoceptors" and P2 receptors which are called "ATP Purinoceptors" (Burnstock, 1978).

Adenosine purinoceptors (P1 receptors)

There are four types of adenosine purinoceptors have been identified (A_1, A_{2A}, A_{2B}, and A_3) (Fredholm et al., 2001). The adenosine receptors subtypes are classified based on their affinity to adenosine molecule, pharmacological profiles, and G-protein coupling (Blackburn et al., 2009). The adenosine A_{2A} and A_{2B} receptors are coupled to $G_{s/olf}$ proteins to stimulate adenylate cyclase and thus the production of cyclic adenosine monophosphate (cAMP). The A_1 and A_3 receptors are coupled to $G_{i/o}$ to inhibit adenylate cyclase (Fredholm et al., 2001, Sheth et al., 2014). The A_1 subtype has been found in human (Tsai et al., 1996) and guinea pig uterine smooth muscles (Smith et al., 1988, Schiemann et al., 1991) and its stimulation lead to contraction of myometrium through adenylate cyclase inhibition (Schiemann et al., 1991).

ATP purinoceptors (P2 Receptors)

There are two subtypes of ATP purinoceptors that present in cell membranes; metabotropic P_{2Y} receptors, which consist of seven membrane-spanning receptors, and ionotropic P_{2X} receptors that are cation-selective channels (Burnstock, 2007). P_{2X} receptors are ligand-gated cation channels that are located on the plasma membrane of most cells in the body including smooth muscles (O'Reilly et al., 2001). They are primarily gated by extracellular ATP, and facilitate the influx of extracellular cations (sodium and calcium) into the cell. Consequently, if they are stimulated, the membrane depolarizes and L-type Ca channels open (Khakh and North, 2006).

The effect of ATP on purinergic receptors in pregnant and non-pregnant myometrium and its contraction has been reported in different species (Gillman and Pennefather, 1998). Studies showed that P_{2X} receptors have a major role in mediating the contractions of uterine smooth muscles during delivery in pregnant rats (Urabe et al., 2009). Furthermore, it was found that ATP was capable of producing myometrial

contractions in women which suggest the presence of ATP purinoceptors in uterine smooth muscles in human (Ziganshin et al., 2006). In addition, ATP triggered tonic and phasic uterine contractions in pregnant myometrium. However, ATP generated phasic contractions in non-pregnant myometrium, which have smaller amplitude and were less frequent compared to pregnant myometrium (Osa and Maruta, 1987).

P_{2x7}, formerly known as P_{2Z}, are ATP-gated cation channels. They are present in different cell types including the myometrium (Urabe et al., 2009, Miyoshi et al., 2010, Miyoshi et al., 2012, Alotaibi, 2018). These channels are similar to the other P_{2x} in that they are permeable to different cations mainly Na^+, K^+, Ca^{2+} (Sluyter, 2017). However, they behave differently in having low affinity to ATP as they are activated by high doses of external ATP (50µM to 2.5mM) and do not desensitize rapidly (Garcia-Marcos et al., 2006, Sluyter, 2017). P_{2x7} are inhibited by copper, zinc, calcium, magnesium and acidification (Virginio et al., 1997, Stojilkovic et al., 2014). External protons act as allosteric modulators as they decrease P_{2x7} current amplitude without affecting their agonist sensitivity (Liu et al., 2009). However, other study reported that protons change the P_{2x7} affinity to bind the external ATP (Virginio et al., 1997). A438079 is the most specific compound used pharmacologically to block P_{2x7} in laboratory settings (Stojilkovic et al., 2014). The signalling cascade of the P_{2x7} channels in uterine myocytes has been little studied. Expression of P_{2x7} in the rat myometrium was found increased in term- and pre-term delivery (Urabe et al., 2009). Recently, it was found that the stimulatory effect of ATP in the uterus was abolished in the presence of A-438079 (Alotaibi, 2018).

1.7 Gestational Differences

Pregnancy induces major physiological changes in the female body generally and the uterus in particular. Furthermore, changes in the expression or activity of some proteins were described between mid-term and labour. I will present here a literature review about the physiological changes happen in the myometrium during pregnancy.

Cells size

Shynlova *et al* work on rat (Shynlova et al., 2006) showed that in the first half of gestation, uterine smooth muscle cells undergo hyperplasia with an increase in anti-apoptotic proteins. This hyperplasia happens as a result of oestrogen activation of IGF1/PI3K/MTOR signalling pathway in the uterine myocytes (Jaffer et al., 2009). While in the second half of the pregnancy, there is an increase in the myocyte size leading to hypertrophy and thickening of the muscle layer and changes in the extracellular matrix (Shynlova et al., 2006). Hypertrophy was linked to the effect of sex hormones, oestrogen and progesterone (Douglas et al., 1988). In addition, mechanical stretch caused by the growing foetus can mediate both hypertrophy and hyperplasia (Douglas et al., 1988, Wu et al., 2008, Shynlova et al., 2010b, Shynlova et al., 2010a).

Blood flow

Blood supply to the growing uterus dramatically increases during pregnancy. This local increase in blood flow was assisted by different hemodynamic changes e.g. increase blood volume and cardiac output with decrease in vascular resistance and blood viscosity (Burton et al., 2009). Angiotensin II activates both angiotensin type-1 receptors to cause vasoconstriction and angiotensin type-2 receptors to mediate vasodilation. In pregnant rat, oestrogen was found to upregulate angiotensin type-2 receptor in the uterine vessels and the vasoconstriction response to angiotensin II was attenuated. Hence, high levels of angiotensin II was found to increase uterine blood flow via enhancing angiotensin type-2 receptor-mediated signalling (Mishra et al., 2018).

Receptor and channel changes

The binding capacity of L-type calcium channels and mRNA expression of α-1 subunit were found to increase throughout pregnancy until term in rats (Mershon et al., 1994, Tezuka et al., 1995) and guinea pig (Collins et al., 2000). Plenty of work on

myometrial oxytocin receptors, including their function and expression, concluded that they are upregulated at the end of pregnancy and during labour (Jurek and Neumann, 2018). Expression of prostaglandin receptors also changes during labour. In rats, prostaglandin receptors (EP_2) which have a relaxant effect on myometrium were found expressed more in day 16 in comparison to parturition and postpartum (Brodt-Eppley and Myatt, 1998). However, expression of FP receptors which have a contractile effect increased significantly during parturition (day 22) in comparison to their low expression at day 16 in pregnant rats (Brodt-Eppley and Myatt, 1998). The β1 subunit of BK_{Ca} channels, which may hyperpolarize the uterus and thus contribute to keeping the pregnant uterus quiescent, was found upregulated in mid-pregnant myometrium in the mouse (Benkusky et al., 2002) and downregulated during parturition in human uterus (Matharoo-Ball et al., 2003). Similarly, expression of K_{ATP} channels was found less in non-pregnant uterus, up-regulated during pregnancy and down-regulated during parturition in human and rat (Curley et al., 2002, Sawada et al., 2005).

Oestrus cycle in mouse and uterine activity

There are four stages of the mouse oestrus cycle lasting for 4-6 days (Cora et al., 2015) (Figure 1.7). They reflect the changes in the concentration of oestrogen and progesterone secreted by the ovarian follicles. Stages are called proestrus, estrus, metestrus, and diestrus (Cora et al., 2015). Some investigators divided oestrus cycle into only three stages; proestrus, estrus, and diestrus depending on the objectives of their study (Goldman et al., 2007). These stages are characterized by the types, numbers and arrangements of four basic cell types present on the slide; namely, neutrophils, small and large nucleated epithelial cells and anucleated keratinized epithelial cells (Cora et al., 2015). In non-pregnant mouse, variabilities in the uterine contractile behaviour in relation to the oestrus cycle were detected (Naderali and Wray, 1999, Dodds et al., 2015). Hence, uterine agonists and antagonists have been found to vary in effect depending on the oestrus cycle (Naderali and Wray, 1999, DeMayo et al., 2002, Kawamata et al., 2004, Griffiths et al., 2006, Dodds et al., 2015).

Given all the changes that occur from the non-pregnant state and also between mid-gestation to term, I therefore investigated and compared, the effects of acidic pH_o in non-pregnant, mid- and term myometrium.

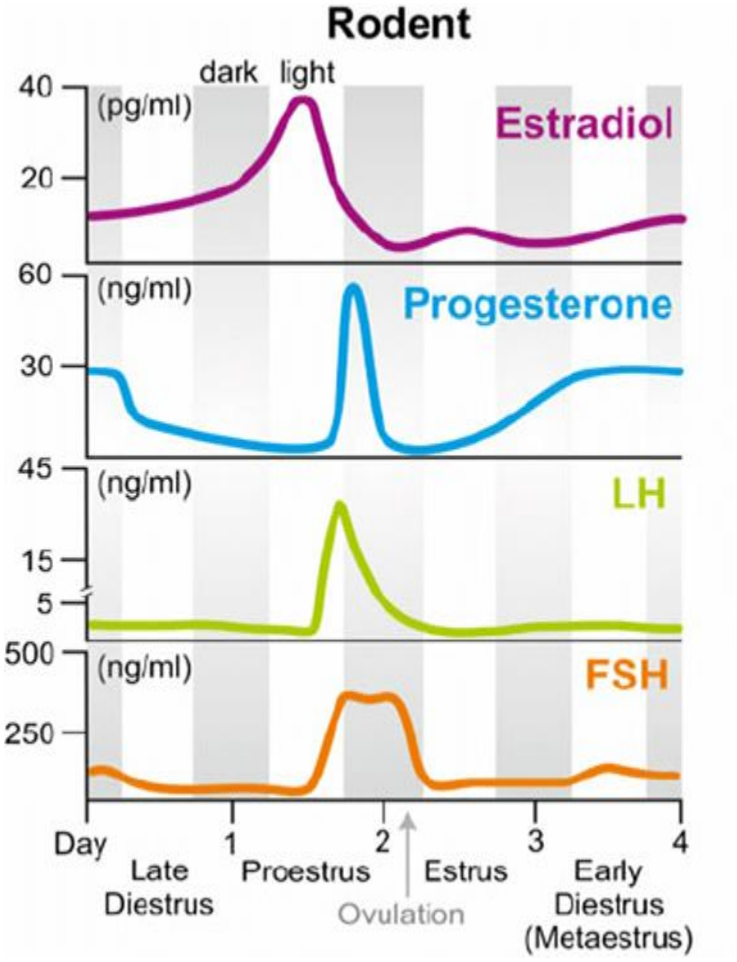

Figure 1.7 shows the oestrus cycle in the mouse over 4 days and the hormonal changes in each stage. (This figure was adapted from Donner 2013)

1.8 Extracellular Space and pH Regulation

The extracellular fluid is the atmosphere surrounding the cells which keeps essential living supplies including nutrition, oxygen, appropriate temperature and elimination of waste. pH is the negative logarithm of the hydrogen ion concentration (Grogono, 1986, Carlson, 1997).

$$pH = -\log[H^+]$$

Thus, the normal pH of extracellular fluid (7.4) is a $[H^+]$ of 40 nM/L while the normal pH inside the cell (7.2) represents $[H^+]$ of 63 nM/L. Thus there is a concentration gradient in favour of proton efflux. However, the cell membrane potential of ≈60 mV favours proton entry into the cell. The proton equilibrium potential (V_H) can be calculated to be (-12.1 mV) at body temperature using Nernst equation as follow:

$$V_H = RT/zF \ In \ [H]_{out} / [H]_{in}$$

Where R is the universal gas constant and is equal to 8.314 $J.K^{-1}.mol^{-1}$, T is the temperature in Kelvin (K = °C + 273.15), z is the valence of the hydrogen ion (+1) and F is the Faraday's Constant = 96485 $C.mol^{-1}$

The plasma membrane is not totally impermeable to protons and thus over several minutes, an elevation of extracellular proton concentration will be transmitted in part to the cytoplasm (Grogono, 1986, Carlson, 1997). Changes in intracellular proton concentration (i.e. pH) are well recognised for causing profound changes in the cellular metabolic processes through changing charge and structure of the proteins inside the cells, e.g. enzymes. Changes of extracellular pH can also alter the structure of ion channels, transporters, receptors and thus their functions (Owicki and Parce, 1992, Zarnowska et al., 2002). Changes of extracelluar pH can also affect cellular immunity by regulating the pro- and anti-inflammatory processes (Lardner, 2001, Okajima, 2013). Currently, targeting tumor extracellular pH by cancer nanomedicines is promising (Tian and Bae, 2012). In the uterus, decreasing the acidic uterine environment in labour, by giving bicarbonate has been shown to improve labour outcome in a small randomized controlled trial (Wiberg-Itzel et al., 2018).

Intracellular acidification may be fatal to the cells and it is prevented by a tight pH regulation (Figure 1.8). Protons will be extruded to the extracellular space, producing extracellular acidification, by different exchangers against their concentration gradient.

Although pH regulation mechanisms are different from cell to cell and its full picture is still under investigation, I will explain it in a broad-spectrum. Regulation of pH_i needs a strict balance between acid extruders and acid loaders. Acid extruders are proteins that send protons to the extracellular space while acid loaders mediate the passive exit of weak bases like bicarbonate (HCO_3^-) (Roos and Boron, 1981, Aickin, 1986, Wray, 1988b, Owicki and Parce, 1992, Boron, 2004). Generally, Na^+/H^+ transports Na^+ into the cell, and H^+ out of the cell and this is balanced by pumping the Na^+ out in exchange of K^+ via Na^+/K^+-ATPase (Owicki and Parce, 1992). Also, H^+/K^+-ATPase transports the proton outside the cell in exchange of K^+ at the expense of ATP (Owicki and Parce, 1992). Protons can be extruded using ATP without the need to be exchanged with other ions through H^+-ATPase (Boron, 2004).

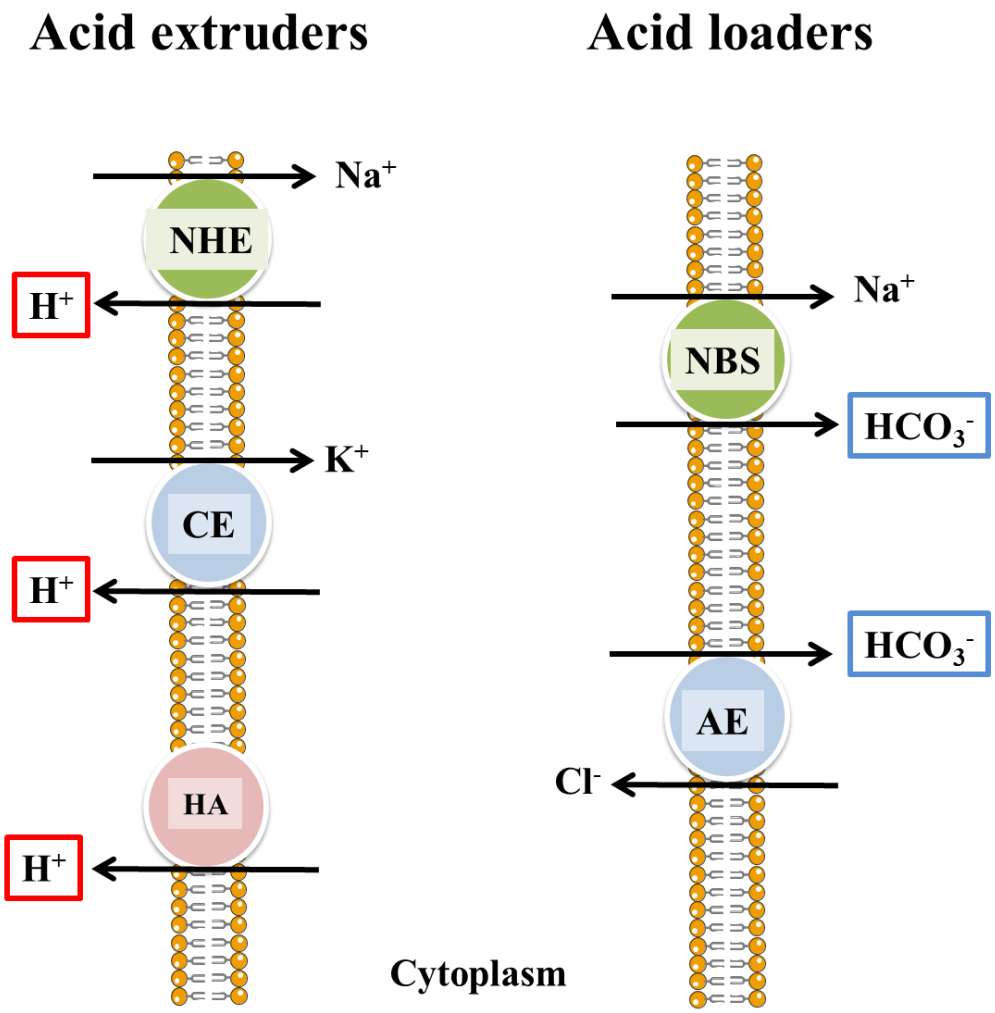

Figure 1.8 Acid extruders and acid loaders.

Regulation of pH_i involves the balance between acid extruders and acid loaders.

NHE= Na^+/H^+ exchange, NBC= Na^+/HCO_3^- cotransporter, CE= Cation exchanger, AE= Anion exchanger, HA= H^+ ATPase

1.9 Extracellular Acidification

Extracellular acidification happens in all living tissues either physiologically or pathologically. It occurs as a result of the cellular metabolic activity, i.e. waste of the aerobic and anaerobic metabolism and proton extrusion due to the regulation of intracellular pH (pH_i) (Roos and Boron, 1981, Boron, 2004). Most mammalian cells excrete around $1x10^8$ H^+ per cell in the form of carbon dioxide and lactic acid as a result of their metabolic activities (Owicki and Parce, 1992).

Extracellular volume is estimated to be around 37% to 57% of the whole myometrial volume which is considered to be high (Miller, 1990). During term, strong myometrial contractions occlude uterine blood vessels resulting in ischemia and hypoxia which produces acidification (Greiss, 1965, Towell and Liggins, 1976, Harrison et al., 1994, Harrison et al., 1995, Larcombe-McDouall et al., 1998, Larcombe-McDouall et al., 1999, Li et al., 2003).

1.9.1 Effects of extracellular acidification on intracellular pH

Changes in extracellular pH (pH_o) will be expected to change intracellular pH (pH_i) but these changes are different in speed, extent and even direction depending on the tissue, animal and even the strain (Table 1.1 and 1.2). In Wistar rats, changes in pH_o produced similar changes in pH_i in the mesenteric artery (Austin and Wray, 1993b, Austin et al., 1996), coronary artery (Ramsey et al., 1994), aorta (Capellini et al., 2013) and portal vein (Taggart et al., 1994). The same results also found in portal vein from guinea pig (Smith et al., 2002b). However, pH_i was not affected by acidifying the pH_o in the aorta from spontaneously hypertensive and Wistar Kyoto rats (Rohra et al., 2003a, Rohra et al., 2005a). Changes in pH_o caused only trivial changes in the pH_i in the ureter from guinea pig (Burdyga et al., 1996).

As other smooth muscles, alteration of extracellular pH changed the intracellular one in the myometrium. Changes in pH_o made same changes in the pH_i in the myometrium from pregnant rat (Wray, 1988a, Taggart et al., 1997b) and non-pregnant guinea pig (Naderali and Wray, 1999). Hence, extracellular acidification is expected to produce intracellular acidification in the myometrium.

1.9.2 Effects of extracellular acidification on smooth muscle contraction

Alteration of the pH has been found to modify the contractility in almost all the muscles; cardiac (Sato et al., 1985, Irisawa and Sato, 1986, Wang et al., 2016, Hu et al., 2017, Macianskiene et al., 2017), skeletal (Lannergren and Westerblad, 1991, Baker et al., 1995, Jubrias et al., 2003, Sood et al., 2014), vascular smooth muscle (Wray, 1988a, Aalkjaer, 1990, Karaki et al., 1992, Smith et al., 1998b, Wray and Smith, 2004) and other smooth muscle (Yamakage et al., 1995, Burdyga et al., 1996, Nakanishi et al., 1999, de Oliveira et al., 2017). The effects of extracellular acidification on smooth muscle contractility are unpredictable. They differ depending on the species and even the strain (Rohra et al., 2003c), muscle types (Yamakage et al., 1995, Burdyga et al., 1995, Nakanishi et al., 1999, Wray and Smith, 2004, de Oliveira et al., 2017) and degree of acidification (Saxena et al., 2012). Effects of pH changes were studied extensively in different smooth muscles. Table 1.1 summarizes the effects of the alteration of extracellular pH on contractility of different smooth muscles. However, only a few studies have been conducted to investigate the effects of changing extracellular pH on uterine contractility. Table 1.2 summarizes the effects of the alteration of extracellular pH on uterine contractility.

1.9.3 Measurement of Intracellular pH

As altering external pH in my experiments was likely to change intracellular pH, I needed to measure pH_i. Techniques used to measure intracellular pH (pH_i) have changed over the past four decades. Early work of Carter *et al* in 1967 used glass microelectrode to measure pH_i in the skeletal muscle (Carter et al., 1967b, Carter et al., 1967a). Twenty years later, this technique was used in smooth muscle, vas deferens by Aickin (Aickin, 1984). Moon & Richards in 1973 used ^{31}P-NMR spectroscopy to quantify pH_i in red blood cells (Moon and Richards, 1973). ^{31}P-NMR spectroscopy provided less invasive method to measure pH_i in myometrial cells (Kushmerick et al., 1986, Wray, 1990). It is however dependent on very expensive NMR spectrometers, and so is not routinely available. More recently, florescent pH indicators were developed and used successfully in measuring pH_i in the uterine myocytes. BCECF (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein) (Tsien et al., 1982) and SNARF (seminaphthorhodafluor) (Whitaker et al., 1991) are the most commonly used pH-sensitive indicator. They are relatively convenient, accurate and compatible with using on thin strips of tissue, allowing simultaneous measurements of contractions.

BCECF is dual-excitation single-emission indicator while SNARF is single-excitation dual-emission one, and compatible with equipment I had access to. Table 1.3 summarizes the characteristics, advantages and drawback of each technique.

Table 1.1 A summary of the experiments done to investigate the effects of extracellular pH alteration on the contractility of different smooth muscles and the mechanisms tested.

All vessels are arteries unless otherwise stated. No change (=); increased (+); decreased (-)

CPA= Cyclopiazonic Acid; **DAF-FM DA**= Nitric Oxide (NO) fluorescent probe; **GP**=Guinea Pig; **Indo-1 & Fura-2** = Calcium fluorescent probes; **MLCP**=Myocin Light Chain Phosphatase; **MTC**= Magnetic twisting cytometry; **NA**=Noradrenaline**; RLIE**=Reference Liquid-Ion Exchanger; **RyR**=Ryanodine Receptors; **SHR**=Spontaneously Hypertensive Rat; **SNARF & BCECF**= pH fluorescent probes; **WKY**=Wistar Kyoto Rat.

References: 1. (Austin and Wray, 1993b) 2. (Austin and Wray, 1995) 3. (Austin et al., 1996) 4. (Smirnov et al., 2000) 5. (Dvoretsky et al., 2000) 6. (Yartsev et al., 2002) 7. (Kim et al., 2005) 8. (Niu et al., 2014) 9. (Rohra et al., 2002a) 10. (Rohra et al., 2002b) 11. (Rohra et al., 2003b) 12. (Capellini et al., 2013) 13. (Klockner and Isenberg, 1994) 14. (Weirich et al., 2004) 15. (Gurney et al., 2003) 16. (Dubuis et al., 2004) 17. (Apkon et al., 1997)18. (Nazarov et al., 2000) 19. (Aoyama et al., 1999) 20. (Kim et al., 2004) 21. (Su et al., 1994) 22. (Rohra et al., 2005b) 23. (Achike et al., 1996) 24. (Taggart et al., 1994) 25. (Smith et al., 2002b) 26. (Arner and Hogestatt, 1990) 27. (Twort and Cameron, 1986) 28. (Yamakage et al., 1995) 29. (Saxena et al., 2012) 30. (Uvelius et al., 1990) 31. (Fry et al., 1994) 32. (Yoon et al., 1995) 33. (Wu and Fry, 1998) 34. (Burdyga et al., 1996) 35. (Nakanishi et al., 1999) 36. (Aickin, 1984) 37. (de Oliveira et al., 2017)

Species	SM	pH_o	Techniques used	Sample used	Buffering and oxygenation	ºC	Findings	Proposed mechanism/s	Ref
Rat	Mesenteric	6.9 7.9	Force SNARF	Muscle strips	HCO_3/ 5% CO_2 and HEPES	37	Acidification (=) or (-) Alkalinization (+) the vascular tone	corresponding changes in pH_i	1
Rat	Mesenteric	6.9	Force Indo-1	Rings	HCO_3/ 5% CO_2 and	37	Acidification (-) Alkalinization (+)	corresponding changes in $[Ca^{2+}]_i$	2

		7.9			HEPES		high-K- contraction and $[Ca^{2+}]_i$		
Rat	Mesenteric	6.9 7.9	Force SNARF Indo-1	Intact muscle strips	HEPES	37	Acidification (-) Alkalinization (+) tone, $[Ca^{2+}]_i$ and pH_i	corresponding changes in pH_i then $[Ca^{2+}]_i$ followed by the muscle tension	3
Human	Mesenteric	6.2 8.2	Voltage clamp	Freshly isolated cells	HEPES		Acidification (-) Alkalinization (+) L-type Ca^{2+} current	alter the membrane potential resulting in changes in the L-type Ca^{2+} channels' gating properties	4
Rat	Mesenteric	6.6 7.0 7.8	Force	Rings			Acidification (-) Alkalinization (+) the vascular tone		5
Rat	Mesenteric	6.6 7.0 7.8	Force	Rings	HCO_3/ 5% CO_2	37	Acidification (=) resting tension Acidification (-) NA- tissue response	Nitric oxide-mediated pathway	6
Rat	Mesenteric	6.4 6.9 7.8	Force Voltage clamp	Rings	HCO_3/ 5% CO_2		Acidification (-) Alkalinization (+) the vascular tone	Altering the Na^+-K^+ pump activity and inwardly rectifying K^+ current	7
Rat	Coronary Renal Mesenteric	6.6 6.8 7.0 7.2	Force BCECF fluo-4 Voltage clamp	Rings Freshly isolated cells	HCO_3/ 5% CO_2	37	Acidification (+) coronary tone Acidification (=) renal or mesenteric tone	Acidification increases $[Ca^{2+}]_i$, enhances L-type Ca^{2+} channels, depresses voltage-gated K^+ channels and activates H^+-K^+-ATPase in coronary arteries	8

SHR and WKY Rat	Aorta	6.5	Force Fura-2	Rings	HCO_3/ 5% CO_2 and HEPES	37	Acidification (+) contraction and $[Ca^{2+}]i$ and that was inhibited by L-VGCC and CaCCs-inhibitors	L-type calcium entry and CaCCs role in depolarization	9
	Aorta	6.5	Force MLCP assay	Rings	HCO_3/ 5% CO_2 and HEPES	37	Acidification (+) contraction and that was inhibited by MLCP blockers	stimulating tyrosine kinases to enhance tyrosine phosphorylation of PI3-kinase, resulting in the MLC phosphorylation	10
WKY Rat	Aorta	6.5	Force	Intact muscle strips	HCO_3/ 5% CO_2 and HEPES	37	Acidification (+) contraction and that was inhibited by ryanodine (RyR) blocker and CPA (a SERCA inhibitor)	Inducing the release of Ca^{2+} from SR through activation of ryanodine- and CPA-sensitive store.	11
Wistar Rat	Aorta	6.5 7.0 8.0 8.5	SNARF DAF-FM DA	Cross sections			Acidification and alkalinization (+) $[NO]_i$ in smooth muscle layer	Nitric oxide-mediated pathway?	12
Bovine Pig	Pial Coronary	6.4 8.4	Voltage clamp	Freshly isolated VSMCs			Acidification (-) Alkalinization (+) L-type Ca^{2+} inward current	Modulating the L-type channel gating and conductance	13
pig	Coronary	6.5 8.0	Force BCECF fluo-4	Intact muscle strips			Acidification (-) Alkalinization (+) $[Ca^{2+}]_i$, BHQ contraction and high-K response	Through SOCE?	14
Rabbits	Pulmonary	6.3	Voltage	Freshly			Acidification depolarized	Modulating the TASK-1	15

		8.3	clamp	isolated cells			Alkalinization hyperpolarized the cells	channels current	
Rat	Pulmonary	6.8	Force Fura-2	Rings	HEPES		Acidification abolishes the effect of repeat applications of ATP on force and $[Ca^{2+}]_i$	alteration in the desensitization-resensitisation characteristics of the ATP receptor	16
Rat	Cerebral	6.9 7.8	MPEG movie sequences	Freshly isolated Cells	HCO_3/ 5% CO_2	37	Acidification (-) Alkalinization (+) contraction	Motor responses of the VSMCs to the changes in pH_o are intrinsic and independent from endothelial or neuronal intervention.	17
Rat	Cerebral	6.9 7.8	BCECF Fura-2	Freshly isolated Cells	HEPES		Acidification (-) Alkalinization (+) $[Ca^{2+}]_i$	Through modulating Ca^{2+} influx.	18
Rabbit	Basilar	6.9 7.9	Force BCECF Fura-2	Circular muscle strips	HEPES	37	Acidification (-) Alkalinization (+) agonist-induced contraction	Corresponding changes in the $[Ca^{2+}]_i$	19
Rabbit	Basilar	6.8 7.0 7.9	Force Voltage clamp	Rings Freshly isolated Cells	HEPES		Acidification (-) Alkalinization (+) high-k^+ - and histamine-induced contractions	By modulating Ca^{2+} inward current	20
Cat	Ophthalmo-ciliary	6.0 8.0	Force	Rings	HCO_3/ 5% CO_2	37	Acidification and alkalinization (-) the NA and high-K^+ response Acidification (-)		21

							$PGF_{2\alpha}$ response		
Rat	Internal Mammary	6.8	Force	Rings	HCO_3/ 5% CO_2 and HEPES	37	Acidification (=) resting tension Acidification (-) agonist-stimulated contraction	activates K_{ATP} channels and inhibits L-type Ca^{2+} entry	22
Rat	Tail artery	7.0 7.6	Force	Intact muscle strips	HCO_3/ 5% CO_2	37	Acidification (-) Alkalinization (+) the vascular tone	Acidotic vasodilatation is through L-type Ca^{2+} channels Alkalotic vasoconstriction involves the ROCCs	23
Rat	Portal vein	6.9 7.9	Force SNARF	Intact muscle strips	HEPES	35-37	Acidification (-) Alkalinization (=/+) force	Effects produced by changing pH_i are more sig. than those produced by changing pH_o	24
Guinea-pig	Portal vein	6.9 7.9	Force SNARF Indo-1	Intact muscle strips	HEPES	35	Acidification (-) Alkalinization (+) agonist-induced tone (KCl and NA), $[Ca^{2+}]_i$ and pH_i		25
Human	Hand vein	6.9 7.6	Force	long ring segments	HCO_3/ 5% CO_2		Acidification (=) Alkalinization (+) NA-contractile response. Both (=) high-K-induced contraction	α1 + α2 adrenoceptor channels.	26
Rat	Trachea	6.7 6.9	Force	Intact muscle	HCO_3/ 5% CO_2	37	Acidification (+) Acetylcholine-induced tension	By modulating Ca^{2+} entry	27

		7.8		strips					
Dog	Trachea	7.0 7.8	Force BCECF Fura-2	Intact muscle strips	HCO_3/ 5% CO_2	37	Acidification (-) Alkalinization (+) high-K^+ - induced $[Ca^{2+}]_i$ Acidification (=) Alkalinization (+) high-K^+-induced contraction.	acidification may sensitize the contractile elements to Ca^{2+} or activate a Ca^{2+}-independent contractile mechanism	28
Human	Trachea and bronchi	6.4 6.8 7.2 7.6	MTC Fura-2	Freshly isolated cells			Acidification (+) contraction, Pi hydrolysis and Ca^{2+} mobilization in ASMCs	Through proton-sensing OGR1 and Ca^{2+} mobilization	29
Rat	Urinary Bladder	6.75 7.85					Acidification (-) response to nerve stimulation	Low pH_o causes prejunctional inhibition of nerve induced contraction	30
Human	Urinary Bladder	7.6 6.8	Voltage Clamp Fura-2	Freshly isolated cells	HCO_3/ 5% CO_2		Acidification (-) $[Ca^{2+}]_i$	Decreasing Ca^{2+} influx	31
Rabbit	Urinary Bladder		Force	Intact muscle strips	HCO_3/ 5% CO_2		Acidification (-) contractility		32
Guinea-pig	Urinary Bladder		Voltage clamp BCECF	Freshly isolated cells	HCO_3/ 5% CO_2	37	Acidification (-) the Ca^{2+} transient in carbachol-induced and high-K^+ induced	Reducing the filling of intracellular Ca^{2+} stores via preventing Ca^{2+} entry through	33

			Fura-2				contractions	cell membrane.	
Guinea -pig	Ureter	6.8 8.0	Force SNARF Indo-1	Intact muscle strips	HEPES	37	Acidification (=/-) force, pH_i and $[Ca^{2+}]_i$		34
Guinea -pig	Vas Deferens	7.0 7.8	Force	Intact muscle strips	HEPES	37	Acidification (+) Alkalinization (-) ATP-induced contraction Acidification (-) Alkalinization (+) NA-induced contraction		35
Guinea -pig	Vas Deferens	6.5 7.7	Force RLIE	Intact muscle strips	HCO_3/ 5% CO_2		Acidification depolarized Alkalinization hyperpolarized Membrane potential	Through altering the membrane potential	36
Rat	Gastric fundus & duodenum	6.0	Force	Intact muscle strips	HCO_3/ 5% CO_2		Acidification (-) gastric muscle response to agonists Acidification (=) duodenal muscle response to agonists.	selectively inhibiting the Gq/11 protein signalling pathway	37

Table 1.2 A summary of the experiments done to investigate the effects of extracellular pH alteration on the uterine contractility and the mechanisms tested. P=pregnant, NP=non-pregnant

References: 38. (Wray, 1988a) 39. (Heaton et al., 1992) 40. (Crichton et al., 1993) 41. (Shmigol et al., 1995) 42.(Taggart et al., 1997b) 43. (Naderali and Wray, 1999) 44. (Pierce et al., 2003) 45. (Hong et al., 2013) 46. (Heyman et al., 2013) 47. (Alotaibi et al., 2015) 48. (Kyeong et al., 2016)

Species	Gest-ation	pH_o	Technique used	Sample used	Buffering and oxygenation	Temp °C	Findings	Proposed mechanism	Ref
Rat	P	6.4	^{31}P NMR	Intact myometrial strip	HCO_3/ 5% CO_2		Changes in pH_o were transmitted within small limit into changes in pH_i which indicates a strict regulation of pH_i in the myometrium.		38
Rat	P & NP	7.6 7.8 8 8.4	FORCE & SNARF	Intact myometrial strip	HEPES or HCO_3/ 5% CO_2	37	Pregnant- alkalinization (-) uterine contraction NP- alkalinization (=) uterine contraction	Reduction of external $[H^+]$ inhibits Ca^{2+} entry.	39
Rat	NP	6.7 7.7	a-toxin permeabilization	Intact myometrial strip	HEPES	20-22	Acidification (+) Alkalinization (-) Ca^{2+}-activated force	Reciprocal changes in $[Ca^{2+}]_i$ may explain the pH_o effect	40
Rat	P	6.9 7.9	Whole cell patch clamp	Single myometrial cell	HEPES	22	Acidification (-) Alkalinization (+) Ca^{2+} current	Changes of the pH affect the membrane Ca^{2+} current but not the outward K^+ currents.	41

Rat	P	7.9 8.4	Force & SNARF & Indo-1	Intact myometrial strip	HEPES or HCO_3/ 5% CO_2 or HEPPSO for 8.4	36	Alkalinization (-) contractions and $[Ca^{2+}]_i$ Alkalinization (=) membrane K^+ conductance.	1. Changes in pH_i do not mediate the effects of pH_o. 2. Alkalinization prevents Ca^{2+} entry. 3. Protons may have no role on the membrane potential	42
Guinea-pigs	NP	6.4 8.4	Force & SNARF	myometrial strip	HEPES	36	Acidification (+) contractions in both day 7 and day 15 myometrium with no difference between them. In day 7, alkalinization (-) contraction massively. In day 15, alkalinization (-) contractions in 40% of preparations while increased them in the other 60%	1. Oestrous cycle has a minor effect on contractile changes induced by the change in pH_o in guinea-pig. 2. the effects of pH_o on force in guinea-pig were not due to the corresponding changes in pH_i	43
Human	P	6.9 7.9	Force & Indo-1	myometrial strip	HEPES	36	Acidification (+) the frequency but Acidification (-) the amplitude of the spontaneous contractions and Ca^{2+} transients. Alkalinization (+) the amplitude and Ca^{2+} transients Alkalinization (-) the frequency of the contraction.	1. Changes in amplitude in response to pH_o may be due to induced changes in L-type Ca^{2+} entry. 2. While causes of changes in frequency in response to pH_o are still unclear.	44
Mouse	P & NP	6.4	Force	Circular myometrial strip	HCO_3/ 5% CO_2	36	Acidification (+) circular uterine spontaneous contraction in pregnant more than NP mice.	By inhibition of TASK-2 channels.	45

Human	P	6.0	Patch clamp	freshly isolated uterine SMCs	HEPES		Acidification (-) Alkalinization (+) TEA-insensitive K^+ currents (TREK-1) in pHUSMC was	Through TREK-1 channels.	46
Rat	P	6.9 7.9	Force	myometrial strip	HEPES	37	Acidification (-) spontaneous contractions. Alkalinization (+) spontaneous contractions.		47
Mouse	P & NP	6.4	Force & Patch clamp	Longitudinal myometrial strip	HCO_3/ 5% CO_2	36	Acidification (+) contractility in pregnant longitudinal myometrium	Through inhibition of TASK-2 and NIOK channels.	48

Table 1.3 Summary of the characteristics of techniques used to measure intracellular pH

References: (Iles, 1981, Wray, 1988b, Szmacinski and Lakowicz, 1993, Chow et al., 1996, Han and Burgess, 2010, Loiselle and Casey, 2010)

	pH-Sensitive Microelectrodes	**^{31}P-NMR**	**Fluorescent pH Indicators**	
			BCECF	**SNARF**
When it was discovered?	1967	1973	1982	1991
pH indicator used		resonant frequency of phosphorus-31 (^{31}P) present in inorganic phosphate (Pi)	Carboxy-fluorescein	Benzoxanthene Dyes
Excitation wavelength			Dual-excitation 490 and 440 nm	Single-excitation between 488nm and 530nm
Emission wavelengths			Single-emission 535 nm	Dual-emission 580/640 nm
Machine needed		Spectrometer	Inverted microscope, xenon lamp, dichroic mirrors, photomultiplier tubes (PMT)	
Useful pH range		Physiological pH limits	6.5–7.5	7.0–8.0
pKa		6.8	7.0	7.5
Used in smooth muscles	Yes	Yes	Yes	Yes
Used in uterus	No	Yes	Yes	Yes
Type of tissue needed	Whole-cell or strip	Whole-tissue	Either individual cell or group of cells.	
Advantages	Can record both H^+ conc. and ion influx	Least-invasive	High signal to noise ratio and more pH-specific	

Drawbacks	Can cause damage when penetrate small cells like SMCs	low time resolution (5 minutes at best)	Needs acetoxymethyl ester (AM) to allow crossing the cell membrane	
		Non-specific pH_i measurement for both cytoplasm and cytoplasmic organelles	Signal may be fading slowly due to leakage of the dye outside the cell or because of the photobleaching	
	Difficult to apply in contracting muscles	low sensitivity and needs a large sample	Their fluorescence emission intensities are dependent on the concentration of the probes	
		Not good for measuring cytoplasmic pH above pH 7.7		
	It could measure other cations	Technically difficult to perform		
Other pH indicators might be used in this technique	• pH-sensitive glass microelectrodes • H+ -selective liquid membrane microelectrodes • recessed tip microelectrode • Double barreled • microelectrodes	• **^{1}H-NMR** used protons in the histidine ring • **^{19}F-NMR** used fluorinated α-methylamino acid	BCECF 4 BCPCF 11	• SNAFLs • SNAFRs • SNARFs

1.10 Aims

Due to the interesting and important role of extracellular acidification in smooth muscle contraction, and the limited work done to investigate this in the uterus, and the possibility of its involvement in parturition, I focused this work to investigate the effect of external acidification on mouse uterine contractility, to study some possible mechanisms by which it occurs and to find if there are any gestational differences. The aims of this study were to:

1. Investigate the effect of extracellular acidification on uterine contraction from mid-, term- and non-pregnant mice.
2. Investigate the effect of extracellular alkalinization on pregnant uterine contraction.
3. Investigate the effect of extracellular acidification in the presence of oxytocin.
4. Investigate the effect of altering intracellular pH on mouse uterus.
5. Investigate the relation between extracellular acidification and intracellular pH.
6. Investigate the relation between extracellular acidification and intracellular Ca^{2+}.
7. Study some mechanisms that might underlie the effect of extracellular acidification.

Chapter Two

General Materials And Methods

Chapter-2
General Materials and Methods

In this chapter, I will generally explain the materials and methods used in this thesis. More details about specific methods and related chemicals will be found in the relevant results chapters, as required.

2.1 Animal Tissue

What?

Female C57BL/6J mice (Charles River, UK) were used in all the work performed in this thesis unless otherwise stated. Pregnant uterine tissue was collected either as mid-gestation from 14 days pregnant mice, or as term from 18 or 19 days pregnant mice. Non-pregnant uterine tissue was taken from virgin female mice at 8-10 weeks of age.

Why?

Animal uterus is more easily available than human samples and it allows testing at different gestational age. It allows easy access to transgenic models if it is needed. Mouse tissue is commonly used as a model for human tissue (Matthew et al., 2004, Matsuki et al., 2017).

Tissue collection

Animals were culled following the code of practice for the humane killing of animals under the UK Home Office guidelines under the UK Animals (Scientific Procedure) Act 1986. Mice were placed in a CO_2 chamber and subjected to a rising CO_2 concentration. Death was confirmed by cervical dislocation. Pups were delivered manually by caesarean section and culled via cervical dislocation. The uterus was then removed and placed straight into fresh buffered physiological saline solution (PSS solution, composition given below). The tissue was used for contractility experiments and/or frozen on day of collection.

Tissue Dissection

The whole mouse uterus was placed on a shallow dissection dish and covered with fresh PSS at a room temperature under a Nikon light dissection microscope (Micro Instruments Ltd, Oxford) and 10x objective for fine dissection. Uterus was cut longitudinally and opened out and cleaned from clotted blood, placenta and any connective tissue. Then, 4-8 strips measuring 2mm x 10mm were dissected containing all the 3 layers of the uterus i.e. the endometrium, the myometrium and the perimetrium. Each strip was tied with surgical silk thread in both ends in preparation for mounting in organ bath. Once dissection was completed the strips were moved to the organ bath in PSS for isometric force measurement (Figure 2.1).

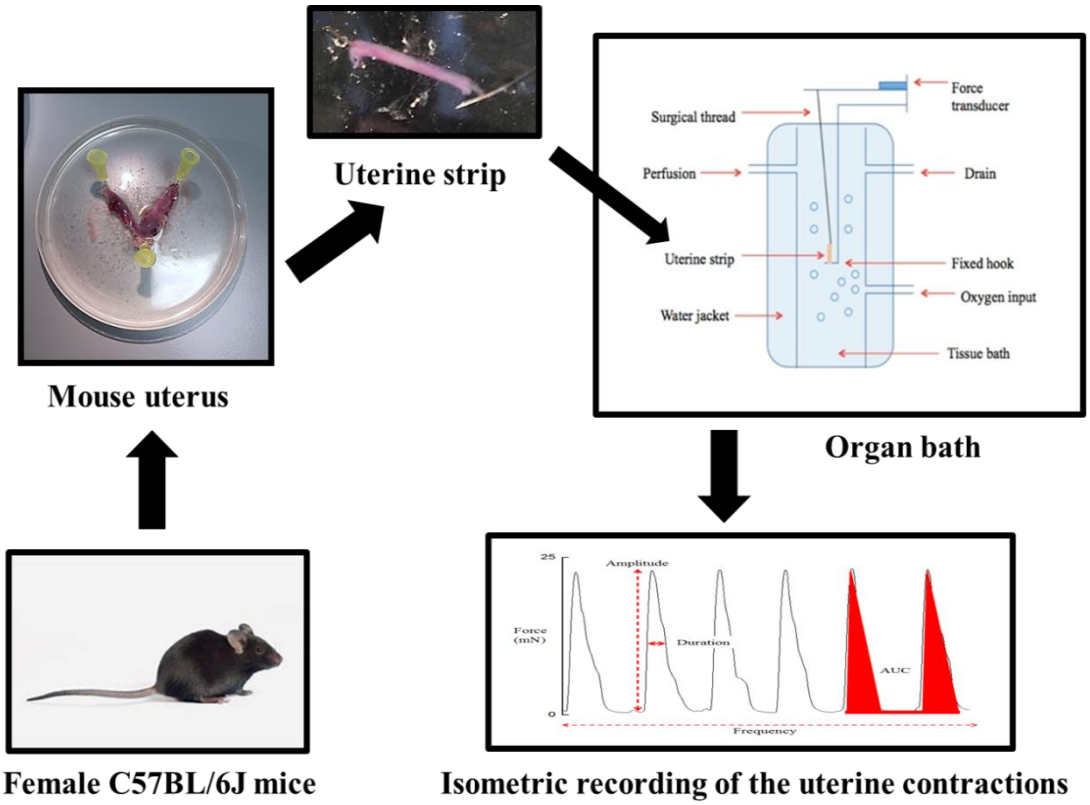

Figure 2.1 Isometric recording of the uterine contraction

Uterine strips, measuring 2mmx10mm, were dissected from mouse and then, each strip was tied with surgical silk thread to both ends. Next, strip was mounted in the organ bath and continuously superfused with PSS (pH 7.4) at 37ºC and bubbled with 100% O_2.

2.2 Solutions and chemicals

2.2.1 Physiological solution

Physiological saline solution was freshly prepared on the day of experiment and buffered to a 7.4 pH in the following composition:

Physiological salt solution (PSS)	**M.W**	**mM**	**g/L**
Sodium Chloride (NaCl)	58.44	154	9.0
Potassium Chloride (KCl)	74.55	5.6	0.42
Magnesium Sulfate ($MgSO_4$)	246.47	1.2	0.29
HEPES	238.30	10.9	2.6
Glucose	180.16	8	1.4
Calcium Chloride ($CaCl_2$)	110.98	2	2 ml/L
Distilled water (dH_2O)			1.0 litre

2.2.2 Test solutions used to alter extracellular pH (pH_o)

Main test solutions were used to investigate the effects of extracellular pH alteration on uterine contractility. For extracellular acidification, pH of the PSS was dropped to 6.9 by adding strong acid (HCl). In some experiments, pH_o 6.5 was needed then, PIPES (10.9 mM = 3.3g/L) was used to buffer PSS instead of the HEPES and the pH_o was decreased to 6.5 using HCl. For extracellular alkalinization, pH of the PSS was increased to 7.9 by adding strong base (NaOH). Previously, it has been shown that similar data were generated regardless of whether the pH was buffered using HEPES or HCO_3/CO_2 system (Heaton et al., 1992, Albrecht et al., 1996, Kilarski et al., 1998, Pierce et al., 2003).

2.2.3 Test solutions used to alter intracellular pH (pH_i)

These solutions were made by isosmotic replacement of NaCl with either weak acids or bases accordingly as follows:

Solution	M.W	mM	g/L	NaCl (M.W)	NaCl (g/L)
Intracellular acidification using sodium butyrate	110.1	20	2.2	134	7.8
Intracellular alkalinization using ammonium chloride (NH_4Cl)	53.5	20	1.0	134	7.8

2.2.4 High potassium PSS solution

High potassium PSS solution (known as High-K^+ solution) was made by isosmotic replacement of NaCl with KCl as follows:

Solution	M.W	mM	g/L	NaCl (M.W)	NaCl (g/L)
High Potassium solution (KCl)	74.5	40	2.9	114	6.9

2.3 Drugs and agents

2.3.1 Oxytocin

Oxytocin (Sigma-Aldrich, UK) is a naturally occurring hormone used for induction of labour in clinical practice. It has been used in this work to augment myometrial contraction. It was aliquoted as 1mM in distilled H_2O at -20^oC. For the experiments here, concentration of 0.5nM was used as a final working concentration.

2.3.2 Nifedipine

Nifedipine was purchased from Sigma-Aldrich (N7634) and used to block L-type calcium channels (Brown et al., 2007). It was aliquoted as 1mM in distilled H_2O at -20^oC and a final concentration of 1nM was used in this thesis.

2.3.3 ATPγS

ATPγS was purchased from Sigma-Aldrich, UK **(**A1388). ATPγS was used as a non-hydrolysed ATP analogue (Chen and Lin, 1997). It was used at a final concentration of 100µM.

2.3.4 A-438079

A-438079 hydrochloride hydrate was purchased from Sigma-Aldrich, UK (A9736). It was used as a selective P_{2X7} purinoceptor antagonist (Miyoshi et al., 2010). It was aliquoted as 10mM in distilled H_2O at -20^oC and a final concentration of 5µM was used to perform the experiments in this thesis.

2.3.5 Niflumic acid

Niflumic acid was purchased from Sigma-Aldrich (N0630) and used to block calcium-activated chloride channel (CaCCs) selectively (Jones et al., 2004). It was aliquoted as 100mM in DMSO at -20^oC and a final concentration of 30µM was used in this thesis.

2.3.6 Amiloride

Amiloride was purchased from Sigma-Aldrich (A7410) and used in this thesis as a pan-ASICs inhibitor (Kellenberger and Grutter, 2015). It was aliquoted as 100mM in DMSO at -20^oC. For the experiments performed here, concentration of 100µM was used as a final working concentration.

2.4 Isometric Force Measurement in the Organ Bath

2.4.1 Calibration of Force

The force transducer converted the uterine contractions into electrical signals which were then amplified (LabTrax 4-Channel Data Acquisition, World Precision Instruments, USA) and transformed into digital signals on the computer using LabScribe 3 software (iWorx Systems, Inc, USA). For calibration of the force in mN, I had to compare the traces of uterine contractions to a known amount of force. This was achieved by suspending a known weight from the force transducer and measuring its deflection then the force was found out using the equation: $N=kg*m/s^2$; i.e. 0.1g = 1mN.

2.4.2 Mounting the tissue in the organ bath

The uterine strips were mounted vertically in a 10 ml organ bath (World Precision Instruments, UK), containing PSS using surgical silk thread that attaching one end to a fixed hook and the other end to an isometric force transducer (Figure 2.2). The strips were continuously perfused with the PSS (pH_o 7.4) at a rate of 5 ml/min using peristaltic pump (Peri-Star Pro, World Precision Instruments, UK), and the temperature was maintained at 37^oC during all the experiments using a water bath (Grant Instruments Ltd, Cambridge). The strips were then stretched by 5 mN resting tension and were allowed to equilibrate in the PSS while bubbled with 100% O_2 for 30-60 minutes before starting the experiments. Experimental protocols were explained in details in the related chapters. Figure 2.3 shows time-control traces of the uterine contractions from the three different gestational stages investigated in this thesis.

2.4.3 Exclusion Criteria for Experiments

In every experiment, four identical tissue strips from the same animal were mounted on four identical organ baths. Unstimulated stripes with irregular or no contractions after 30-60 minutes were eliminated.

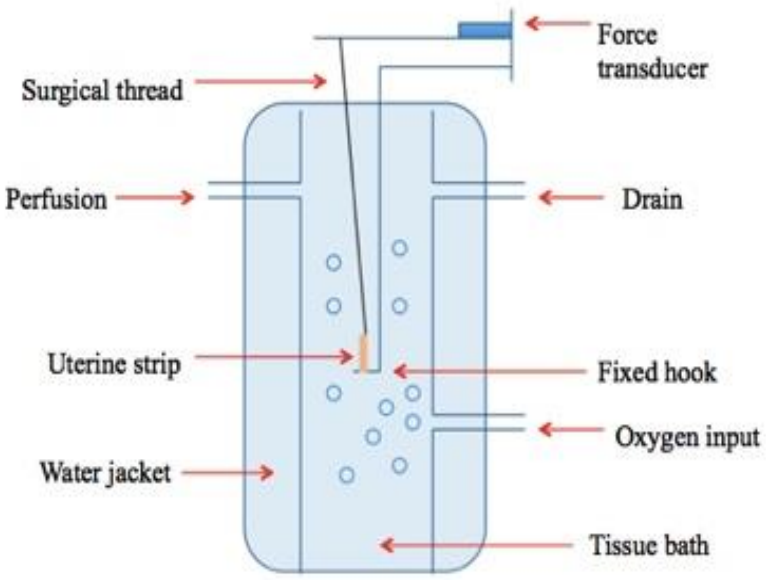

Figure 2.2

The organ bath (10ml) used for isometric force measurement.

2.5 Measurement of Contractile Parameters and Statistical Analysis

2.5.1 Data Analysis

Recorded data were analysed using Origin Pro 2015 software (OriginLab Corporation, USA). Four contractile parameters were measured and normalized against percentage of control (100% control). Specifically, they are amplitude, duration, frequency, and area under the curve (Figure 2.3).

Amplitude

The amplitude (mN) of the contraction was measured from the baseline of a contraction to the peak of the same contraction. It was determined by averaging all measured amplitudes for every contraction in the control period (i.e. 10 minutes before applying the test solution) and the test period (i.e. 10 minutes of applying the test solution).

Frequency

The frequency of contractions was measured, over 10 minutes time, during control and test periods.

Duration

The duration of contraction is represented by how long each contraction lasts for (in minutes) and was measured at half-maximal height of the amplitude.

AUC

The area under the curve (AUC) of the uterine contractions represents the overall uterine activity in a certain period of time. It was measured, during the 10mins of the test period and compared to its equivalent during the 10mins of the control period (i.e. just before the test period)

2.5.2 Statistical Analysis

Origin Pro 2015, Microsoft Excel 2010 and GraphPad Prism-5 were used to analyse the data and produce graphs. Data are presented as Mean± SEM using paired or unpaired Student's t-test for comparing two groups and One-way ANOVA test were used as appropriate and detailed in the related chapters. The Bonferroni test was used as a post hoc test. $P < 0.05$ was accepted as statistically significant. (n) is the number of uterine strips used in each experiment, one from each animal.

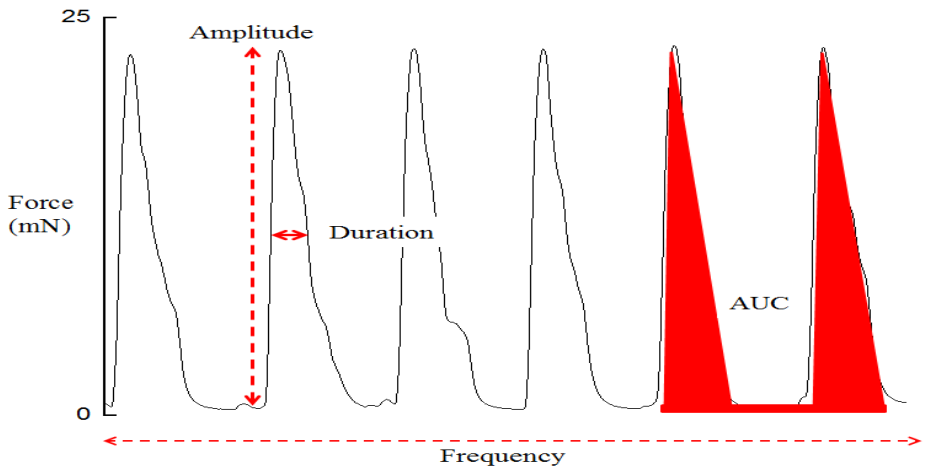

Figure 2.3 Measurement of the uterine contractile parameters

A diagram showing how the elements of contraction were analysed from the uterine contraction as the amplitude, duration (half-maximal the amplitude), frequency and area under the curve (AUC, red triangles).

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2.6 Simultaneous Measurement of the Force and Intracellular pH

Measuring intracellular pH (pH_i) in relation to the changes in the extracellular pH was of a paramount importance. In some experiments, I measured the effect of extracellular acidification on the uterine contractions simultaneously with the corresponding changes in the pH_i.

2.6.1 Carboxy-SNARF-1 AM

Changes in intracellular pH were recorded using a ratiometric cell-permeant pH indicator 5-(and-6)-Carboxy SNARF™-1, Acetoxymethyl Ester, Acetate (Carboxy SNARF-1 AM, C-1272, Invitrogen, Molecular Probes, Life Technologies Ltd, UK) (Taggart and Wray, 1993a, Parratt et al., 1995a, Naderali et al., 1997, Hanley et al., 2015). Carboxy SNARF-1 AM is single excitation-dual emission fluorochrome which is excited at one wavelength, between 488nm and 530nm and the emission at two wavelengths, 580nm and 640nm are recorded. It is a pH sensitive fluorophore, its acidic form emits at 580nm while its basic form emits at 640nm (Figure 2.4) (Buckler and Vaughan-Jones, 1990, Han and Burgess, 2010). Carboxy-SNARF is not able to cross the cell membrane on its own due to its charges. Therefore, acetoxymethyl ester (AM) covers the carboxylic group and makes SNARF neutral so, it can cross the cell membrane. When carboxy-SNARF enters the cell, esterases will cleave the AM groups (Figure 2.5).

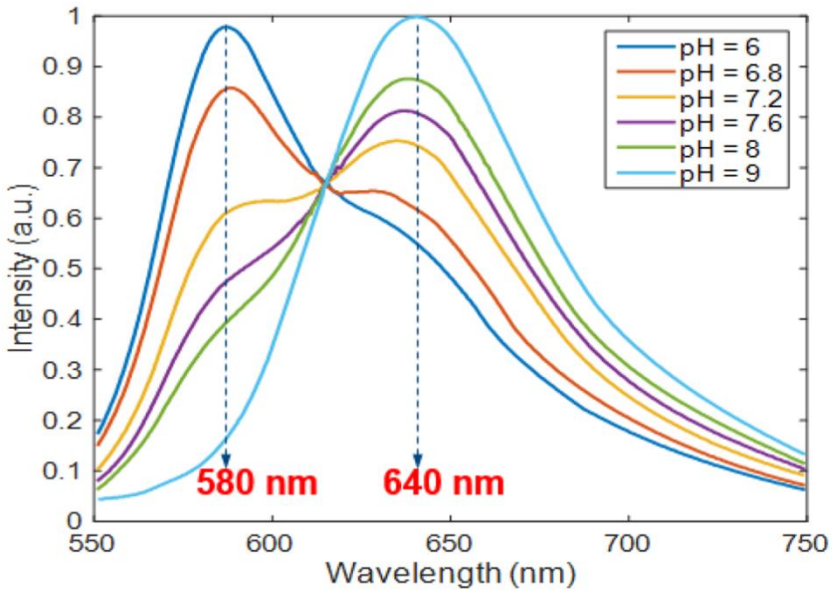

Figure 2.4

Emission spectra of carboxy SNARF-1 AM at various pH values. (Adapted from (Kateklum, 2018)

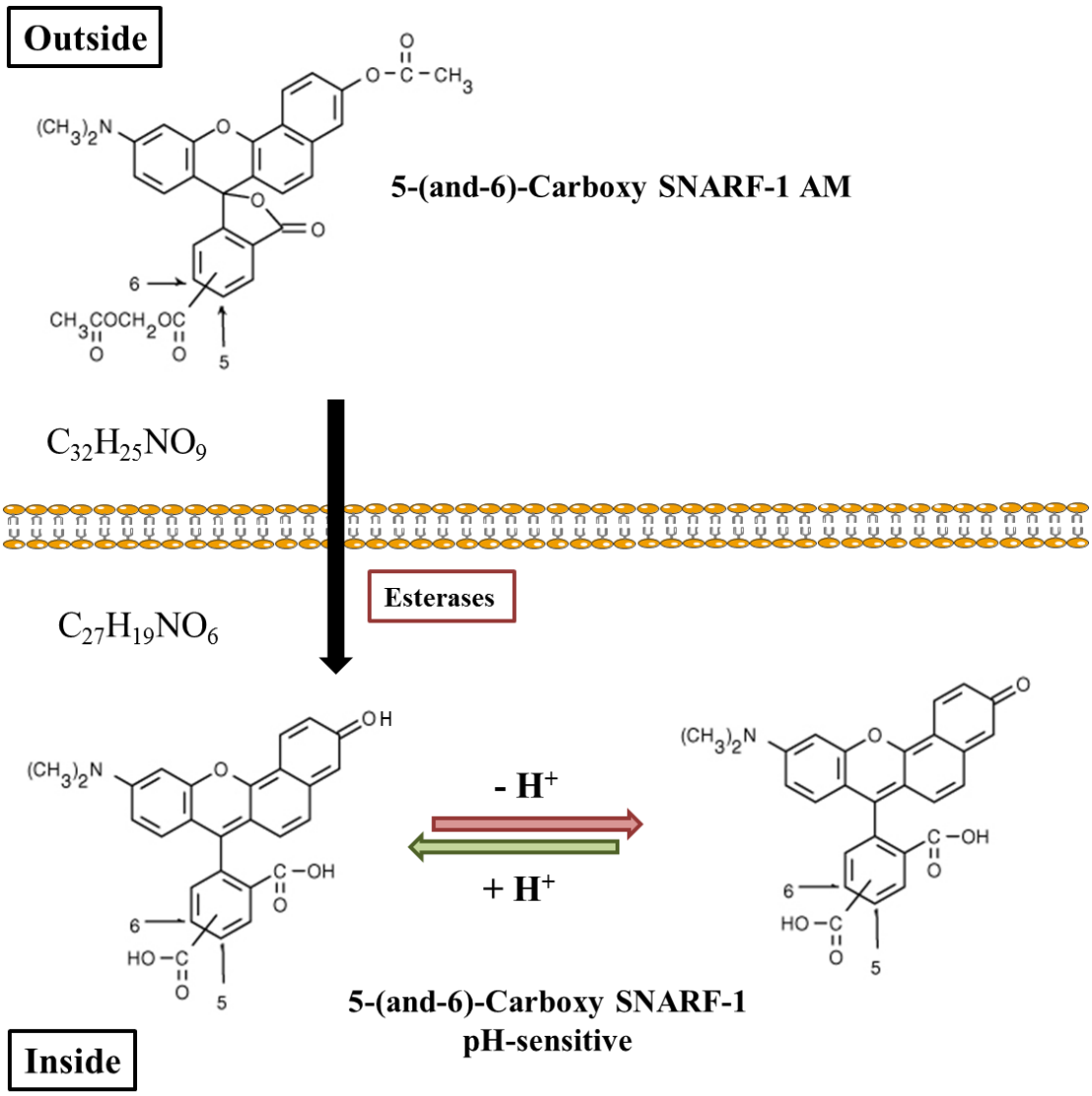

Figure 2.5 A scheme showing carboxy-SNARF-1 structure before and after crossing the cell membrane

AM esters protect the carboxylic groups and makes it neutral, then it can cross the cell membrane. Once enters the cell, esterases will cleave AM groups. This process release the charged indicator and make it pH-sensitive.

2.6.2 Dissection of the myometrial strips

Dissecting the uterine tissue for measuring pH_i is different from previously described (section 2.1). Strips should be smaller and thinner to provide good signaling of the pH fluorescent. In the dissection dish under the light microscope, uterus was cut longitudinally and opened out and cleaned from clotted blood, placenta and any connective tissue. Endometrial layer was gently rubbed off with cotton buds then 7-10 longitudinal myometrial strips (1mm x 1mm x 5mm) were dissected avoiding any underlying circular smooth muscles.

2.6.3 Loading uterine strips with carboxy-SNARF-1 AM

DSMO-Pluronic mix solution previously prepared by adding 0.05g Pluronic acid to 200µL of an anhydrous dimethylsulfoxide (DMSO), both from Sigma-Aldrich, UK. Pluronic acid dissolving into DMSO was assisted by gentle warming. Next, 50µg of carboxy-SNARF-1 AM was added to 50µL of this mix and vortexed to form a final concentration of 12.5µM in the loading solution. Then, the loading solution was diluted into 4ml PSS containing no more 6 myometrial strips in each of the 5ml vials. Vials were kept on a rotator plate protected from light for 2 hours at room temperature. Loaded strips were then washed with fresh PSS for at least 10-15 minutes before used in the experiments to allow complete hydrolysis of the AM esters to avoid artefacts.

2.6.3 Simultaneous measurement of the force and intracellular pH

Loaded strips were then transferred back into dissection dish and aluminium clips were applied to their ends. Next, strips were taken to a dark room in which all intracellular pH experiments were performed in to lessen photobleaching. Then, strips were mounted on an inverted microscope (Nikon Diaphot, World precision Instruments Ltd.) in a 1ml horizontal tissue bath and viewed with 20× objective lens. They were mounted with one clip attached to the fixed hook and the other clip attached to the transducer with PSS (pH7.4) perfusing continuously at a rate of 1ml/min and at 35-36ºC. Axoscope software was used for data acquisition from both the force transducer and the photomultiplier tubes (PMT).

The mounted strip was stretched to a resting tension of 2mN and was allowed to equilibrate for at least 30-60 minutes. The objective was focused onto the myometrial

strip which was then excited with a light of wavelength 530nm by UV illumination from a xenon lamp. Emitted light collected at the wavelength of 580nm and 640nm by PMT. Light path of the carboxy SNARF-1 is described in the legend of Figure 2.6.

When myometrial contraction reached a steady state, acidic solution pH 6.9 was added for 10 minutes. Then the perfusate was returned to PSS (pH 7.4) for 20-30 minutes recovery period. The changes in intracellular pH were measured by the changes in carboxy-SNARF-1 AM ratio that was accompanied by a shift in the opposite direction of both 580nm and 640nm emission signals.

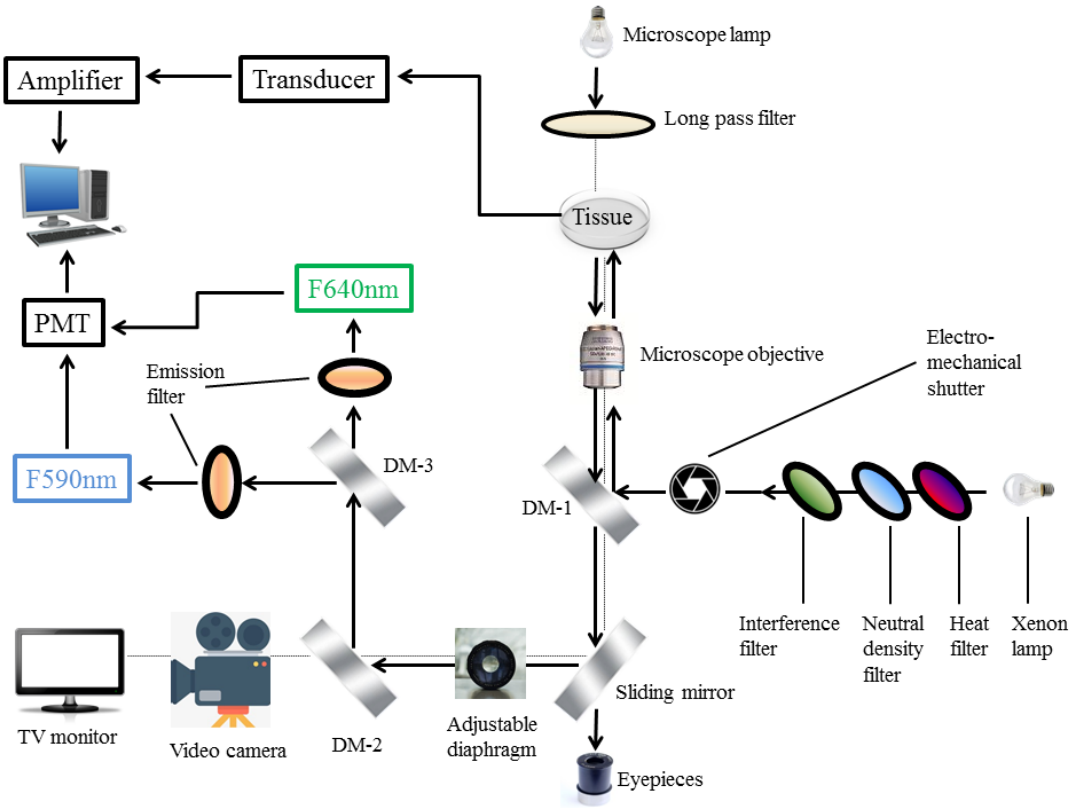

Figure 2-6: Schematic diagram for the mechanism used for simultaneous measurement of force and intracellular pH. In order to excite the pH fluorescent dye (Carboxy-SNARF-1 AM), illumination is provided by 50W xenon lamp. Both heat filter and neutral density filter are placed in the way of the light to reduce the amount of heat and light to decrease photobleaching. Excitation wavelength of 530 nm is produced by using an interference filter. Electromechanical shutter is used to keep the illumination time of the tissue to the minimum. The light is reflected upward by the dichroic mirror (DM1) onto the myometrial strip loaded previously with carboxy-SNARF-1 through the focused microscope objective. The emitted light by SNARF then goes back through DM-1 and reflected by the sliding mirror to pass by the adjustable diaphragm then the longer wavelength emitted light (>610nm) will pass the DM2 to form images on the video camera and shows later on the TV monitor. While the shorter wavelength light is reflected by the DM-2 then split by DM-3 to go to either 640nm filter or 580nm filter of the photomultiplier (PMT). A long pass filter is placed in front of microscope lamp to avoid interference from microscope light with the fluorescence measurements.

2.7 Western blotting

Western blot is used to detect protein of interest and determine its size using antibodies. In the current work, we used immunoblotting to determine the ASICs (1, 2a, and 3) expression in the mouse term uterus. In this work, proteins were separated on SDS-PAGE gels (MINI-PROTEAN TGX 12%, Cat# 456-1045, BIORAD, UK) and transferred to a PVDF membrane (Immobilon-P-Cat# IPVH00010-Pore size 0.45µm) through wet transfer method only. Samples used were term mouse uterus (both full thickness and myometrium only). Mouse brain and urinary bladder were used as positive controls.

The WB was executed as follows:

1. **Day1;** an appropriate, calculated, volume of each sample (1-2.2 µl) was mixed with the sample buffer (Sigma-S3401), in order to achieve a satisfactory dilution level.
2. Samples were heated at 100°C for 3 min on a heating block.
3. Prepare gel as instructed for precast gel.
4. Load gels with protein samples. Use 5uL protein ladder (BIORAD- Cat#161-0375) ladder in first lane.
5. Gel was run at 200V for 50 minutes.
6. Transfer of the proteins to a PVDF membrane was carried out using the manufacturer's manual, at 60V for 110 minutes.
7. The membrane was then blocked with 5% non-fat milk for 2 hours for the ASICs (and 5% BSA for β-actin)
8. After blocking, the membrane was blotted with the primary antibody solution at 4°C for overnight. The recommended dilution of each antibody was obtained from the supplier's records (1:200) (Anti-ASIC1, Anti-ASIC 2α and Anti-ASIC 3 all purchased from Alomone labs, Israel)
9. **Day2;** after primary antibody incubation, the membrane was washed 3 times for 5 min then 1x for 30 mins, each time with fresh 1x TTBS.
10. After washing steps, membrane was blotted with a secondary antibody solution (Goat anti-rabbit 1:10'000) (and Goat anti-mouse 1:40'000 for β-actin) for 1 hour at room temperature.
11. The membrane was then washed 5 times for 5 min, each time with fresh 1 x TTBS.

12. For detection, membrane was incubated with chemiluminescent reagent (Thermo Scientific 34078) for 5 min at room temperature. Then, in the dark room, a Hyperfilm ECL (Sigma-Aldrich, GE28-9068-35) was exposed for 1 min.

Table 2.1 Primary, secondary and housekeeping antibodies used in the western blot in this thesis. NFM=non-fat milk, BSA=Bovine Serum Albumin, Conc.=concentration used

		Source		Conc.	Blocker used
Anti-ASIC1	Primary	Rabbit	Polyclonal	1:200	5% NFM
Anti-ASIC2α	Primary	Rabbit	Polyclonal	1:200	5% NFM
Anti-ASIC3	Primary	Rabbit	Polyclonal	1:200	5% NFM
β-actin	House-keeping	Mouse	Monoclonal	1:4000	5% BSA
Anti-rabbit	Secondary	Goat	Polyclonal	1:10’000	5% NFM
Anti-mouse	Secondary	Goat	Polyclonal	1:40’000	5% BSA

Table 2.2 Concentrations of proteins loaded for the western blot analysis

Sample (all extracted from term-pregnant mouse)	**Concentration**
Bladder	1.419
Brain	1.039
Myometrium only	2.203
Full thickness uterus	1.390

2.8 Immunohistochemistry (IHC)

Immunohistochemistry is a commonly used technique to detect and locate targeted antigens or proteins in tissue sections. In the current work, due to the need to locate ASICs (1, 2α, 3) in the myometrium, immunohistochemistry was done and all immunostaining was conducted by the author unless otherwise stated. All tissue used is term-mouse uterus. Mouse urinary bladder was used as a positive control.

The IHC was conducted as follows:

1. **Day1:** Sections were heated in an oven maintained at 60^{o}C for 60 mins.
2. Dewaxed in xylene (2 x 10mins followed by submersion in 100% ethanol (2 x 5mins). Then slides were submersed in 90% then 70% ethanol (1 min each).
3. The heat mediated antigen retrieval was performed by immersing the sections in boiling 1x citric buffer for 2 minutes.
4. Sections were then incubated in 0.3% H_2O_2 /TBS solution for 10 mins at room temperature to block any endogenous peroxidase activity then incubated in TBS for 5 mins at room temperature.
5. Then non-specific binding sites were blocked by incubating slides in 2.5% Normal Horse Serum (MP-7401, ImmPRESS™ HRP, Vector labs, UK) for 20 mins at room temperature in a humidified chamber.
6. The appropriate concentration of primary antibody (Anti-ASIC 1, 2α, 3 were purchased from Alomone labs, Israel) solution (made in blocking solution) was added to sections which were then incubated overnight at 4^{o}C.
7. **Day 2:** Sections were washed in TBS (2 x 5 mins) and then incubated with anti-rabbit IgG reagent (made in horse) (MP-7401, ImmPRESS™ HRP, Vector labs, UK) for 30 mins at room tempratue in the humidified chamber.
8. Then, slides were washed in TBS (2 x 5 mins) and later incubated with DAB solution to visualise antibody binding (SK-4105, Vector Labs, UK) at room temp for 10 mins then immediately back in TBSand finally into tap water.
9. Sections then were incubated with filtered Gill 2 haematoxylin to visualise antibody binding. The colour reaction was stopped after 1.5 mins by immersion of the sections in running water until the waterran clear.
10. Then, slides were dipped briefly in acid alcohol, and immediately back into tap water for 5 mins at RT

11. Sections were then dehydrated through a series of ethanol; 70% then 90% (1 min each.), 100% (2 x 3 mins) and then xylene (5 then 10 mins) before being mounted and cover slipped in DPX.

12. After 24hrs, slides were viewed under the 4, 10, 20 and 40x lenses and analysed using Image Capture Software (NIS-Elements F).

Table 2.3 Primary antibodies used in the IHC in this thesis. NFM=non-fat milk

Anti-ASIC1	Rabbit	Polyclonal	1:100/250	5% NFM
Anti-ASIC2α	Rabbit	Polyclonal	1:100/250	5% NFM
Anti-ASIC3	Rabbit	Polyclonal	1:100/250	5% NFM

Chapter Three

The Effects of Extracellular Acidification on Mouse Uterine Contraction

Chapter-3

The Effects of Extracellular Acidification on Mouse Uterine Contraction

3.1 Abstract

Introduction:

Adequate uterine contraction is crucial to safe delivery of the foetus. Acidity can play an important role in affecting myometrial contraction but the data concerning the effects of external acidification, have been inconsistent. This study was therefore designed to determine the effects of acidic external pH (pH_o) on mouse uterus and investigate if there are any gestational differences,

Methods:

C57BL/6J mice were used either on day 14 (as mid-term) or day 18-19 (as term) of gestation or at 8-10 weeks non-pregnant. Changes in pH_o to 6.9 or 6.5 (in term uterus) were made by adding HCl to physiological saline. For the sake of comparison, the effects of extracellular alkalinization were also investigated, by increasing the pH of the test solution to 7.9 using NaOH. The perfusion rate was 5mL/min and the physiological saline and experimental solutions were maintained at 37^oC and oxygenated with 100% O_2. The physiological solutions were buffered with HEPES (for pH_o 6.9) and PIPES (for pH_o 6.5). Oxytocin (0.5nM) was used in some experiments. Spontaneous contractions were measured (frequency, amplitude, duration and area under the curve, AUC) and data analysed using LabScribe 3 and OriginPro 2015. Statistical differences were tested with Student's t test (n=6-12 animals), and taken at $P<0.05$.

Results:

- At 14 days, extracellular acidification to 6.9 significantly increased amplitude and frequency and therefore increased AUC.
- At term, extracellular acidification to pH 6.9 and 6.5 both significantly increased AUC, due to the increase in both amplitude and frequency.

- In non-pregnant uterus, extracellular acidification to pH 6.9 produced no significant change in contractility.
- Oxytocin mitigated the effect of extracellular acidification.
- In contrast to pH_o 6.9, pH_o 7.9 decreased amplitude and frequency significantly in all preparations.

Discussion:

My work is the first to study the effect of extracellular acidification on C57BL mouse uterus and it is the first to study the difference between gestations. In pregnant uterus from mouse, a decrease of pH_o stimulated term pregnant uterine contraction, particularly by increasing frequency (55% more than the control) and amplitude. These data are consistent with older studies in our lab found that extracellular acidification increased the frequency in pregnant human myometrium. The data are however, different from what was found in pregnant rat. Alkalinization had the opposite effect in mouse uterus with a decrease in the amplitude and frequency of contractile force. Gestational differences were found as extracellular acidification did not produce a stimulation of force in non-pregnant mouse. Possible reasons for the species and gestational differences are discussed. The stimulatory effects of oxytocin signalling might explain the abolition of the stimulatory effect of extracellular acidification when oxytocin is present. More studies are needed to investigate this theory.

3.2 Introduction

Parturition is a critical stage of pregnancy which needs powerful and synchronised uterine contraction to deliver the foetus safely to the world. These strong contractions have been long known to be accompanied by occlusion of the uterine small blood vessels (Greiss, 1965, Towell and Liggins, 1976, Janbu and Nesheim, 1987, Li et al., 2003). This occlusion leads to episodes of ischemia and therefore acidification (Harrison et al., 1994, Larcombe-McDouall et al., 1999).

For over three decades, it has been known that extracellular pH (pH_o) can modulate vascular smooth muscle tone (Wray, 1988a, Aalkjaer, 1990, Karaki et al., 1992, Smith et al., 1998a, Wray and Smith, 2004). Generally all mammalian blood vessels, apart from pulmonary artery (Pinto-Plata et al., 1995, Sweeney et al., 1998, Hyvelin et al., 2004), relax in response to acidic pH_o, and constrict with alkaline pH_o (Apkon and Boron, 1995, Austin and Wray, 1995, Aalkjaer and Poston, 1996, Aalkjaer and Peng, 1997, Apkon et al., 1997, Smith et al., 1998a, Austin and Wray, 2000, Dvoretsky et al., 2000, Nazarov et al., 2000, Weirich et al., 2004, Wray and Smith, 2004). It is thought that the muscle relaxation caused by extracellular acidification in vascular smooth muscle is due to decreased intracellular calcium concentration (Austin and Wray, 2000) due to inhibition of the Ca^{2+} influx (Chen et al., 1997). Additionally, Weirich *et al*, found that store-operated Ca^{2+} entry (SOCE) was significantly enhanced by extracellular alkalinization and decreased by extracellular acidification (Weirich et al., 2004). Other study shows an inhibitory effect of pH_o 6.6 on the response of the rat mesenteric artery to electrical field stimulation while pH_o 7.8 slightly increased that response (Dvoretsky et al., 2000). There are however, studies which have documented in blood vessels that acidic pH_o induced contraction. These include studies on rat thoracic aorta (Furukawa et al., 1996, Rohra et al., 2002b, Rohra et al., 2003b, Rohra et al., 2004) and rat coronary artery (Niu et al., 2014).

In the trachea, changes in extracellular pH affected Ca^{2+} uptake by the preparations; extracellular acidification decreased Ca^{2+} uptake and hence, decreases the contraction of tracheal smooth muscle strips from rats (Twort and Cameron, 1986). More recent studies found an acidic pH_o increased contraction in human cultured ASM cells by increasing intracellular calcium and activating various signalling pathways (Yamakage et al., 1995, Ichimonji et al., 2010, Saxena et al., 2012). It has been found that in canine tracheal smooth muscle under high K^+ stimulation, extracellular pH alteration affects

both intracellular pH and Ca^{2+} concentration. They reported increases in both $[Ca^{2+}]_i$ and muscle tension with alkaline pH_o while acidic pH_o diminishes the $[Ca^{2+}]_i$ without affecting muscle contraction. This led them to suggest that acidification may increase the sensitivity of the myofilaments to calcium (Yamakage et al., 1995). Thus there appears to be no consensus in the literature about how acidic pH affects airway contractility.

In detrusor smooth muscle cells from rat urinary bladder, acidic pH_o depressed the muscle response to the nerve stimulation (Uvelius et al., 1990). In the same cells from guinea-pig urinary bladders, dropping extracellular pH decreased the Ca^{2+} influx and also decreased the force (Wu and Fry, 1998). In the ureter, extracellular pH alteration has little effect on the contractile force in guinea-pig (Burdyga Th and Magura, 1986, Burdyga et al., 1996). One study found that, in guinea-pig, slight decreases of pH_o increases the contractile response of the vas deference to ATP, while changing the pH_o to the other direction decreases that response (Nakanishi et al., 1999).

If as in other tissues, extracellular pH (pH_o) has effects on uterine smooth muscle contraction, then this may have consequences for parturition. Studies however on the effects of pH_o on myometrial contractions are limited, and the effects of gestation appear no to have been studied. Naderali and Wray investigated the effect of extracellular acidification on non-pregnant guinea-pig myometrium, and found acidic pH_o caused a large increase in force irrespective to the oestrous cycle (Naderali and Wray, 1999) . An increase in frequency (but not amplitude) was found in pregnant human myometrium (Pierce et al., 2003). On the other hand, the opposite effect was found in pregnant rat uterus; pH_o 6.9 decreased contractile force (Shmigol et al., 1995, Alotaibi et al., 2015). On the alkaline side, Heaton *et al* found that in rats, pregnant uterus is more sensitive than the non-pregnant uterus, to the changes in extracellular pH. Alkaline pH_o had little or no effect on non-pregnant rat uterus while it reduced or abolished force in pregnant myometrium (Heaton et al., 1992). Despite the use of mouse uterine tissue as a model for studies on uterine contractility, my literature review revealed only two studies from the same group, undertaken of pH alteration and its effect on contractility in mouse uterus. Dropping pH_o to 6.4 was found to enhanced uterine contraction in pregnant mouse through inhibiting both two pore domain acid-sensitive K^+ channels (TASK-2) and non-inactivating outward K^+ currents (Hong et al., 2013, Kyeong et al., 2016). More details are tabulated in Chapter 1. Given the possible

similarity of responses of human and mouse uterus to external acidification, and the paucity of studies in mouse, I considered it important to study this. In addition, although one study in rats compared effects in pregnant and non-pregnant animals, none have systematically investigated if gestation affects the contractility response to reduced pH. Given all the changes that occur between mid-gestation to term, discussed in chapter 1, to promote contractility, including increases in expression of L-type Ca^{2+} channels, it may be hypothesized that the effects of external pH will be more in term myometrium compared to mid-term and non-pregnant. I therefore investigated and compared, the effects of pH 6.9 in non-pregnant, mid- and term myometrium

Oxytocin is a natural stimulant of the uterus; produced in the hypothalamus and secreted by the posterior pituitary gland as a neuropeptide hormone. It has been used as the first line management to induce labour. Oxytocin works to increase contractility by increasing calcium concentration inside myometrial cells through enhancing Ca^{2+} entry from L-type Ca^{2+} channels, store-operated calcium entry (SOCE) and calcium release from sarcoplasmic reticulum, and increasing membrane excitability (Batra, 1986, Coleman et al., 1988, Wray, 1993, Parkington et al., 1999, Arrowsmith and Wray, 2014, Ferreira et al., 2019). Oxytocin also increases Ca^{2+} by decreasing calcium efflux through inhibiting the plasma membrane Ca^{2+}-ATPase, PMCA (Soloff and Sweet, 1982, Popescu et al., 1985, Anwer et al., 1990, Magocsi and Penniston, 1991, Fernandez et al., 1992). How oxytocin may affect the effects of acidification is unclear, but important to study, as both will be present during the course of labour. A previous study in our laboratory showed that in rat myometrium, lactic acid decreases uterine contractility by lowering pH_i and oxytocin mitigates the effect by limiting the pH_i drop through blocking PMCA therefore preventing protons counter transport inside the cell (Austin and Wray, 2000, Floyd and Wray, 2007, Hanley et al., 2015). Researchers have also found that in human myometrium acidification to pH_o 6.9 decreased the amplitude and increased the frequency of contractions in the presence of oxytocin (Pierce et al., 2003). Due to the presence of oxytocin naturally during labour, I wanted to investigate the relation between oxytocin and extracellular acidification, and see if it might also help in understanding the mechanism of how extracellular pH affects force. Specifically, if they share parts of a common pathway to stimulate force, then it may be hypothesized that the effects of pH will be reduced if oxytocin has already stimulated the uterus.

To the best of my knowledge, my work is the first to investigate the effect of extracellular acidification on spontaneous uterine contraction in different stages of pregnancy in any species and in non-pregnant mice.

Aims

Due to the interesting and important role of extracellular acidification in smooth muscle contraction, and the limited work done to investigate this in the uterus, and the possibility of its involvement in parturition, I focused in this chapter to:

- investigate the effect of extracellular acidification on spontaneous uterine contractions in pregnant mouse.
- Investigate the effect of extracellular alkalinization on pregnant uterus?
- Investigate the effect of extracellular acidification on non-pregnant mouse?
- check if there are any gestational differences in the effects of extracellular acidification?
- Investigate the effect of extracellular acidification in the presence of oxytocin?

3.3 Methods

3.3.1 Tissue

C57BL/6J mice were used either on day 14 (as mid-term) or day 18-19 (as term) of gestation or at 8-10 weeks non-pregnant. They were humanely killed using CO_2 anaesthesia and cervical dislocation. Pups were delivered manually by caesarean section and culled via cervical dislocation. All were in accordance with UK Home Office rules for Schedule 1 killing. The uterus was removed, cleaned and uterine strips (10mm X 2mm) dissected. Individual strips were mounted between a fixed hook and force transducer using surgical silk threads in a 10ml bath and were continuously superfused with physiological saline solution (PSS) (pH 7.4) at a rate of 5mL/min and maintained at 37 ^{o}C and 100% O_2 (more details are given in chapter 2).

3.3.2 Experimental protocol

After 30-60 minutes, the mouse uterine spontaneous contraction reached a steady state in physiological saline. Forthwith, experimental solutions were applied for 10 minutes (the experimental period). Then the perfusate was returned to PSS (pH 7.4) for 20-30 minutes recovery period. The immediate 10 minutes preceding the application of experimental solution was used as the control (100%) period (Figure 3.1). In some experiments, oxytocin (0.5nM) was applied for 2 hours and within that time pH_o 6.9 was applied for 10 minutes twice with 30 minutes control and recovery periods.

3.3.3 Solutions

Buffered physiological saline solution (PSS) was composed of (mM): NaCl 154, KCl 5.6, MgSO4 1.2, $CaCl_2$ 2, Glucose 8 and HEPES 10.9 (PIPES, 10.9 mM, for buffering solutions of pH_o 6.5). All chemicals were obtained from Sigma-Aldrich, Dorset, unless otherwise stated. Changes in pH_o to 6.9 and 6.5 were made by adding HCl to PSS. To achieve extracellular alkalinization, NaOH was used to increase pH_o to 7.9. Oxytocin (0.5nM) was used in some experiments.

3.3.4 Analysis and statistics

Data was recorded and analysed as mentioned previously in Chapter 2. Data was normalised against percentage of control (100%) and found to be normally-distributed and statistical differences were therefore tested using Student's t-test or ANOVA as

appropriate. The Bonferroni test was used as a post hoc test. Significance was taken as $P<0.05$

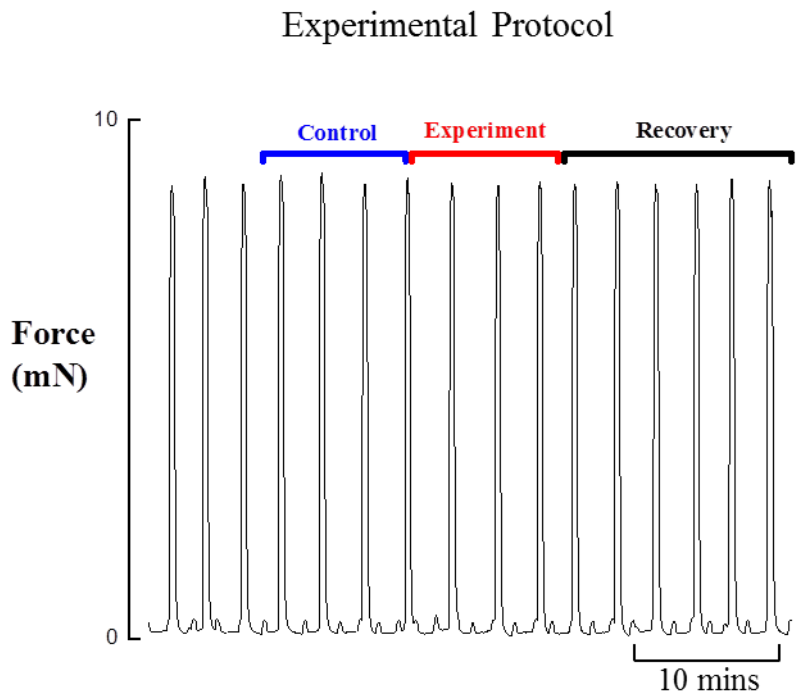

Figure 3.1

Spontaneous uterine contractions were allowed to equilibrate and then 10 minutes control period taken; indicated by blur bar. This was followed by the experimental intervention which is, in this chapter, a pH_o alteration for 10 minutes; indicated by the red bar. Recovery was then followed for at least 20 minutes; indicated by black bar. Control values for the parameters of contraction were designated 100% and changes relative to this (mean± SEM) were measured in the experimental period.

3.4 Results

3.4.1 Control Data

Spontaneous contractions generated from the uterine strips from term- and mid-pregnant and non-pregnant mice start immediately upon mounting the strips and usually last for hours without fatigue when superfused with the appropriate physiological solution and bubbled with 100% O_2 at 37 ºC. Figure 3.2 shows a typical trace for the uterine contractions produced by term-pregnant mice (A, n=30), mid-pregnant (B, n=13) and non-pregnant black mice (C, n=14). Uterine strips used in this chapter contain both endometrium and myometrium, Figure 3.3 shows no difference between contractions generated from full-thickness uterine strips and those generated from strips containing myometrium only. HEPES was used to buffer solutions with pH > 6.8 while PIPES was used as buffer for solutions with pH < 6.8. PIPES did not affect uterine contractility as shown in Figure 3.4.

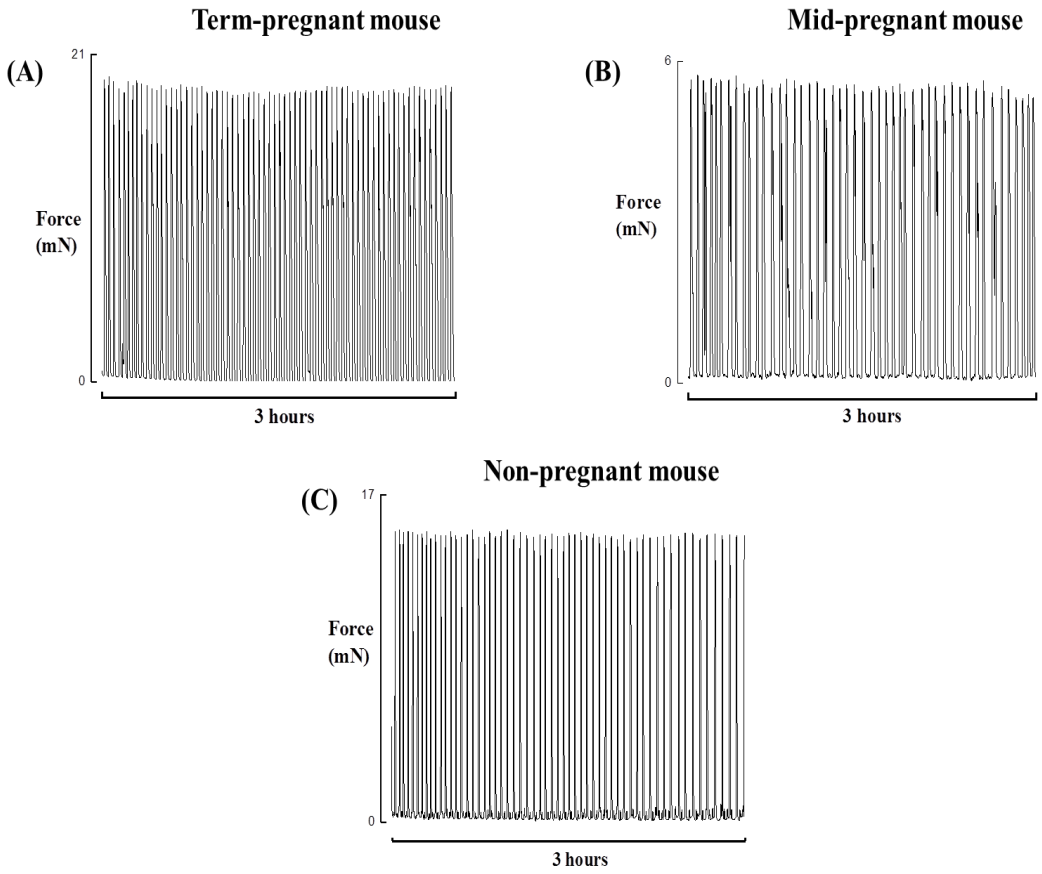

Figure 3.2 Time control contractions from mouse uterus

Typical isometric recordings of spontaneously contracting uteri from A-term-pregnant (top-left trace), B-mid-pregnant (14 days, top-right trace), and C-non-pregnant (bottom trace) mice. Tissue was continuously perfused with physiological saline solution at pH 7.4. The perfusion rate was 5ml/min and preparations were bubbled with 100% O_2 at 37°C in this and subsequent traces in this chapter, unless stated otherwise.

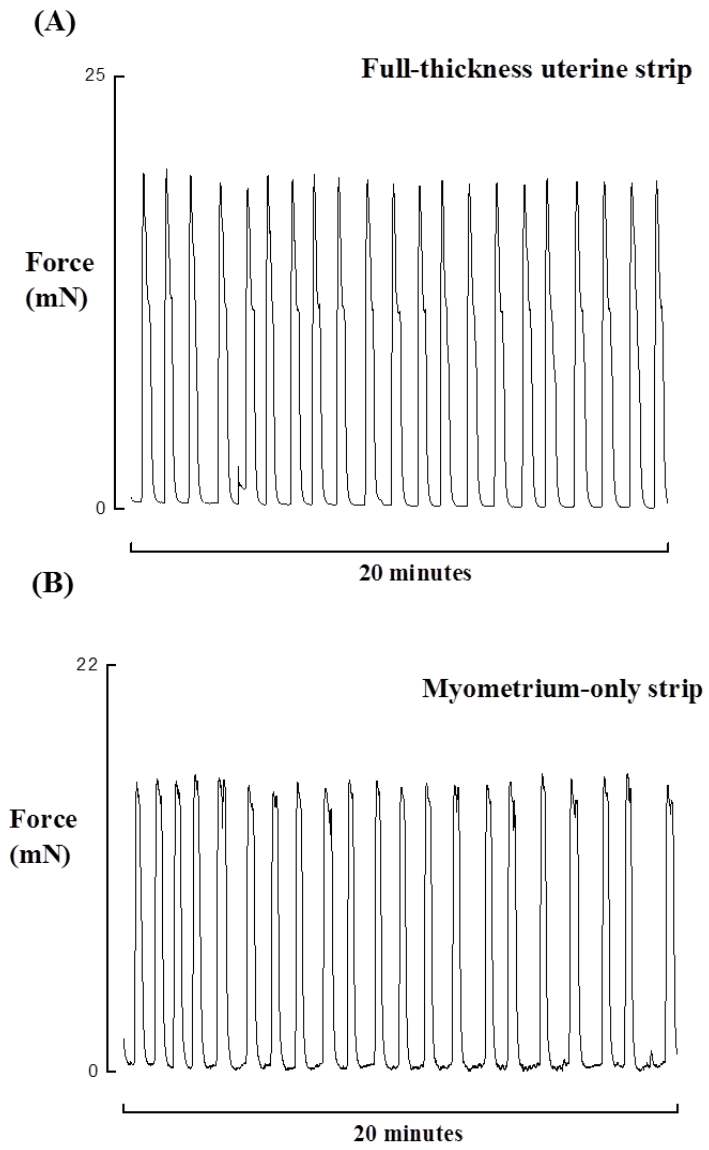

Figure 3.3 Contractions from pregnant mouse uterus

Typical isometric recordings of spontaneously contracting uterus from pregnant mouse. Top trace (A) shows 20 minutes of uterine contractions obtained from a strip containing both endometrium and myometrium. Bottom trace (B) shows uterine contractions for the same period of time generated from a strip containing myometrium only.

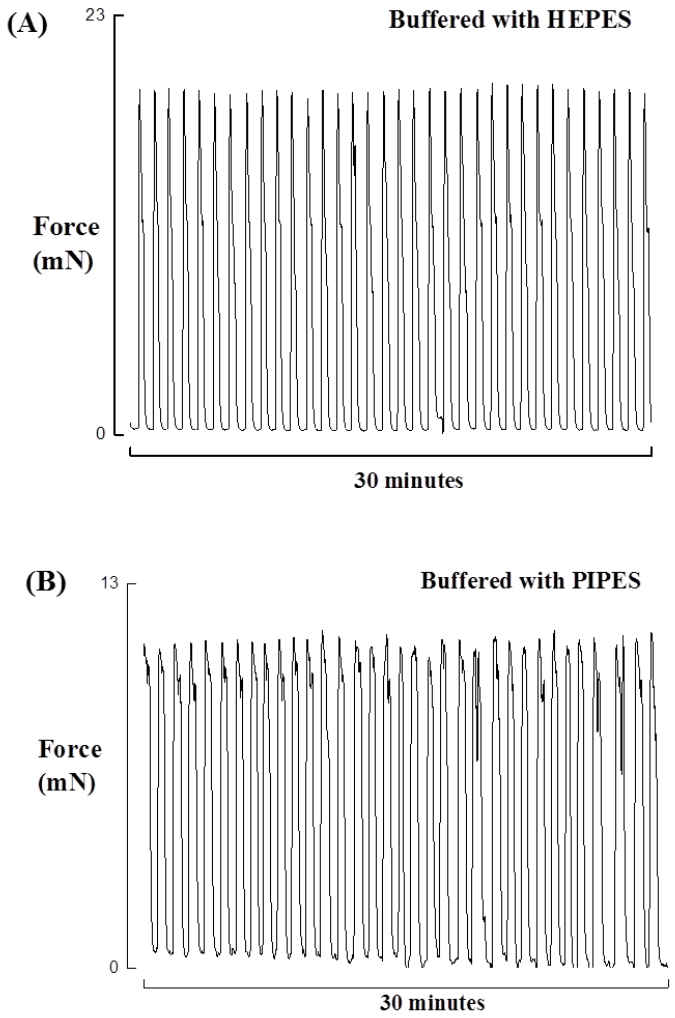

Figure 3.4 Contractions from pregnant mouse uterus shows similar response to different buffering systems

Typical isometric recordings of spontaneously contracting uterus from pregnant mouse. Top trace (A) shows 30 minutes of uterine contractions obtained from a strip superfused with PSS buffered with HEPES. Bottom trace (B) shows uterine contractions generated from a strip superfused with PIPES buffered PSS.

PSS=physiological saline solution

3.4.2 Effect of Extracellular Acidification on Term-Pregnant Mouse Uterus.

After 30-60 minutes of steady contractions, acidic solution (pH 6.9) was applied for 10 minutes to the spontaneously contracting uterus and the parameters of contraction were compared to the values of the control period (n=12). As shown in Figure 3.5 (representative of 11 others), the response to the acidic solution started immediately after it reached the tissue, increased over the first few minutes then plateaued. Analysis showed that the amplitude reached its peak (131.4 ± 8.7%, $p<0.005$) after 3.2 ± 0.3 minutes. Upon returning to control, pH_o 7.4, force returned to the previous control levels. Frequency also increased significantly in response to extracellular acidification (155.3 ± 10.9%, $p<0.0005$), and this was maintained throughout the test period. There was no significant change in the duration of the contractions (101.9 ± 14.11%, $P = 0.9$). The area under the curve, which represents the sum of the myometrial contractile activity, increased significantly with pH 6.9 application (178.5 ± 22.1%, $p<0.005$). The mean data of the effect of extracellular acidification on term-pregnant uterus are summarized in Table 3.1. Return of normal spontaneous contractions to control levels occurred 3.1 ± 0.4 mins after removal of acidic solution and no change in the force during recovery period was detected when resuming PSS (pH 7.4) when compared to control period, i.e. before applying pH_o 6.9.

As the effects of pH 6.9 especially on amplitude appeared transient, the significant data, amplitude and frequency were further analysed to quantify this. The first, middle and last third of the application of the pH_o 6.9 were analysed. As shown in Figure 3.6, the effect on frequency was maintained throughout the test period. On the other hand, the effect on amplitude took a few minutes to reach the maximal effect and so effects are maximal between 3.33 and 6.66 and then it reduced in the last few minutes.

To probe the reproducibility in individual preparation the effects of pH 6.9, I applied it for 10 minutes in a repetitive manner with 20-30 minutes recovery period between each application. As shown in Figure 3.7 (representative of 8 others), each application of pH_o 6.9 produced similar increases in force with no noticeable fatigue of the tissue which recovered well between each exposure to the acidic pH_o.

Table 3.1 Mean values (normalised to 100% control), standard error of the mean (SEM) and p-values of the contractile parameters resulting from the 10 minutes application of extracellular pH_o 6.9 on term-pregnant uterus. Paired Student's t-test was used here.

	Amplitude	**Maximal Amplitude**	**Frequency**	**Duration**	**AUC**
Mean (n=12)	126.9%	131.4%	155.3%	101.9%	178.5%
SEM	7.2	8.7	10.9	14.1	22.1
P-value	**0.003**	**0.004**	**0.0003**	**0.9**	**0.004**

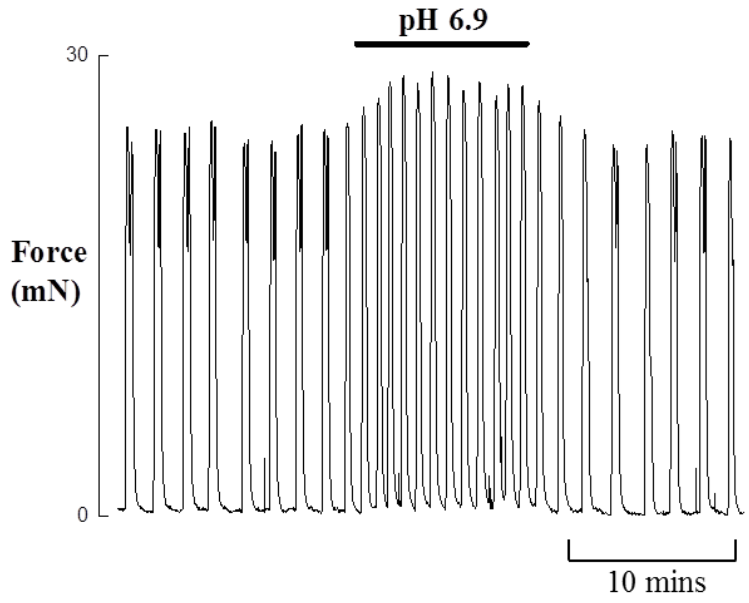

Figure 3.5 Effect of the application of pH_o 6.9 on term mouse uterus.

Isometric recording of 10 minutes application of acidic solution (pH_o 6.9) to spontaneously contracting term-pregnant mouse uterus (n=12).

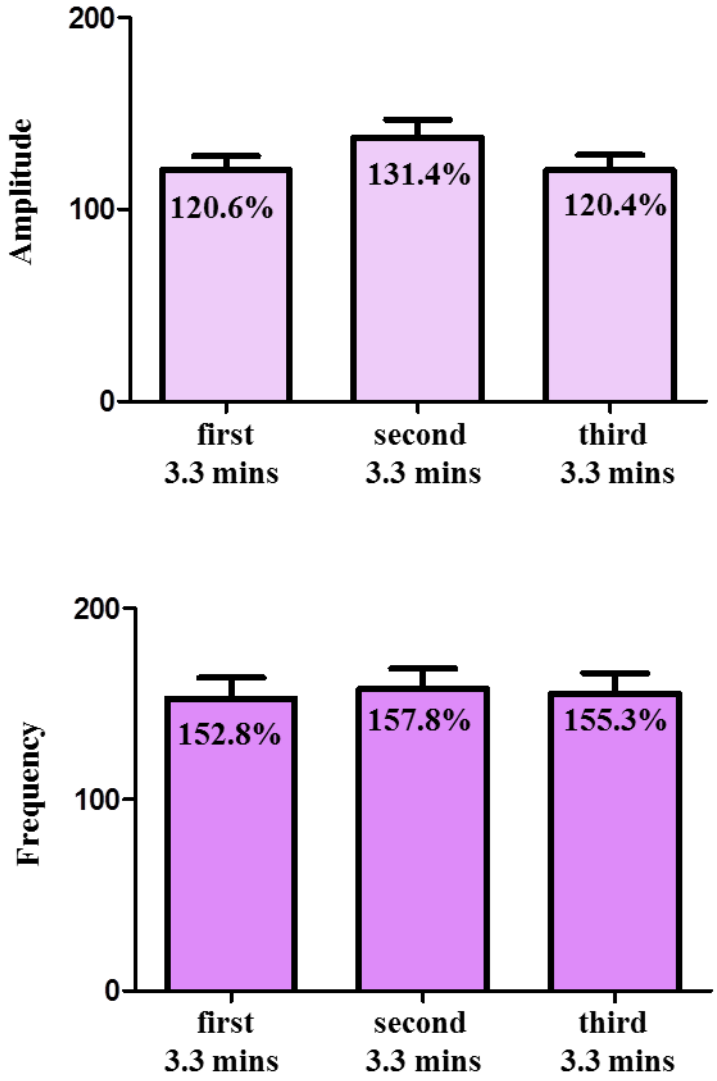

Figure 3.6 Mean data of the effect of pH_o 6.9 on each third of the application period

Bar charts showing the effects of pH_o 6.9 on the amplitude and frequency of contractions; by time. The data were divided into three equal parts of the ten minute application, 3.33 minutes each.

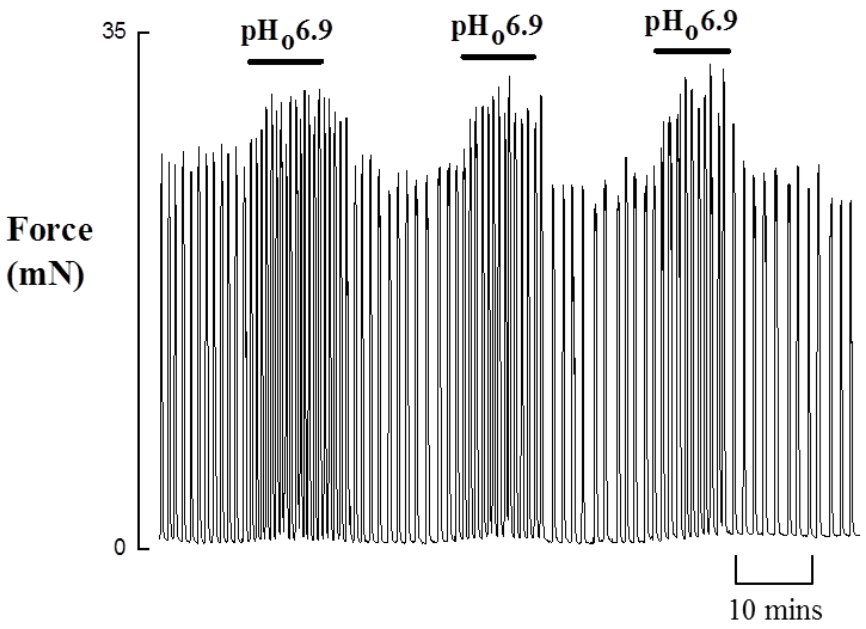

Figure 3.7 Effect of the repetitive application of pH_o 6.9 on term mouse uterus.

Isometric recording of frequent 10 minutes application of acidic solution (pH_o 6.9) to spontaneously contracting term-pregnant mouse uterus (n=12).

3.4.3 Effect of pH_o 6.5 on Spontaneous Uterine Contractions in Term-Pregnant Mouse

To investigate if a higher external proton concentration will result in a stronger effect on uterine contraction in term-pregnant mouse the, pH_o was reduced to 6.5.As with pH_o 6.9, pH_o 6.5 produced an increase of contraction (n=11). Amplitude, frequency and AUC were all significantly increased : 107.6% (p=0.0001), 147.9% (p=0.005) and 155.6% (p=0.008); respectively. As with pH_o 6.9, reduction of pH_o to 6.5 did not change the duration of contraction (p=0.7). Return of normal spontaneous contractions to control levels occurred 2.9 ± 0.7 mins after removal of acidic solution and no change in the force during recovery period was detected when resuming PSS (pH 7.4) when compared to control period, i.e. before applying pH_o 6.5. In addition, there is no difference in time needed for the tissue to return to control levels after discontinuing either pH_o 6.9 or pH_o 6.5. The mean data are summarized in Table 3.2.

When compared to the effect of pH_o 6.9, pH_o 6.5 showed significant less stimulatory effect on amplitude (p=0.01, unpaired Student's t-test) while its effect on the other contractile elements was similar to pH_o 6.9. These data are compared statistically at the foot of Table 3.2. The mean data for pH_o 6.5 and 6.9 are directly compared for each of the parameters of contraction in Figure 3.8. Therefore, pH_o 6.9 was used to investigate extracellular acidification throughout this thesis.

Table 3.2 Mean values (normalised to 100% control), standard error (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification pH_o 6.5 and p-values of comparing pH_o 6.9 to 6.5. . Paired Student's t-test was used here.

		Amplitude	**Maximal Amplitude**	**Frequency**	**Duration**	**AUC**
6.5 (n=11)	Mean	107.6%	109.1%	147.9%	96.8%	155.6%
	SEM	1.2	1.5	13.5	9.3	17.1
	P-value	0.0001	0.0001	0.005	0.7	0.008
6.9 vs 6.5	**P-value**	**0.01**	**0.02**	**0.6**	**0.7**	**0.4**

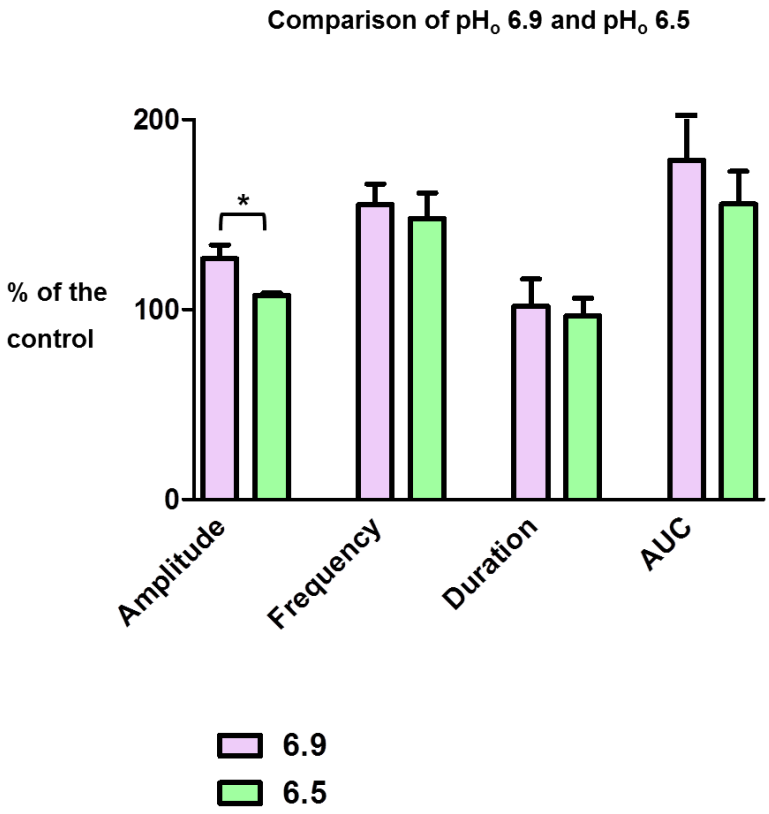

Figure 3.8 Mean data comparing the effects of pH$_o$ 6.9 vs 6.5 on uterine contraction

The bar charts show the mean effects of pH$_o$ 6.9 vs 6.5 (n=12 and 11, respectively). A significant difference in comparison to the control was found using unpaired Student's t-test. * denotes *$p<0.05$ significance only in the amplitude of contraction.

3.4.4 Effect of Extracellular Alkalinization on Term-Pregnant Mouse Uterus

External pH 7.9 (n=7) decreased the force of contraction in all preparations. In 4 out of 7 of the samples, contractions were abolished, and a typical trace of the abolition effect of pH_o 7.9 is shown in figure 3.9-A. In the other three samples, external alkaline pH caused smaller decrease of the force of contraction and a typical example is shown in the trace in Figure 3.9-B. The mean data are present in Table 3.3. The decrease in force amplitude, frequency of contraction and AUC were all significantly different from their paired controls. As with extracellular acidification, the effect on duration of the contraction was not significant. Figure 3.10 shows the effects of extracellular acidification and alkalinization side by side.

Return of normal spontaneous contractions to control levels occurred 2.4 ± 0.8 mins after removal of alkaline solution and no change in the force during recovery period was detected when resuming PSS (pH 7.4) when compared to control period, i.e. before applying pH_o 7.9.

Table 3.3 Mean values (normalised to 100% control), standard error (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular alkalinization. . Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
pH_o 7.9 (n=7)	Mean	39.5%	38.9%	95.4%	41.9%
	SEM	18.7	18.7	4.6	12.9
	P-value	**0.01**	**0.03**	**0.4**	**0.002**

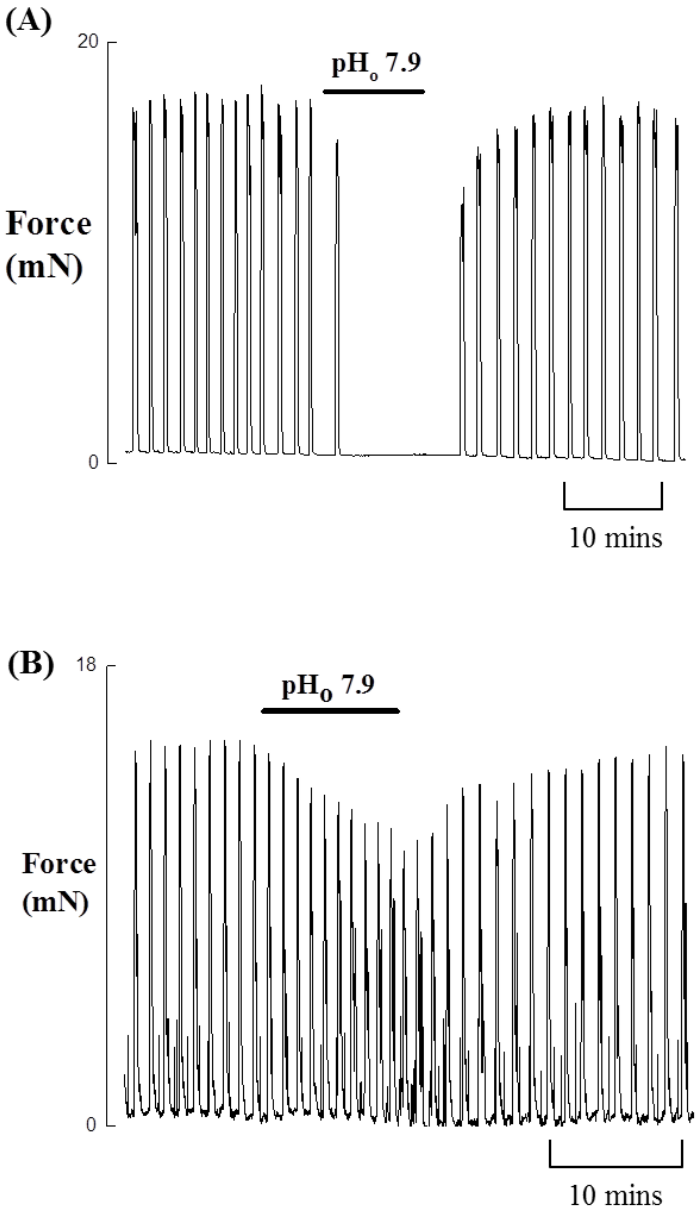

Figure 3.9 Effect of extracellular alkalinization on spontaneous uterine contraction

Isometric recording of the spontaneous uterine contraction in term-pregnant mouse shows two various effects of extracellular alkalinization between abolition of the contraction (n=4, A) and a decrease of the force (n=3, B)

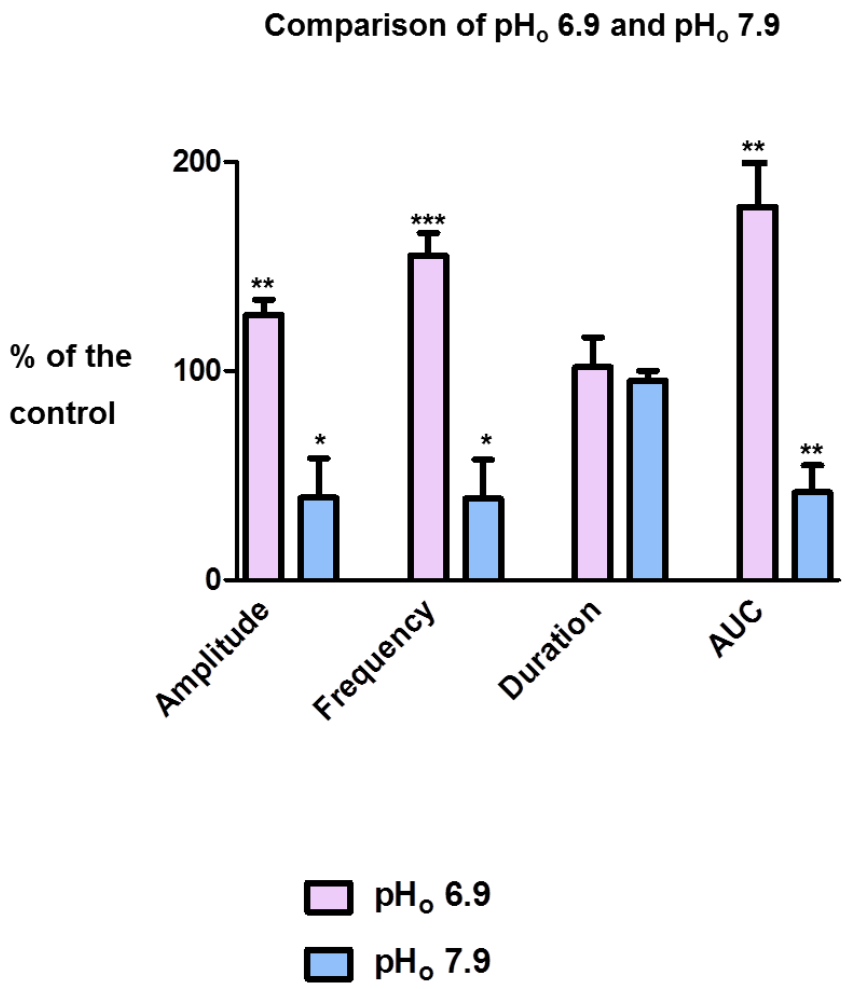

Figure 3.10 Mean data comparing the effects of pH$_o$ 6.9 vs 7.9 on uterine contraction in term pregnant mouse

The bar charts show the mean effects of pH$_o$ 6.9 vs 7.9 (n=12 and 7, respectively). Extracellular acidification increased, while extracellular alkalinization decreased, the force. Significant differences (normalised to 100% control) were found using unpaired Student's t-test. * denotes *p<0.05 **p<0.005 and *** p<0.0005 significance

3.4.5 Effect of Acidification on Mid-Pregnant Mouse Uterus

Uterine strips from 14 days pregnant mice were used to test the effect of extracellular acidification on the uterine contraction in the mid-pregnancy period. Extracellular acidification (n=9) significantly increased the amplitude of force by 16% (p=0.04) and its frequency by 52.6% above control (p=0.003) and consequently AUC was also significantly increased (206.1%; p=0.002). There was no change in the duration (Figure 3.11). The mean data are summarized in Table 3.4.

Return of normal spontaneous contractions to control levels occurred 2.7 ± 0.9 mins after removal of acidic solution and no change in the force during the recovery period was detected when resuming PSS (pH 7.4) when compared to control period, i.e. before applying pH_o 6.9.

Table 3.4 Mean values (normalised to 100% control), standard error (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification on mid-term pregnant uterus from mouse and p-value of comparing the effect of extracellular acidification on 19 days to 14 days pregnant mice.

		Amplitude	**Frequency**	**Duration**	**AUC**
pH_o 6.9 (n=9)	Mean	116.0%	152.6%	106.7%	172.8%
	SEM	4.484	10.99	10.03	13.94
	P-value	0.007	0.001	0.5	0.001
TP vs MP	**P-value**	**0.2**	**0.8**	**0.7**	**0.8**

TP=term-pregnant, , MP=mid-pregnant. Unpaired Student's t-test was used here.

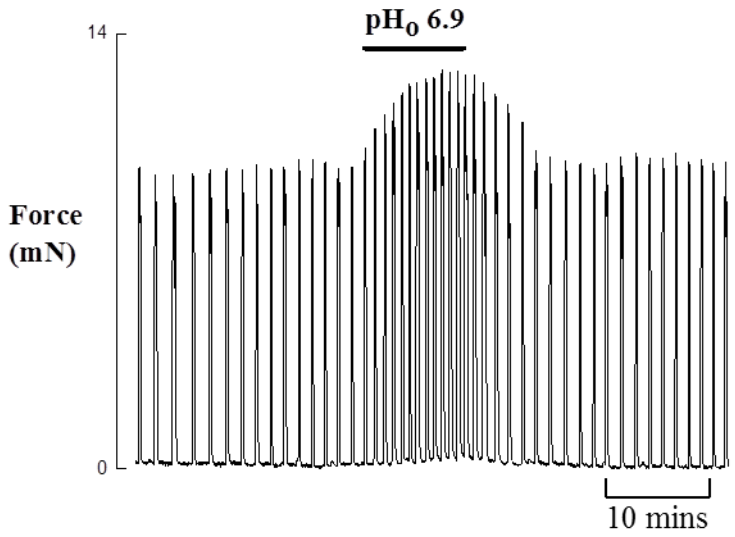

Figure 3.11 Effect of extracellular acidification on 14 days mouse pregnant myometrium.

Isometric recording of 10 minutes application of acidic solution (pH 6.9) to spontaneously contracting 14 days pregnant mouse uterus (n=9).

3.4.6 Effect of Extracellular acidification on Non-Pregnant Mouse Uterus

Uterine strips from non-pregnant mice were used to test the effect of extracellular acidification on uterine contraction. Interestingly, pH_o 6.9 caused insignificant effect on the contraction of non-pregnant uterus (n=7; Figure 3.12). The mean data are summarized in Table 3.5

Table 3.5

Mean values (normalised to 100% control), standard error (SEM) and p-values of the contractile parameters after 10 minutes application of extracellular acidification on non-pregnant mouse and comparing it to its effect on the pregnant mice.

		Amplitude	**Frequency**	**Duration**	**AUC**
pH_o 6.9 (n=7)	Mean	94.7%	117.5%	108.0%	99.3%
	SEM	3.9	14.5	7.2	14.7
	P-value	0.2	0.3	0.3	0.9
TP vs NP	**P-value**	**0.004**	**0.05**	**0.9**	**0.02**

TP=term-pregnant, , NP=non-pregnant. Unpaired Student's t-test was used here.

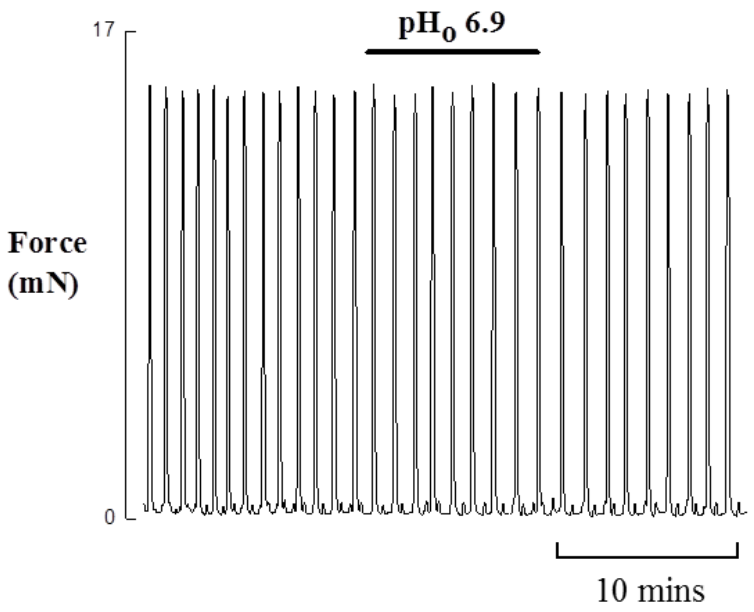

Figure 3.12 Effect of extracellular acidification on non-pregnant myometrium from mouse.

Isometric recording of 10 minutes application of acidic solution (pH_o 6.9) to uterus from non-pregnant mouse (n=7).

3.4.7 Comparison of the Effects of Extracellular Acidification at Different Gestation

Comparing the effects of extracellular acidification, using one-way ANOVA test, at the three different gestations showed significant difference in the effects of pH_o 6.9 on the amplitude and AUC ($p=0.005$ and 0.02; respectively). Acidic pH_o showed more stimulation in the term- and mid-pregnant uteri while it didn't change the contractility of the non-pregnant one. On the other hand, there is no difference in the effects of acidic pH_o on the frequency and duration between pregnant and non-pregnant preparations. The mean data are summarised in Figure 3.13

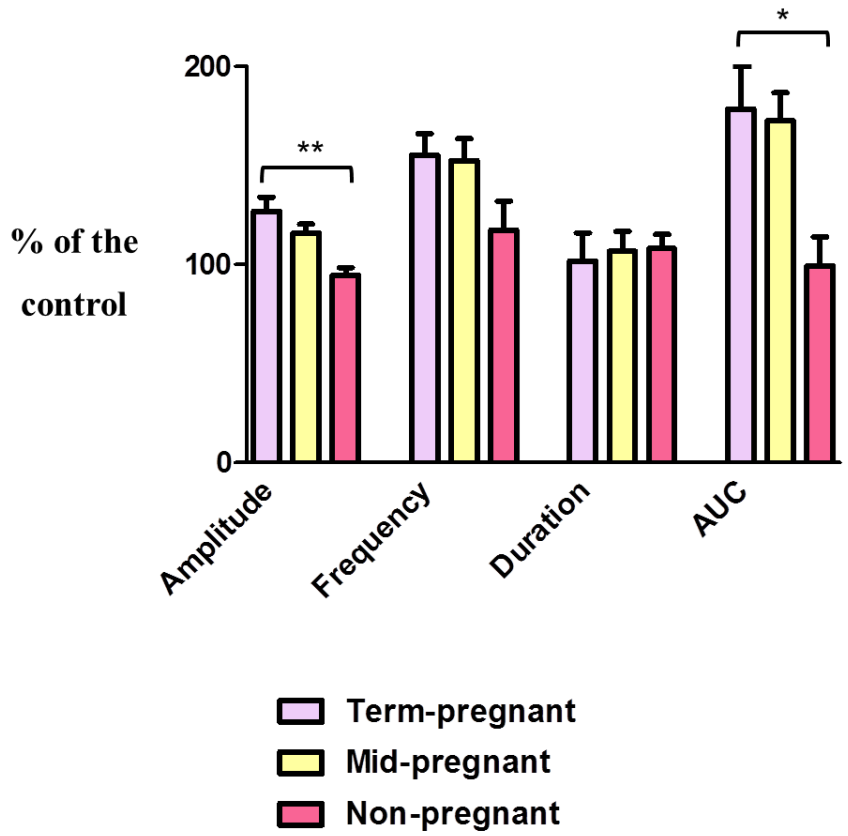

Figure 3.13 Mean data of the different effects of extracellular acidification on uterine contraction between term-pregnant (n=12), mid-pregnant (n=9) and non-pregnant mice (n=7).

Bar chart shows significant increase in the amplitude and AUC of extracellular acidification in the pregnant uterus more than its effect in non-pregnant one. No differences were detected between the other contractile elements, frequency and duration. A significant difference in comparison to the control was found using ANOVA test. * denotes *$p<0.05$, **$p<0.005$

3.4.8 Effect of Extracellular Acidification on Oxytocin-Induced Uterine Contractility

A test solution of pH_o 6.9 was applied to the uterine strips to ensure a stimulatory effect was produced. Oxytocin was then added after washout of the pH 6.9 solution. The concentration of oxytocin was chosen to give clear stimulation that could be maintained. A control trace showing the presence of oxytocin for 2 hours (Figure 3.14-A), shows that 0.5nM of oxytocin was a suitable concentration (Table 3.6). Adding the acidic solution (pH_o 6.9) to the oxytocin-induced contracting uterus produced little or no effect on contractility (Figure 3.14-B, Table 3.6). In some experiments, the acidic solution (pH 6.9) was repeated to confirm that there was no effect on contractility when oxytocin was stimulating the myometrium. As can be seen in Figure 3.14-C, the uterine preparations returned to control values when oxytocin was removed, and a final application of pH_o 6.9 solution, produced stimulation of contraction.

Table 3.6

Mean values (normalised to 100% control), standard error (SEM) and p-values of the contractile parameters after application of oxytocin and extracellular acidification (n=9). Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
Oxytocin	Mean	122%	180.3%	101.6%	202.3%
Vs	SEM	2.8	24.4	2	24
Spontaneous	**P-value**	**0.001**	**0.03**	**0.5**	**0.01**
pH_o6.9 with oxytocin vs oxytocin alone	Mean	108.7%	87.2%	127.4%	101.9%
	SEM	7.8	4	15.1	10.6
	P-value	**0.3**	**0.06**	**0.1**	**0.8**

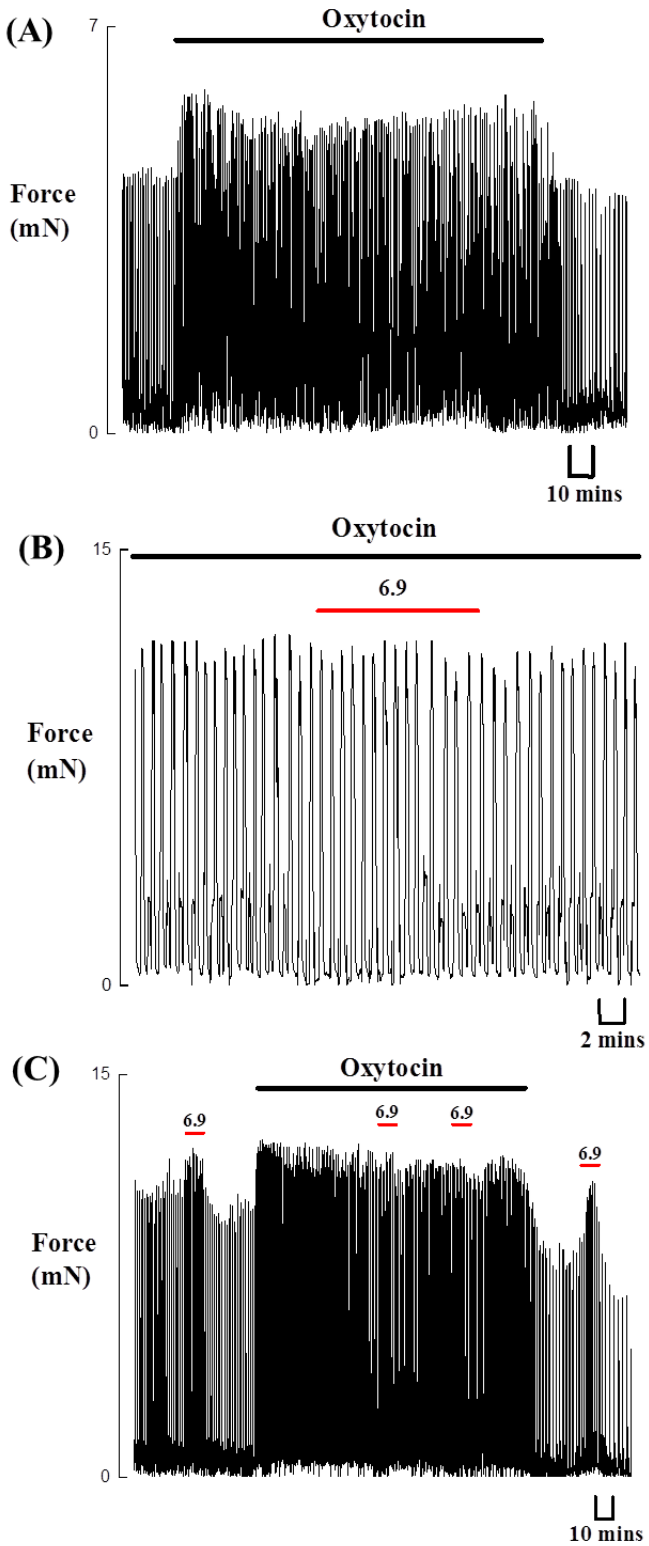

Figure 3.14 Effect of extracellular acidification on uterine contractility in the presence of oxytocin

A. Isometric recording of 120 minutes application of oxytocin.

B. Close-up view of the effect of pH_o 6.9 in the presence of oxytocin.

C. Other recording of the effect of 10 minutes applications of acidic solution pH_o with and without oxytocin (represents 8 others)

3.5 Discussion

The main findings of this chapter are: a) extracellular acidification increases uterine contraction in pregnant mouse; b) extracellular alkalinization decreases uterine contraction in pregnant mouse; and c) gestation and oxytocin change the effects of extracellular acidification.

Parturition is a critical stage of pregnancy which needs powerful and synchronised uterine contraction to deliver the foetus safely to the world. These strong contractions are accompanied by occlusion of the uterine small blood vessels (Greiss, 1965, Li et al., 2003) which leads to episodes of ischemia and therefore acidification intra- and extracellularly as the blood vessels within the myometrium are compressed (Brotanek et al., 1969, Harrison et al., 1994, Larcombe-McDouall et al., 1999). Cellular acidity plays a vital role in the metabolism and contraction in the myocytes. Previous studies in our laboratory showed changes in intracellular pH modulate uterine contraction in different species (Heaton et al., 1993, Phoenix and Wray, 1993, Taggart and Wray, 1993a, Pierce et al., 2003). On the other hand, the limited literature shows no consensus on the effect of extracellular acidification on uterine smooth muscle contractions. Therefore, in this thesis chapter, I focused my work on determining the effect of pH acidification on uterine contractility. I used mouse as the tissue is accessible and it allows testing at different gestational stages. The mechanism of force production and excitation-contraction coupling are considered to be the same in mouse, rat and human uterus. Also, as with human and rat myometrium, changes in mouse uterine contractions are preceded by changes in the intracellular calcium concentration (Matthew et al., 2004).

These data show that mouse uterine tissue, regardless gestation, is spontaneously active and responds to changes in extracellular pH by changing its force. As expected, full thickness strips, containing endometrium and myometrium produced the same pattern of contractions as strips containing only myometrium. I therefore proceeded to use full thickness strips throughout this chapter (and thesis unless otherwise mentioned) to increase the physiological relevance of my work, as intact uterus is the *in vivo* condition. The need to change buffer according to external pH used also did not affect the quality of uterine contractions and thus ruled out any buffer interference with pH_o effect.

My data clearly showed that in term-pregnant mouse myometrium, decreasing external pH_o to 6.9 will significantly affect force production. These effects were most

notable on frequency and amplitude of contractions. As mentioned in my introduction, the previous findings concerning extracellular acidification on the pregnant myometrium have not been consistent; with several studies describing an increase in some aspects of contractility i.e. frequency (Pierce et al., 2003), while others reported no change or a decrease in contractile parameters (Shmigol et al., 1995, Alotaibi et al., 2015). Of particular note, my data are consistent with that of Lee's group, who included an examination of external acidification (to 6.4) in mouse myometrium, in their studies of two-pore domain acid sensitive potassium channels (Hong et al., 2013, Kyeong et al., 2016). Their traces showed pH_o 6.4 increasing contractility but little further detail is included. In addition, in the 2013 study circular muscle only was studied. It is perhaps surprising that the effect of acidic pH_o in mouse myometrium different from those reported on another rodent, namely rat myometrium. Authors have consistently reported that in rat reducing pH_o leads to a decrease in force (Shmigol et al., 1995, Alotaibi et al., 2015).

The stimulatory effect of extracellular acidification in mouse uterus was surprising; most published data reports extracellular acidification decreasing force. This was the case in rat myometrium (Shmigol et al., 1995, Alotaibi et al., 2015). I; therefore conducted a small number of experiments on term-pregnant rat, myometrium to allow me to directly verify these opposite effects. In these three experiments, decreasing pH_o to 6.9 resulted in a decrease in uterine force, in agreement with the previous literature (Figure 3.15). This strongly suggests that my data showing increased contractility in the pregnant mouse myometrium is not due to any experimental differences, but rather represents a novel finding and different response between species. Species differences in the biological characteristics between rats and mice have been reported widely in the literature. For example, epinephrine was found to inhibit TSH-stimulated cAMP formation in the rat thyroid tissue but not in the mouse thyroid preparation (Mills and Sherwin, 1985). Other study showed that the different actions of the cholecystokinin (CCK) agonist JMV-180 in rat and mouse is due to species-specific differences in the structure of CCK receptors (Ji et al., 2000). In addition, strain-differences in the rats were noticed in relation to the aortic smooth muscle response to extracellular acidification (Rohra et al., 2003c).

Given that calcium signalling and electro-mechanical coupling have no major differences between rats and mice, I can only speculate as to why there should be this

difference in functional effects of acidic external pH. In the next chapters, I investigate the possible mechanisms producing the functional effects.

Extracellular acidification significantly increased the amplitude and frequency in term-and mid-term pregnant uterine tissue from mouse. Frequency was influenced significantly by extracellular acidification and this is in consistent with what was found in human myometrium previously (Pierce et al., 2003). It also increased contractility in non-pregnant guinea pig (Naderali and Wray, 1999). Thus it may be that the rat is the anomalous species not the mouse.

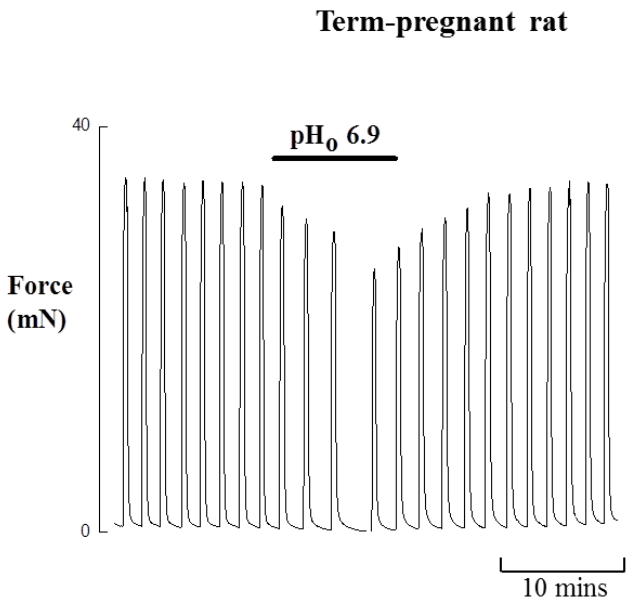

Figure 3.15 Effect of extracellular acidification of term-pregnant rat

Isometric recording of the effect of 10 minutes application of pH_o 6.9 on spontaneously contracting uterine strips from term-pregnant rat. Same protocol was followed, perfusion rate was 5ml/min and preparations were bubbled with 100% O_2 at 37 °C. Note the decrease of the amplitude and frequency as a result of extracellular acidification.

Effect of proton concentration

Previous work done on different species to test the effect of extracellular acidification on the uterus is limited; a literature review is summarized previously in Chapter-1, table 1.2; and did not investigate the effect of different proton concentrations on myometrial contraction. To the best of my knowledge, this is the first work analysing that effect. Interestingly, pH_o 6.9 caused 10% more increase in amplitude in comparison to pH_o 6.5, and there were no other significant differences. As explained in chapter 1 and explored further in the next chapter, changing pH_o will be expected to change intracellular pH (pH_i). If the effects of pH_i are different from pH_o, then these effects may be stronger at pH 6.9. It may also be that the stimulatory effects are maximal with pH_o 6.9 and there is no capacity to further increase force.

Effect of gestational status

Many physiological and molecular changes happen to the uterus during pregnancy in order to prepare it for the developing foetus and birth (Finn, 1982, Maltsev et al., 1983, Yamada, 1988). Specifically, gestational status changes the expression of many receptors and channels (Soloff et al., 1979, Martin et al., 1999, Mazzone and Buxton, 2003, Dickmann et al., 2008, Floyd et al., 2017, Platonova et al., 2017, Berisha et al., 2018, Osaghae et al., 2019). Investigating the effects of extracellular acidification on the force of mid- and non-pregnant murine uteri was an important step to compare the gestational differences. In mouse, extracellular acidification enhanced uterine contraction in pregnant uterus more than non-pregnant one. Pregnant uterus is known to be more sensitive to the changes in pH than the non-pregnant among different species; in mouse (Hong et al., 2013, Kyeong et al., 2016), rat (Heaton et al., 1992) and guinea-pig (Naderali and Wray, 1999). These different responses could be explained by the increase in L-type calcium channels expression during pregnancy (Mershon et al., 1994). Therefore, I tested the relation between extracellular acidification and L-type calcium channels in Chapter-5.

What the effects of extracellular acidification on force suggest about underlying mechanism?

Given the difference I have already described between my findings on mouse and rats, it cannot easily be assumed what happens to pH_i in intact mouse myometrium when the external $[H^+]$ is increased or what this must do to contractility. While the

purpose of this chapter was to elucidate and characterize the effect of external pH change on mouse myometrium; the data analysis was also used to guide my subsequent mechanistic investigation into how external protons affect contractility. This is because, the frequency of uterine contractility can most usually be related to frequency of firing of action potential and membrane potential changes, whereas contraction amplitude usually reflects the changes in intracellular [Ca^{2+}] (Wray et al., 2015).

Effect of extracellular acidification on amplitude

Briefly, the increase in force of contraction of the pregnant myometrium, in response to acidification, is very likely due to an increase in intracellular [Ca]. This can be due to an increase in the Ca^{2+} entry into the cell through L-type Ca^{2+} channels (Shmigol et al., 1998), or other non-voltage sensitive channels; e.g. SOCE (Arrowsmith et al., 2014). Previously, Shmigol *et al* showed that external pH alteration affects Ca^{2+} current across the cell membrane in the uterus (Shmigol et al., 1995). Their work on freshly isolated myometrial cells using the whole cell patch clamp technique showed acidic pH_o decreases, while alkaline pH_o increases, the Ca^{2+} current across myometrial membrane in pregnant rat. As explained above functionally the rat responses differ from those in the mouse, but this study does demonstrate that external pH can affect L-type Ca entry in myometrial cells. Increase in the Ca^{2+} release from intracellular store may also make a contribution to an increase in Ca^{2+} and thus contraction amplitude. Acidification increases calcium sequestration inside the myometrial SR and that loads it with Ca^{2+} ready to release as the SR Ca^{2+}-ATPase (SERCA) can be activated by acidic pH (Pierce et al., 2003). As explained in the introduction however, in myometrium a full SR has been shown to limit contractions and an empty SR bring Ca^{2+} into the myometrial cell, via SOCE, and so a role for the SR is unlikely to explain my data. Gardner & Diecke found an increased Ca^{2+} sensitivity with decreasing pH in the skinned vascular smooth muscle (Gardner and Diecke, 1988). However, in the myometrium there is no direct evidence from many studies to support a role for Ca^{2+} sensitivity in the myometrium (Crichton et al., 1993, Kupittayanant et al., 2001). Therefore, I do not further consider investigating calcium sensitivity in this work. Thus a focus for mechanism seeking should be on whether [Ca^{2+}] increases with acidic pH_o, and if it does, what underlies this increase.

Effect of extracellular acidification on frequency

The increase in frequency, caused by acidic pH_o is likely to be mainly due to increase the frequency of firing of action potential, as this is what drives frequency in the myometrium (Heaton et al., 1992, Wray et al., 2015). The relation between protons and action potential has been under investigation for the past four decades. Kurachi in 1982 found that increasing H^+ inside single ventricular cells shortened the action potential and depressed its plateau (Kurachi, 1982). In the uterus, the relation between protons and the excitability of the myometrial cells is not as well established. Garfield and Maner found that direct agonists and antagonists of the uterine contractions affect the excitability and/or conductivity of the myometrial cells (Garfield and Maner, 2007). Extracellular acidification (pH 6.4) was found to enhance the contraction in ICR mouse myometrium and that was referred to the inhibitory effect of acidic pH_o on TASK-2 channels, one of the two-pore domain K^+-channels (Hong et al., 2013, Kyeong et al., 2016) and if those channels are inhibited, then action potentials will be fired more frequently.

Thus to increase the frequency of uterine contractions, extracellular pH may be expected to increase the excitability of the cells. This can be studied using electrophysiological techniques, or more easily, by altering $[K^+]$.

What is the role of intracellular pH in the effects of extracellular acidification on force?

Intracellular pH is well known to cause multiple changes within cells and their signalling pathways and functional outcomes. The myometrium is no exception (Phoenix and Wray, 1993, Parratt et al., 1995a, Parratt et al., 1995b). The question therefore arises if changes of pH_i are involved in the effects of pH_o. When investigated in other tissues, alteration of pH_o induces changes of the pH_i in the same direction and the time course for these induced changes is dependent on the tissue (Taggart et al., 1997b). In the rat, myometrium, when measured, extracellular acidification is slowly transmitted to and then acidifies the cytoplasm (Taggart et al., 1997b). I examined the time course of my data in detail to distinguish between immediate effects of pH_o change and slower changes, which may be due to induced changes in pH_i. When the time course of functional effects on amplitude and frequency were analysed by the first,

middle and last third of the application of the pH_o 6.9, or the traces were simply examined by eye, it is clear that the effects, particularly on frequency, are rapid. Amplitude peaks after around three minutes and both parameters are then more or less maintained through the application period. There are few measurements in myometrium and none in mouse measuring intracellular pH. Simultaneous measurement of force and intracellular pH when pH_o is changed to 6.9 is therefore required for a better understanding of the mechanisms whereby extracellular pH can affect force. I performed these experiments using carboxy SNARF-1 AM, a florescent pH indicator, in the next Chapter.

Stimulatory effect of extracellular acidification was abolished by oxytocin

As discussed earlier, oxytocin is a potent stimulus of the uterus and is widely used to augment labour (Alfirevic et al., 2009). Its receptors are upregulated markedly near labour (Gimpl and Fahrenholz, 2001). Oxytocin increase calcium concentration inside myometrial cells mainly through Ca^{2+} entry through L-type calcium channels, store-operated calcium entry (SOCE) and calcium release from sarcoplasmic reticulum (SR). (See recent review by Arrowsmith & Wray for further details) (Arrowsmith and Wray, 2014).

My work showed that pH_o 6.9 caused no change in uterine force under the effect of oxytocin i.e. oxytocin abolished the stimulatory effect of pH_o 6.9. The relation between oxytocin and external protons is not clear. Similar effect of oxytocin on abolishing the effect of acidification was noticed in uterine strips from pregnant women (Pierce et al., 2003). On one hand, oxytocin lessened the pH_i drop produced by the weak acid lactate (Hanley et al., 2015) but on the other hand, cellular acidification *per se* was found to prevent oxytocin stimulatory effect on the myometrium (Quenby et al., 2004) and its antidiuretic effect in the bladder (Parisi et al., 1981b, Parisi et al., 1981a). These latter researchers found that oxytocin caused cellular alkalinization in the frog's bladder (Parisi et al., 1981a).

The exact relation between oxytocin and hydrogen ions needs further investigation but given that oxytocin acts mainly by increasing Ca^{2+} entry, and I have suggested that this will be the main way that external acidification increases force, it could be that both agents are using the same pathways. Thus if oxytocin has already stimulated force, pH_o 6.9 can produce no further increase. Due to abolition of the effect of extracellular

acidification by oxytocin, I performed the experiments in the next chapters on spontaneously contracting mouse uterus only.

Relevance to labour

Metabolic acidosis and respiratory alkalosis develop during pregnancy. These metabolic changes are increased during labour (Sjostedt, 1962). In addition, I and others showed that extracellular acidification modulate uterine contraction (Table 1.2). My experiments *in vitro* on pregnant uterus from mouse have shown that when extracellular pH is reduced, uterine force will increase. Thus it could be a mechanism to try and maintain or even increase force during labour. Hypoxia has been shown to act in a similar manner, an increase in force when hypoxia is occurring (Alotaibi et al., 2015). When I applied pH_o 6.9 in a brief and repetitive manner, acidic pH_o caused increases in force with each application (Figure 3.7), which suggests it could be functional in labour, where three are many repeated contractions. Disturbance of the extracellular pH might result in labour complications e.g. dystocia, preterm labour or uncontrolled postpartum bleeding.

Chapter Four

The Effects of Intracellular pH Alteration on Mouse Uterine Contraction

Chapter-4

The Effects of Intracellular pH Alteration on Mouse Uterine Contraction

4.1 Abstract

Introduction: Acidity can play an important role in affecting myometrial contraction. My work showed that extracellular acidification enhanced the amplitude and frequency of uterine contraction in pregnant mouse. One key potential mechanism by which extracellular acidification may be affecting force, is by inducing changes in intracellular pH (pH_i). In this chapter, I therefore investigated i) the relation between extracellular acidification and intracellular pH; and ii) the effects of intracellular pH alteration on contractility of mouse uterus.

Methods: C57BL/6J mice were used either on day 14 or day 18-19 of gestation or at 8-10 weeks non-pregnant. Changes in pH_o to 6.9 were made by adding HCl to physiological saline. Intracellular acidification at constant external pH (7.4) was made by adding Na-butyrate (20mM), isosmotically substituted for NaCl. Intracellular alkalinization was produced by isosmotically substituting with ammonium chloride (NH_4Cl, 20mM). The perfusion rate was 5mL/min and the physiological saline and experimental solutions were maintained at 37^oC and oxygenated with 100% O_2. The physiological solutions were buffered with HEPES (10.9 mM). For simultaneous measurement of force and intracellular pH, longitudinal myometrium strips were loaded with carboxy-SNARF-1 AM (fluorescent pH indicator). Strips were then mounted on an inverted microscope between a fixed hook and a transducer and continuously perfused with the physiological saline at 1ml/min at 35^oC. Determination of intracellular pH was calculated following calibration, using the proton ionophore, nigericin. Preliminary attempts were made to measure intracellular Ca^{2+} using Indo-1, a fluorescent, Ca sensitive indicator. Spontaneous contractions were measured (frequency, amplitude, duration and area under the curve, AUC) and data analysed using LabScribe 3 and OriginPro 2015. Statistical differences were tested with Student's t test (n=6-10) animals, and taken at $P<0.05$.

Results: Intracellular acidification significantly increased uterine force in pregnant uterus from mouse but not in non-pregnant preparations. Intracellular alkalinization was found to decrease uterine contractility. Intracellular pH began to measurably decrease in response to the application of acidic pH_o after approximately one minute. It continued to steadily decrease, and then plateaued between 9-10 minutes after application. It was found that 0.1 pH change was induced. Preliminary attempts to measure intracellular Ca^{2+} were not fruitful.

Discussion: Intracellular acidification caused significant increase in uterine contractility in pregnant, but not the non-pregnant, mouse. Intracellular alkalinization produced the opposite effect. Measurements of pHi showed that, consistent with findings in rat myometrium, changes of pH_o induces a slow change in pHi, but that this is too slow to explain the initial stimulation of force, especially frequency, produced by extracellular acidification. I was unsuccessful in measuring intracellular $[Ca^{2+}]_i$ in mouse myometrium, but was kindly granted access to data obtained by a postdoctoral fellow in our group. My analysis of this $[Ca^{2+}]_i$ data, shows that extracellular acidification increases intracellular calcium, which mirrored the changes in force.

4.2 Introduction

Extracellular acidification in the myometrium develops as a result of repeated hypoxia due to forceful uterine contractions during labour which cause constriction of the uterine blood vessels (Greiss, 1965, Li et al., 2003). In the previous chapter, I showed that extracellular acidification significantly increased uterine contractility in pregnant mouse, but not in non-pregnant mouse. The effect of pH and its mechanism on uterine smooth muscle are poorly understood and have been little investigated. In this chapter, I studied the role of intracellular pH in the stimulatory effect of extracellular acidification on murine uterine contractility.

Effects of changes in intracellular pH (pH_i) on myometrial contraction have been studied previously in different species but not in the mouse. Intracellular acidification decreases spontaneous contractions in pregnant and non-pregnant uteri in rats (Wray et al., 1992, Taggart and Wray, 1993a) and women (Phoenix and Wray, 1993, Parratt et al., 1994, Parratt et al., 1995b) while intracellular alkalinization increased contractile activity (Heaton et al., 1992, Phoenix and Wray, 1993, Taggart et al., 1997b). Similar findings were found in the laying hen's uterus (which is the shell gland of the oviduct) (Kupittayanant and Kupittayanant, 2010). Thus the available literature shows that myometrium is sensitive to even small changes in pH_i as they change its force.

The same functional effects have been found in some blood vessels e.g. mesenteric arteries (Austin and Wray, 1993a) and coronary arteries (Nagesetty and Paul, 1994). However, another study investigated the effects of extra- and intracellular acidification on the contraction of thoracic aorta in rats (Furukawa et al., 1996). Furukawa *et al* measured the isometric tension and intracellular calcium concentration in relation to changes in pH_o and pH_i. They found that acidifying external pH caused a rapid and sustained decrease in pH_i and increase in $[Ca^{2+}]_i$ followed by a contraction in rat thoracic aorta. Similar results were found in rabbit's aorta (unpublished work for the same group). The same study ruled out the involvement of α-adrenergic receptors and Na^+ channels (Furukawa et al., 1996). Arnal *et al* found that in cultured aortic vascular smooth muscle cells from rats, there is an increase in intracellular Ca^{2+} from the SR due to intracellular acidification (Arnal et al., 1993), although cultured cells are so phenotypically altered that caution has to be used when considering them.

Salts of weak acids like butyrate, propionate, pyruvate and lactate are used to induce intracellular acidification at a constant pH_o of 7.4. Butyrate has been most used in

myometrial studies. The undissociated butyric acid enters the cell and dissociates causing release of proton ions and therefore acidification. So, intracellular acidification can be achieved without changing extracellular pH (Figure 4.1). Similarly, salt of weak bases like ammonium, diethylamine (DEA) and trimethyl-amine (TMA) are used to induce intracellular alkalinization at a constant pH_o of 7.4 (Wray, 1988a). Previous work on rat myometrium suggested that 20mM butyrate or NH_4Cl will produce a change in the pH_i of around 0.15 pH unit (Taggart et al., 1997b). Recovery of the tissue during the application of butyrate indicates that the acidosis affects uterine contraction rather than the butyrate itself, as pH_i start to be regulated and moves towards less acid values (Phoenix and Wray, 1993). In tissues, including the uterus, removal of the weak acid produced a rebound intracellular alkalinization (Heaton et al., 1992, Taggart and Wray, 1993a, Pierce et al., 2003, Hanley et al., 2015). Similarly, withdrawal of the weak base produced rebound intracellular acidification (Heaton et al., 1993, Taggart and Wray, 1993a, Parratt et al., 1994, Parratt et al., 1995b, Taggart et al., 1996, Naderali and Wray, 1999, Pierce et al., 2003). Acid and base rebound indicate that pH regulation is occurring in the uterine myocytes.

Simultaneous measurement of force and intracellular pH was therefore a necessary step toward understanding the relation between acidifying the pH outside the myometrial cells and its impact on the cytoplasmic pH. Measurements of intracellular pH in the uterus used either ^{31}P-NMR spectroscopy (Wray, 1988a, Wray, 1990, Harrison et al., 1994, Larcombe-McDouall et al., 1998, Larcombe-McDouall et al., 1999), BCECF (Eiesland et al., 1991, Danylovych Iu and Tuhai, 2005) or carboxy SNARF-1 (Taggart and Wray, 1993a, Taggart and Wray, 1993b, Parratt et al., 1995a, Parratt et al., 1995b, Taggart et al., 1997a, Taggart et al., 1997b, Naderali et al., 1997, Hanley et al., 2015). I chose to use carboxy SNARF-1 because it has been more widely used in the myometrium. Although ^{31}P-NMR spectroscopy can provide valuable information, including that of key metabolites, such as ATP along with the measurement of intracellular pH, it is not easy to master and requires access to a suitable spectrometer. Fluorescent indicators, such as SNARF and BCECF are more amenable to routine laboratories studies, in the same way that the Ca-sensitive probes, Indo-1 and Fura-2 are. These indicators, in their acetoxymethyleater (AM) forms, can cross the cytoplasmic membrane and then the ester bond is cleaved by intracellular esterases, releasing the ion-sensitive and selective indicators. Previous work has shown that the indicators will be retained in the cytoplasm for several hours and are non-toxic

and do not contribute significantly to the buffering, if used at reasonable i.e. 5-20 uM, concentrations. The advantages of Indo-1 and SNARF over Fura-2 and BCECF is that they are dual emissions ratiometric indicators which lessen the amount of artefact created in detecting the changes in $[Ca^{2+}]_i$ and pH_i, respectively (Chow et al., 1996, Bootman et al., 2013).

Spontaneous uterine contractions have been shown to cause small 0.04 pH unit decrease in intracellular pH. This was first published by Taggart & Wray (Taggart and Wray, 1993b). They simultaneously measured the relation between uterine contractions, from non-pregnant rats, and intracellular pH using 5µM carboxy-SNARF-1 at 20ºC with loading time 1-1.5 hours.

Limited work has been done to show the relation between changes in extracellular pH and intracellular pH in the uterus. Wray's early work on the pregnant rat, using ^{31}P-NMR spectroscopy, showed that a 1 pH unit decrease in the extracellular pH caused 0.29 ± 0.02 pH unit drop in intracellular pH (Wray, 1988a). A decade later, carboxy-SNARF-1 was used to find that a 1 pH increase in extracellular pH caused 0.58 ± 0.10 increase in pH_i (Taggart et al., 1997b). So, between 30 and 60% of the changes in pH extracellularly is transmitted inside the myometrial cell and could be affecting the force.

Resting pH_i has been found to be different between different species and gestational status. For example, resting pH_i was found to be significantly lower in non-pregnant human uterus (7.07 ± 0.04) than in the pregnant uterine tissue (7.15 ± 0.09) (Parratt et al., 1995b). While in the rat uterus, gestation does not seem to have a significant effect on resting pH_i, i.e. intracellular pH in non-pregnant rat uterus was 7.16 ± 0.01 while in pregnant rat uterine tissue was 7.15 ± 0.05 (Wray, 1988a, Wray, 1990, Taggart and Wray, 1993a, Taggart and Wray, 1993b). In post-partum uterine tissue from rats however, resting pH_i was higher (7.27 ± 0.05) (Harrison et al., 1994, Larcombe-McDouall et al., 1998, Larcombe-McDouall et al., 1999).

Aims

In this chapter, I focused the work on the role of intracellular pH in influencing the stimulatory effect of extracellular acidification on mouse uterine contraction. So, I aimed to:

- Determine the effects of intracellular acidification at constant external pH on spontaneous uterine contraction in term-pregnant, mid-pregnant and non-pregnant mouse.
- Determine the effect of intracellular alkalinization on pregnant uterus.
- Develop the best protocol for measuring pH_i in mouse myometrium.
- Determine the effect of acidic $pH_{o,}$ and its time course, on intracellular pH in the mouse myometrium.

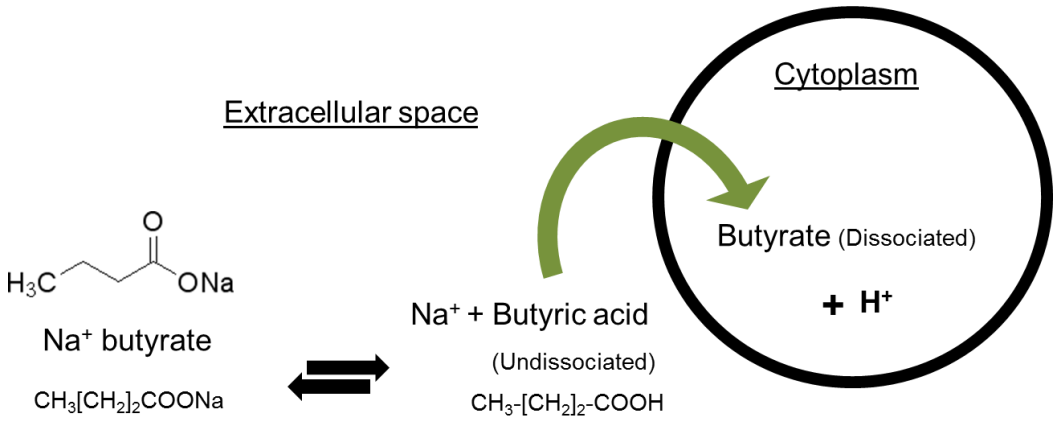

Figure 4.1 Dissociation of butyric acid

Butyric acid is a weak acid with a pKa (dissociation constant) of 4.82. It enters the cell and dissociates causing release of proton ions and therefore acidification. So, intracellular acidification can be achieved without changing extracellular pH.

4.3 Methods

4.3.1 Tissue

C57BL/6J mice were used either on day 14 or day 18-19 of gestation or non-pregnant. They were humanely killed using CO_2 anaesthesia and cervical dislocation, in accordance with UK Home Office rules for Schedule 1 killing. The uterus was removed, cleaned and uterine strips (10mm X 2mm) dissected. Individual strips were mounted between a fixed hook and force transducer using surgical silk threads in a 10ml bath and were continuously superfused with physiological saline solution (PSS) (pH 7.4) at a rate of 5mL/min and maintained at 37 ^{o}C and 100% $O_{2.}$

4.3.2 Experimental protocol

I followed same protocol mentioned in chapter 3. Briefly, after 30-60 minutes of spontaneous contraction, mouse uterine contraction reached the steady state. Then, experimental solutions were applied for 10 minutes (the experimental period). Next, PSS (pH 7.4) was resumed for 20-30 minutes recovery period. The immediate 10 minutes preceding the application of experimental solution was used as the control period. In some experiments, the effect of weak acid or base withdrawal was measured by analysing the immediate 10 minutes following the experimental period.

4.3.3 Solutions

Buffered physiological saline solution (PSS) composed of (mM): NaCl 154, KCl 5.6, $MgSO_4$ 1.2, $CaCl_2$ 2 and Glucose 8. All chemicals were obtained from Sigma-Aldrich, Dorset, unless otherwise stated. Changes in pH_o to 6.9 (buffered by HEPES 10.9) were made by adding HCl to PSS. Intracellular acidification was obtained by isosmotically substitute NaCl with the weak acid salt, sodium butyrate (20mM). Intracellular alkalinization was obtained by isosmotically substituting with ammonium chloride (NH_4Cl, 20 mM).

4.3.4 Measurement of intracellular pH

No previous protocol existed for measuring intracellular pH in mouse myometrium. I experimented to determine best loading conditions by varying time, temperature and concentration, and details are given in the results section. For best signal, strips of mouse longitudinal myometrium (5mm x 2mm x 1mm) were loaded with 12.5µM carboxy SNARF-1 AM for 2 hours in room temperature. After loading, strips were washed for 30 minutes in PSS and aluminium clips were attached at each end. Then, strips were mounted between a fixed hook and force transducer in a 1 ml horizontal bath and were continuously superfused with Physiological saline solution (PSS) (pH 7.4) at a rate of 1mL/min and maintained at 35 ^{o}C. Previous work has shown that in these small strips, there is no need to increase the PO_2 beyond ambient (Hanley and Wray 2015). More details on the protocol used in this chapter are given in Chapter-2.

4.3.5 Nigericin calibration of carboxy SNARF-1 AM for pH_i measurements

Nigericin is a K^+-H^+ antiporter. It is a proton ionophore that works in high K^+ medium to equilibrate intra- and extracellular pH. When abolishing K^+ gradient across cell membrane, protons will be moving freely in and out of the cell making pH equal inside and outside of the cell (Thomas et al., 1979).

Nigericin calibration solution:

KCl	140 mM	The calibration solution was dissolved in ethanol
$MgCl_2$	117 mM	(EtOH)
Nigericin	10 µM	

Then the desired pH was achieved by adding either HCl or NaOH accordingly.

pH	Buffer used (20 mM)
5.5	MES (2-N-MorpholinoEthaneSulfonic acid)
6.5, 7.0, 7.5, 8.0	HEPES
9	CAPSO (N-Cyclohexyl-2-hydroxyl-3-AminoPropaneSulfOnic acid)

Then, 1 μM free acid carboxy SNARF-1 (cell-impermeant pH indicator) was added to the calibration solutions. After measuring the fluorescence, fluorescence/pH ratio was plotted as shown in (Figure 4.2, n=5). From this calibration graph, pH can be calculated using equation below:

$$pH = pKa + \log [R\text{-}Rmin/(Rmax\text{-}R)]$$

Where R = (F580-background) / (F640–background); R stands for ratio and F stands for raw fluorescence. Rmax was recorded at low pH 5.5, while Rmin was recorded at high pH 9.0

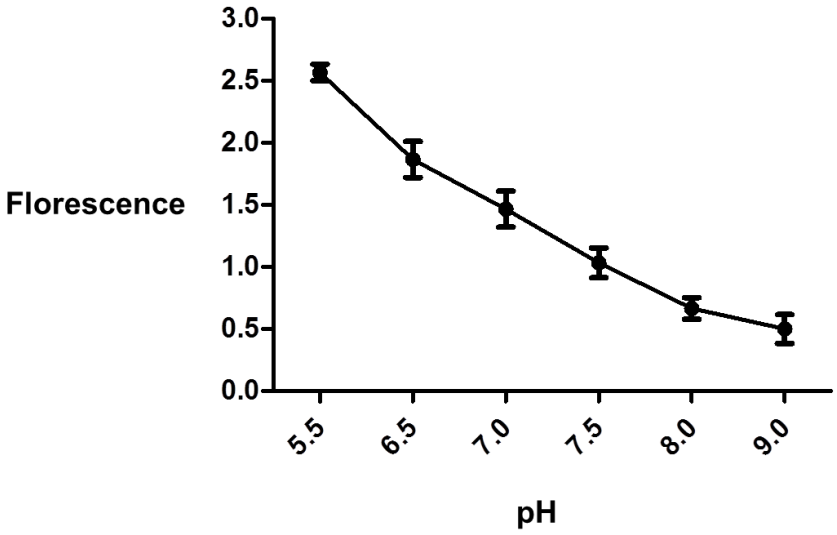

Figure 4.2

Fluorescence / pH ratio curve was obtained using nigericin calibration method (n=5).

4.3.6 Analysis and statistics

Data was recorded and analysed as mentioned previously in Chapter 2. Statistical differences were tested using parametric statistical tests either Student's t-test or ANOVA as appropriate in GraphPad Prism 5. The Bonferroni test was used as a post hoc test. Significance was taken as $P<0.05$.

4.4. Results

4.4.1 Effect of Intracellular Acidification on Term-Pregnant Mouse Uterus

Sodium butyrate was used to induce an intracellular acidification at constant external pH (pH 7.4). A typical trace (representative of 8 others) is shown in Figure 4.3. Intracellular acidification increased the amplitude of contractions significantly (112.7±3.4%, p=0.006). The frequency was also significantly increased (122.2 ± 8.8%, P = 0.03) while no difference in the duration of contractions was found. The area under the curve increased significantly (136.1 ± 11.7%, P = 0.01). Mean data are summarized in Table 4.1. It can also be seen in the trace that the effects of butyrate are not sustained, and the amplitude of contractions, and their frequency, start to decline in the continued presence of butyrate (red arrow in Figure 4.3). As discussed later this indicates that pH regulation is occurring.

Upon removal of the butyrate, there is a rebound decrease in amplitude (88.7 ± 3.1%, p=0.01) and AUC (80.1 ± 7.7%, p=0.04) in comparison to the control period, i.e. before application of butyrate. This is likely to be due to rebound intracellular alkalinization. The mean data are summarized in Table 4.1.

I have also compared the effects of extra- and intracellular acidification in Table 4.1. This shows that, frequency increased more with extracellular acidification in comparison to intracellular one (155.3% vs 122.2%, respectively) while no differences were noted with the other parameters of contraction.

Table 4.1 Mean values (compared to 100% control), standard error (SEM) and p-values of the contractile parameters of 10 minutes addition of Na-butyrate (20mM) and its removal. Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
Na-Butyrate	Mean	112.7%	122.2%	102.6%	136.1%
(n=9)	SEM	3.4	8.8	15.4	11.7
	P-value	**0.006**	**0.03**	**0.8**	**0.01**
Rebound force on butyrate removal	Mean	88.7%	89.2%	99.1%	80.1%
	SEM	3.1	6.9	1.8	7.7
	P-value	**0.01**	**0.1**	**0.6**	**0.04**
pH_o 6.9 vs Butyrate	**P-value**	**0.1**	**0.04**	**0.9**	**0.1**

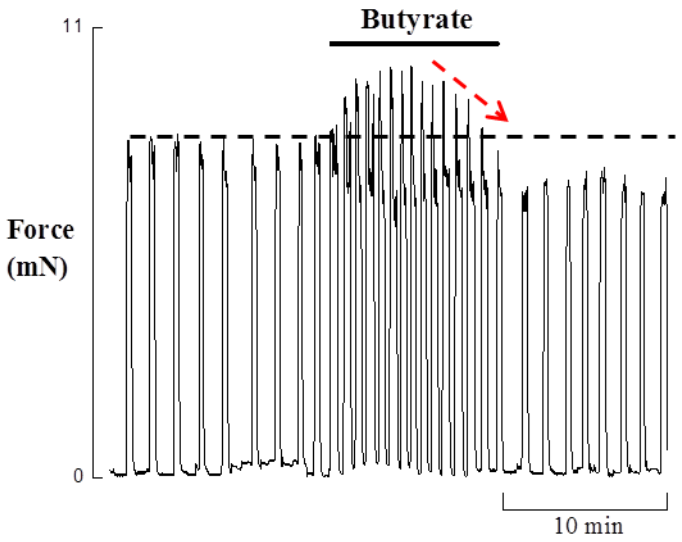

Figure 4.3 Effect of intracellular acidification on mouse myometrium.

Isometric recording of 10 minutes addition of Na-butyrate (20mM), at a constant extracellular pH 7.4, of spontaneously contracting term-pregnant mouse uterus (n=9). Tissue was continuously perfused with physiological saline solution at pH 7.4. The perfusion rate was 5ml/min and preparations were bubbled with 100% O_2 at 37 °C. It can be noted that the force of contractions starts to decline in the continued presence of butyrate (red arrow). Effect of removal of butyrate, which is expected to produce a bound alkalinization, can be noted to be associated with a decreased in contraction. The dotted line shows control amplitude.

4.4.2 Effect of Intracellular Acidification on Mid-Pregnant Mouse Uterus

Uterine strips from 14 days pregnant mice were used to test the effect of extra- and intracellular acidification on the uterine contraction in the mid-pregnancy period. The effects of extracellular acidification were shown in Chapter-3. Here I present the effect of intracellular acidification (Figure 4.4). Intracellular acidification (n=6) significantly increased amplitude by 15%, and frequency by 30% more than the control with no change in duration. The increase in amplitude and frequency led to increase in AUC to 66% more than the control. The mean data are summarized in Table 4.2.

Withdrawal of butyrate in 14 days pregnant uterus led to immediate decrease in frequency (61.1±5.6%) in comparison to the control period before applying the butyrate (Table 4.2)

I have also compared the effects of extra- and intracellular acidification on uterine contractions from mid-pregnant uterus (Table 4.2). This shows no differences in the parameters of contraction between them. Similarly, no differences were found when the effect of butyrate was compared between term- and mid-pregnant tissues (Table 4.2)

Table 4.2 Mean values (compared to 100% control), standard error (SEM) and p-values of the contractile parameters of the 10 minutes application of Na-butyrate (20mM) on mid-pregnant uterus from mouse. Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
Na-Butyrate (n=6)	Mean	115.1%	129.8%	133.3%	166.3%
	SEM	6.2	12.0	21.5	25.7
	P-value	**0.04**	**0.04**	**0.2**	**0.04**
Rebound force on butyrate removal	Mean	84.9%	61.1%	98.0%	69.8%
	SEM	9.5	5.6	4.6	15.8
	P-value	**0.2**	**0.02**	**0.6**	**0.1**
pH_o 6.9 vs Butyrate	**P-value**	**0.3**	**0.4**	**0.2**	**0.9**
Term vs Mid-term	**P-value**	**0.7**	**0.6**	**0.6**	**0.2**

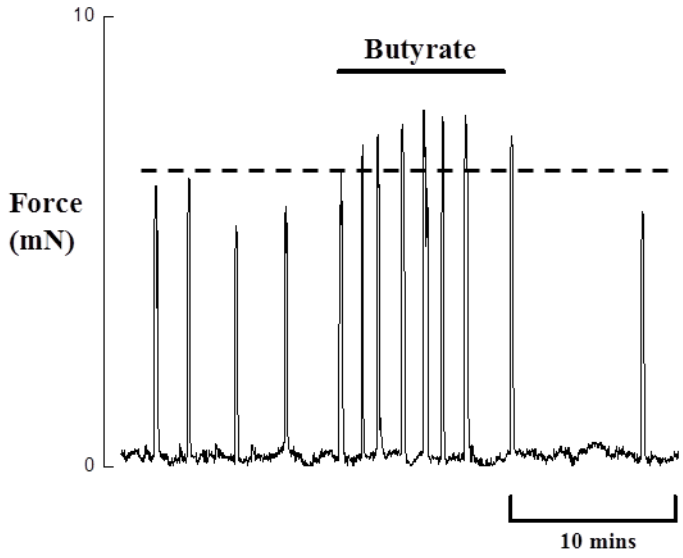

Figure 4.4 Effect of intracellular acidification on 14 days mouse pregnant myometrium.

Isometric recording of 10 minutes application of the weak acid sodium butyrate (20mM) at a constant external pH of 7.4 (n=6). A clear enhancement of the contractility can be notice when Na-butyrate is applied. Effect of removal of butyrate, which is expected to produce a bound alkalinization, can be noted to be associated with a decreased in contraction. The dotted line shows control amplitude.

4.4.3 Effect of Intracellular Acidification on Non-Pregnant Mouse Uterus

Uterine strips from non-pregnant mice were used to test the effect of intracellular acidification on uterine contraction. Intracellular acidification was again induced using the weak acid Na-butyrate (20 mM)**.** Butyrate did not change the contractions of non-pregnant uterus; Figure 4.5 is typical of other 6 traces. In addition, there was no rebound decrease in contractions upon butyrate withdrawal. The mean data for the effects of intracellular acidification on non-pregnant uterus are summarized in Table 4.3

I have compared the effect of intracellular acidification on term-pregnant and non-pregnant uteri in Table 4.3. Differences were found in the effect of butyrate on the amplitude (112.7% vs 100.3%, respectively). While no differences were found in the other parameters (Table 4.3)

Table 4.3 Mean values (compared to 100% control), standard error (SE) and p-values of the contractile parameters after 10 minutes application of Na-butyrate (20mM) on non-pregnant mouse and comparing it to term-pregnant one.

		Amplitude	**Frequency**	**Duration**	**AUC**
Na-butyrate (n=7)	Mean	100.3%	95.4%	108.0%	104.9%
	SEM	3.5	12.6	7.2	10.9
	P-value	**0.9**	**0.7**	**0.3**	**0.6**
TP vs NP	**P-value**	**0.03**	**0.09**	**0.2**	**0.08**

TP=term-pregnant, NP=non-pregnant. Unpaired Student's t-test was used here.

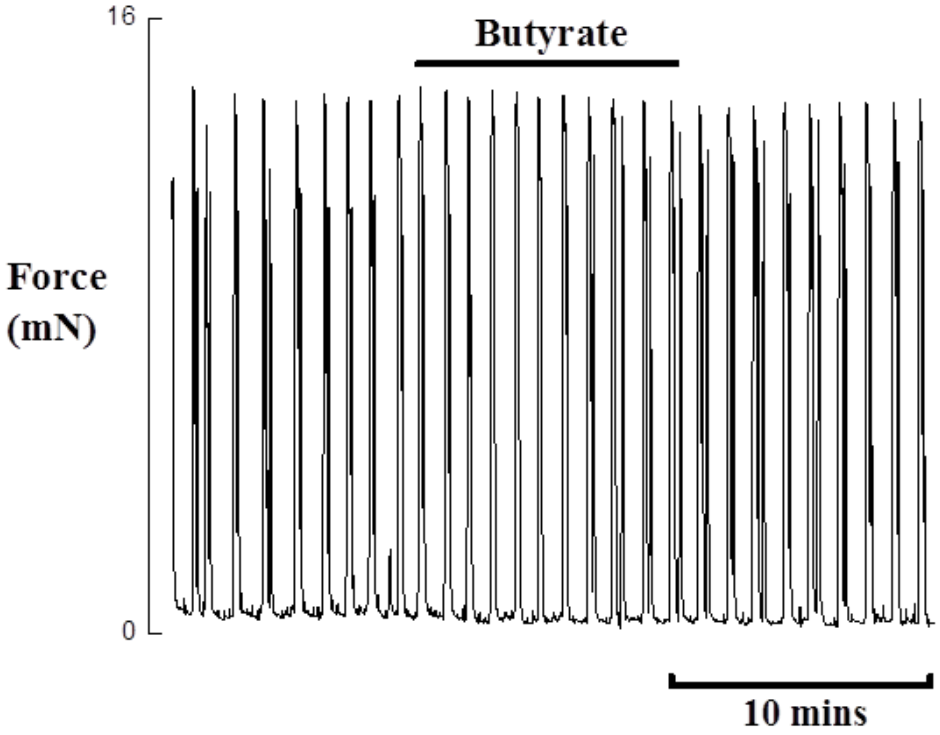

Figure 4.5 Effect of intracellular acidification on non-pregnant myometrium from mouse.

Isometric recording of 10 minutes application of Na butyrate at constant external pH (7.4) to uterus from non-pregnant mouse (n=7).

4.4.4 Comparison of the Effects of Intracellular Acidification at Different Gestation

Comparing the effects of intracellular acidification, using one-way ANOVA test, at the three different gestations showed significant differences in the effect of Na-butyrate (20mM) on the amplitude and AUC ($p=0.04$ and 0.02; respectively). Intracellular acidification showed more stimulation in the term- and mid-pregnant uteri while it didn't change the contractility of the non-pregnant one. On the other hand, there is no difference in the effects of intracellular acidification on the frequency and duration between pregnant and non-pregnant preparations. The mean data are summarised in Figure 4.6

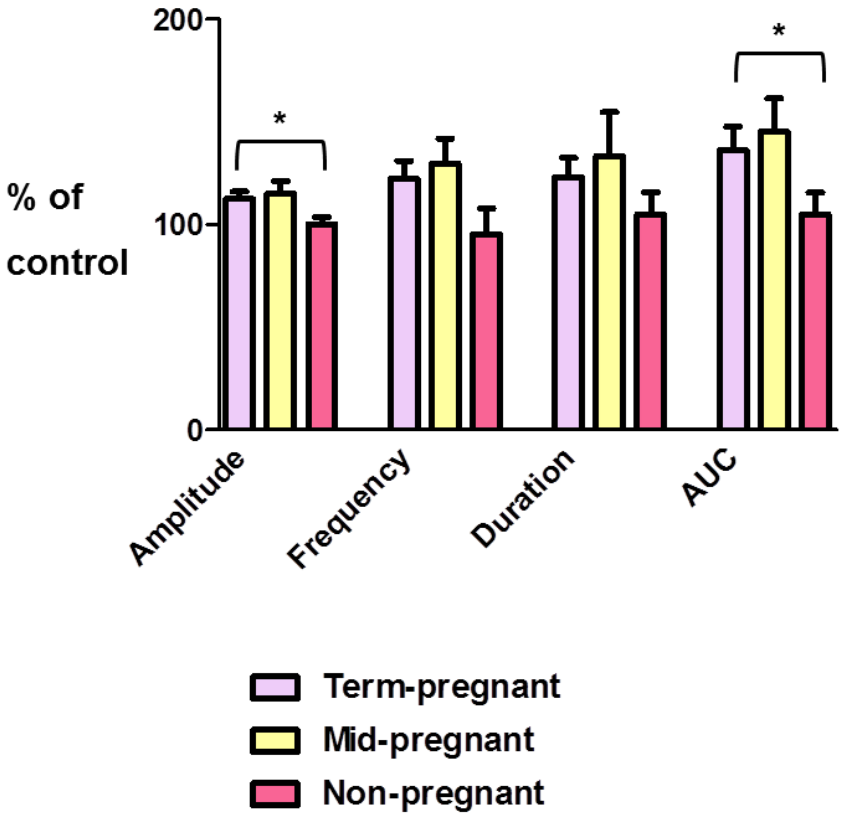

Figure 4.6 Mean data of the different effects of intracellular acidification on uterine contraction between term-pregnant (n=9), mid-pregnant (n=6) and non-pregnant mice (n=7).

Bar chart shows significant increase in the amplitude and AUC of intracellular acidification in the pregnant uterus more than its effect in non-pregnant one. No differences were detected between the other contractile elements, frequency and duration. A significant difference in comparison to the control was found using ANOVA test. * denotes *$p<0.05$.

4.4.5 Effect of Intracellular Alkalinization on Spontaneous Uterine Contraction in Term-Pregnant Mouse.

Intracellular alkalinization was induced using the weak base ammonium chloride (NH_4Cl) at a constant external pH of 7.4. Intracellular alkalinization (n=7) significantly decreased the amplitude by 17% and frequency by 43% of the spontaneous contractions in the term-pregnant uterus. Subsequently, AUC was significantly decreased by 51% with no change in duration. A typical trace is shown in Figure 4.7. An ongoing increase in the force was noticed (red arrow in Figure 4.7) during the continued presence of the ammonium chloride (indicating intracellular regulation of pH is occurring and discussed later). There is also a small overshoot of force when ammonium chloride is removed, indicative of an acid rebound (dotted black line). The mean data are summarized in Table 4.4. When comparing to extracellular alkalinization, pH_o 7.9 significantly depressed amplitude and duration (Table 4.4).

Table 4.4 Mean values (compared to 100% control), standard error (SEM) and p-values of the contractile parameters of the 10 minutes application of intracellular alkalinization (paired Student's t-test was used) and p-values for the comparison to extracellular alkalinization (unpaired Student's t-test was used).

		Amplitude	**Frequency**	**Duration**	**AUC**
NH_4Cl	Mean	82.6%	57.3%	104.2%	49%
	SEM	3.4	9.9	21.7	7.6
	P-value	0.002	0.005	0.8	0.0005
pH_o 7.9 vs NH_4Cl	**P-value**	**0.04**	**0.4**	**0.05**	**0.6**

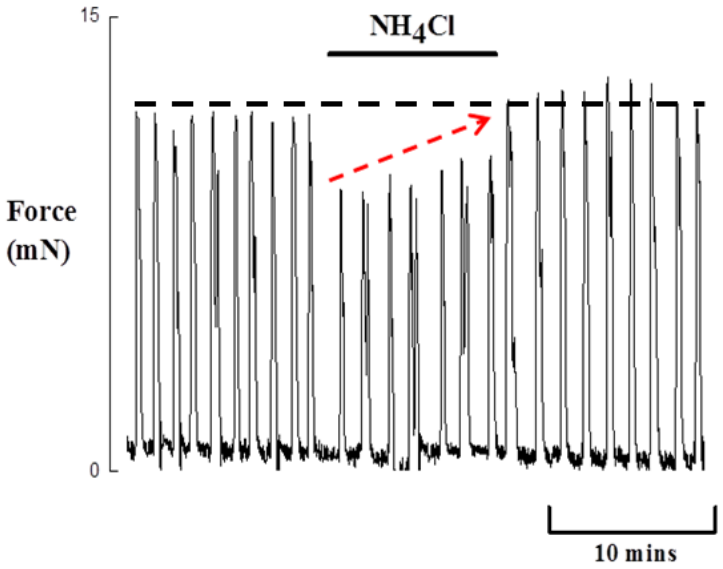

Figure 4.7 Effect of intracellular alkalinization on mouse term-pregnant myometrium.

Isometric recording of 10 minutes application of NH_4Cl (20mM) at a constant external pH of 7.4 (n=7). A slight increase in force is noticed here during the continued presence of NH_4Cl (red arrow). There is also a small overshoot of the force when ammonium chloride is removed, indicative of an acid rebound. The dotted line shows control amplitude.

4.4.6 Effect of Extracellular Acidification on Intracellular pH in Term-Pregnant Mouse: Simultaneous Measurement of Force and pH_i

4.4.6.1 Optimising loading and contractility

No previous protocol for measuring intracellular pH in mouse myometrium existed. For best signal, strips of longitudinal myometrium (5mm x 1mm x 2mm) were loaded with 12.5µM carboxy SNARF-1 AM and kept on a rotator plate protected from light for 2 hours at room temperature. Loaded strips were then washed with fresh PSS for at least 10-15 minutes before used in the experiments to allow complete hydrolysis of the AM esters to avoid artefacts. The experiments were performed on strips that were spontaneously and regularly contracting. Myometrial contractions recorded in these experiments were similar to those recorded in Chapter-3 and lasted more or less unchanged for several hours.

4.4.6.2 Resting pH and changes with contraction

Once both contractions were regular and the baseline pH signal was stable, I calculated the resting pH in the strip. This measurement was made during the control period and averaged over one minute from a period between contractions. In ten myometrial strips the resting pH was 7.18 ± 0.02 (n=10). As shown in Figure 4.8, each contraction was associated by a small decrease in pH_i (0.02 ± 0.005) pH unit.

4.4.6.3 Effect of pH_o 6.9

When pH_o 6.9 was applied for 10 minutes to spontaneously contracting myometrium, force increase and the pH_i decreased. A typical example from 10 preparations is shown in Figure 4.9. There is not an obvious correlation with pH_i changes and force, perhaps suggesting that it is pH_o and not pH_i that is the main driver of the force changes, but that as pH_i decreases it reinforces the pH_o changes.

Intracellular pH started to decrease in response to extracellular acidification 1±0.5 minute after pH_o 6.9 started its effect on the contraction. Then, it continued to steadily decrease, and plateaued between 9-10 minutes after application. It was found that 0.09 ± 0.02 pH decrease was induced (Figure 4.10). The pH_i increased again after resuming PSS at pH_o7.4.

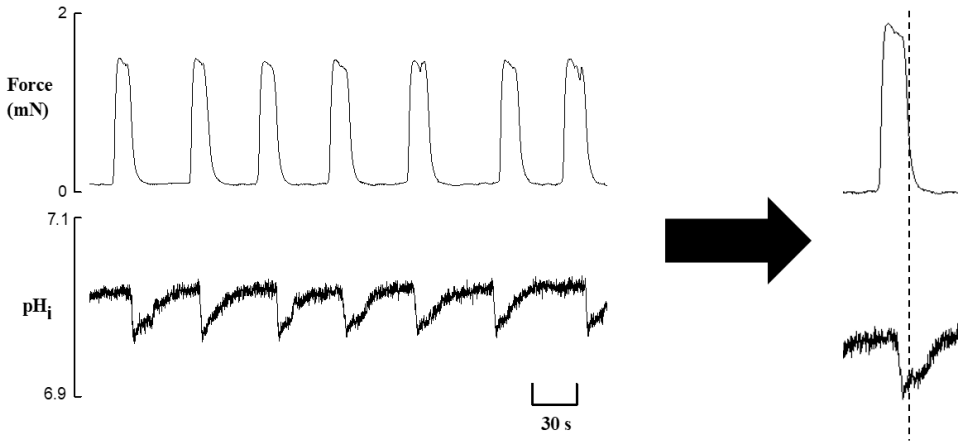

Figure 4.8 Continuous recording of spontaneous contraction (top trace) and pH_i (bottom trace). The close-up contraction and pH_i is shown on the right (n=10). The dotted line indicates the peak of the acidification. It can be noted that the contraction resulted in a small decrease in pH_i. Movement artifacts resulted in changes preceding the increase in force (see expanded traces).

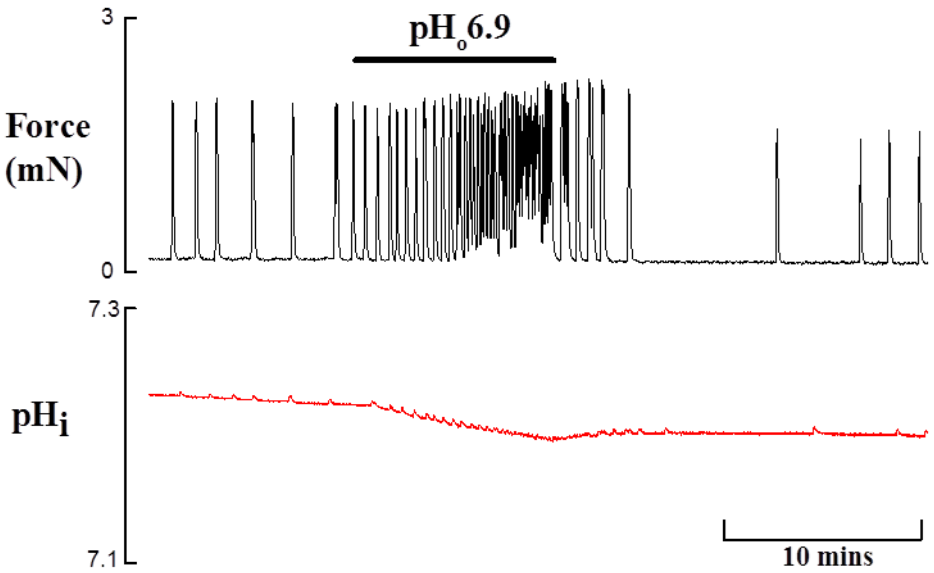

Figure 4.9 Effect of extracellular acidification on intracellular pH

Top trace shows the effect of pH_o on uterine contractility while bottom trace shows the effect of extracellular acidification on the intracellular pH (n=10).

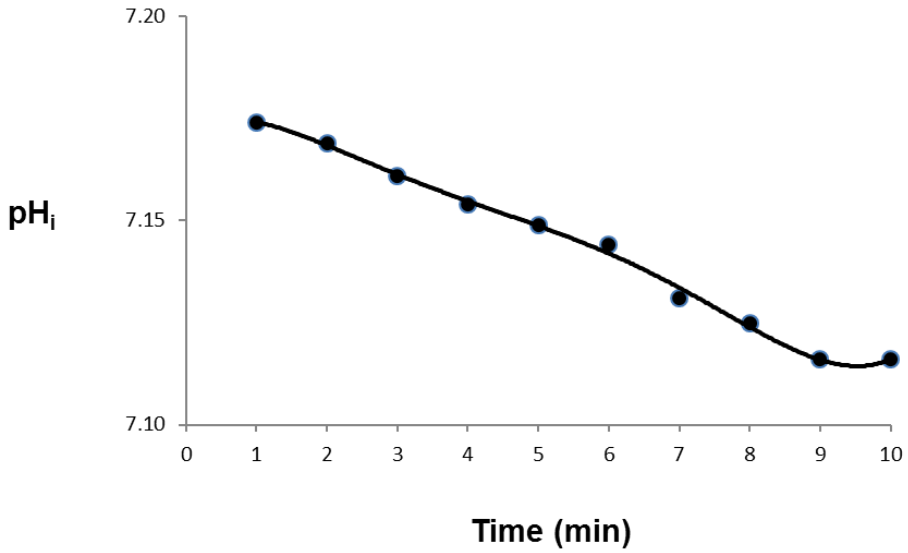

Figure 4.10 Intracellular pH against time (in minutes) in the mouse uterus.

The graph shows the mean intracellular pH_i found over a range of 10 minutes of the application of acidic solution pH_o 6.9. Standard error is within the data point (n=10).

4.4.7 Effect of Combining Extracellular Acidification and Intracellular Alkalinization on Spontaneous Uterine Contractions

I have just shown that pH_o 6.9 over 10 minutes caused a slow and continuous decrease in the pH_i by 0.09 ± 0.02 pH unit. Other studies showed that NH_4Cl increased pH_i in a dose-dependent manner. Taggart *et al* (1993) showed that 30mM of NH_4Cl caused a rapid (<1 min) increase in pH_i in the range of 0.15-0.19 pH unit while smaller concentration (5mM) increased pH_i by 0.02-0.08 pH unit. Thus 20 mM NH_4Cl should reliably abolish the intracellular 0.09+-0.02 pH unit change induced by external pH_o 6.9 Thus, to determine if extracellular acidification produces the same effect on contraction when it does not acidify the cytoplasm, I applied pH_o 6.9 while neutralising any internal pH using NH_4Cl (20mM).

All samples (n=7) showed the initial increase in force as occurred with pH_o 6.9 alone, also occurred in the presence of NH_4Cl. However, with NH_4Cl this was followed by a return to control levels, and then force started to decrease below control values. Indeed in two out of seven preparations, spontaneous contractions were abolished. Typical trace is shown in Figure 4.11.

Data were further analysed by the first, middle and last third of test time (Figure 4.12). As can be seen the tissue immediately responded by increasing the force mainly frequency (140 ± 15%, p=0.03) and AUC (145.4 ± 11.5%, p=0.007) during the first 3.3 minutes. Then force start to decrease in the next 3.3 minutes (AUC, 130.1±12.8%, p=0.05). During the last third of the test period (last 3.3 minutes), there was a significant decrease in amplitude by (41.03 ± 17.5%, p=0.05), frequency by (56.2 ± 14.5%, p=0.008) and AUC by (44.6 ± 14.5%, p=0.02). Mean data are summarized in Table 4.5.

I compared the effect of pH_o 6.9 alone (n=12) for the whole 10 minutes of its application versus the effect of the combination of extracellular acidification and intracellular alkalinization (n=7) on the same period of time, i.e. for the whole 10 minutes of its application (referred to as 'both' in Table 4.5). Acidic pH_o on its own showed a significant stimulatory effect, particularly on the amplitude and frequency, in comparison to combining both extracellular acidification and intracellular alkalinization which indicates the need for acidifying the cell in order to achieve the full stimulatory effect of the acidic pH_o. The mean data are given in Table 4.5.

I also compared the effect of pH_o 6.9 alone (n=12) on the first 3.3 minutes versus the effect of the combination of extracellular acidification and intracellular alkalinization (n=7) on the same period of the application and no difference was noticed between the contractile parameters, amplitude (p=0.1), frequency (p=0.6) which suggests that extracellular acidification in first few minutes acts through a different mechanism other than acidifying the cytoplasm.

Table 4.5 Mean values (compared to 100% control), standard error (SEM) and p-values of the comparison between contractile parameters of the 10 minutes application of extracellular acidification alone (n=12) and in association with intracellular alkalinization (n=7) using unpaired Student's t-test. 'Both' is referred to the combination of extracellular acidification and intracellular alkalinization. Paired Student's t-test was used here.

	Amplitude			**Frequency**	
	pH_o 6.9 alone	**Both**		**pH_o 6.9 alone**	**Both**
Mean	126.9%	91.8%		155.3%	98.6%
SEM	7.2	8.6		10.9	12.3
P-value	**0.007**			**0.004**	
	Duration			**AUC**	
	pH_o 6.9 alone	**Both**		**pH_o 6.9 alone**	**Both**
Mean	101.9%	90.8%		178.5%	99.3%
SEM	14.1	8.7		22.1	11.2
P-value	**0.6**			**0.04**	

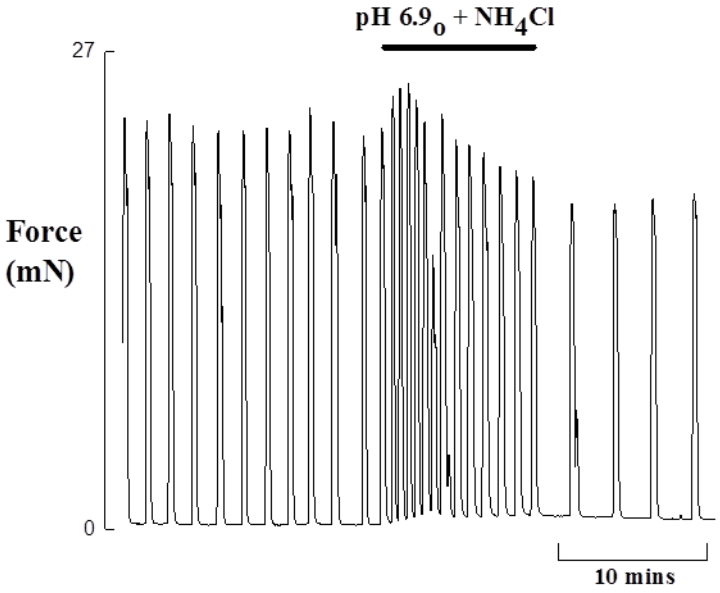

Figure 4.11 Effect of combining extracellular acidification and intracellular alkalinization on uterine contractions in pregnant mouse.

Isometric recording of 10 minutes application of acidic solution pH_o in association with 20 mM NH_4Cl (n=7).

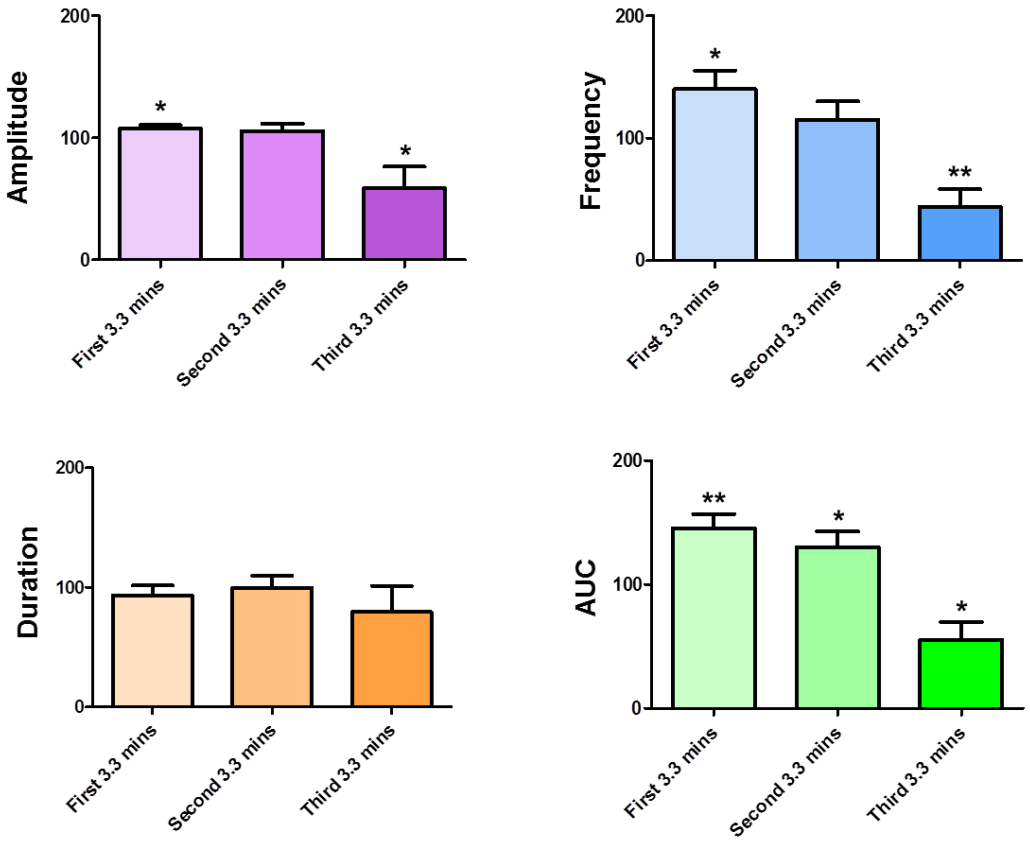

Figure 4.12 Effect of Combining Extracellular Acidification and Intracellular Alkalinization on each third of the application period

Bar charts show the effects on amplitude and frequency of contractions, by time of the test solution. The data were divided into three equal parts of the ten minute application, 3.33 minutes each (n=7). A significant difference in activity was found using paired Student's t-test. * denotes $p<0.05$ **$p<0.005$ significance.

4.4.8 Measurements of Intracellular Calcium

As discussed, it is likely that changes in intracellular [Ca^{2+}] play a part in engendering the force changes produced by extracellular acidification. I tried using Indo-1 to obtain such signals. Unfortunately, in the time available I was not successful in obtaining signals with sufficient quality e.g. signal to noise ratio and stability, for data analysis. My colleague in the group, Dr. Karen Noble, however, had obtained some measurements in mouse, while working a few years previously on another project. These Ca^{2+} traces had not been analysed or published. In consultation with my supervisor and Dr. Noble, it was considered reasonable for me to have access to these data, to determine how pH_o 6.9 affects Ca^{2+} signalling. What follows therefore is my analysis and interpretation of that data. Measuring intracellular calcium was performed in the same experimental apparatus as I used to measure intracellular pH, but with Indo-1 loaded preparations, and filters changed for Indo-1 excitation and emission recordings.

Figure 4.13 shows example traces, from 7 experiments with term pregnant mouse myometrium, with a range of stimulation of force produced by external acidification. It can be seen that during control activity the phasic myometrial contractions, are produced by rises and falls in intracellular Ca^{2+}. Figure 4.14 is a close up view showing that, as expected, the increase in $[Ca^{2+}]_i$ always precedes contraction. As $[Ca^{2+}]_i$ falls, so too does the amplitude of force. That in all the traces included in this figure, pH_o 6.9 caused increase in the uterine force of pregnant mouse (black traces) and caused similar underlying changes in calcium (red traces). It can be seen that when the PSS is returned to pH 7.4, then the Ca^{2+} signals return to control levels along with force. The figures also show that baseline Ca^{2+} rises in the presence of external pH 6.9.

From my analysis, it is clear that the changes in force produced by external acidification are mirrored by changes in intracellular Ca^{2+}.

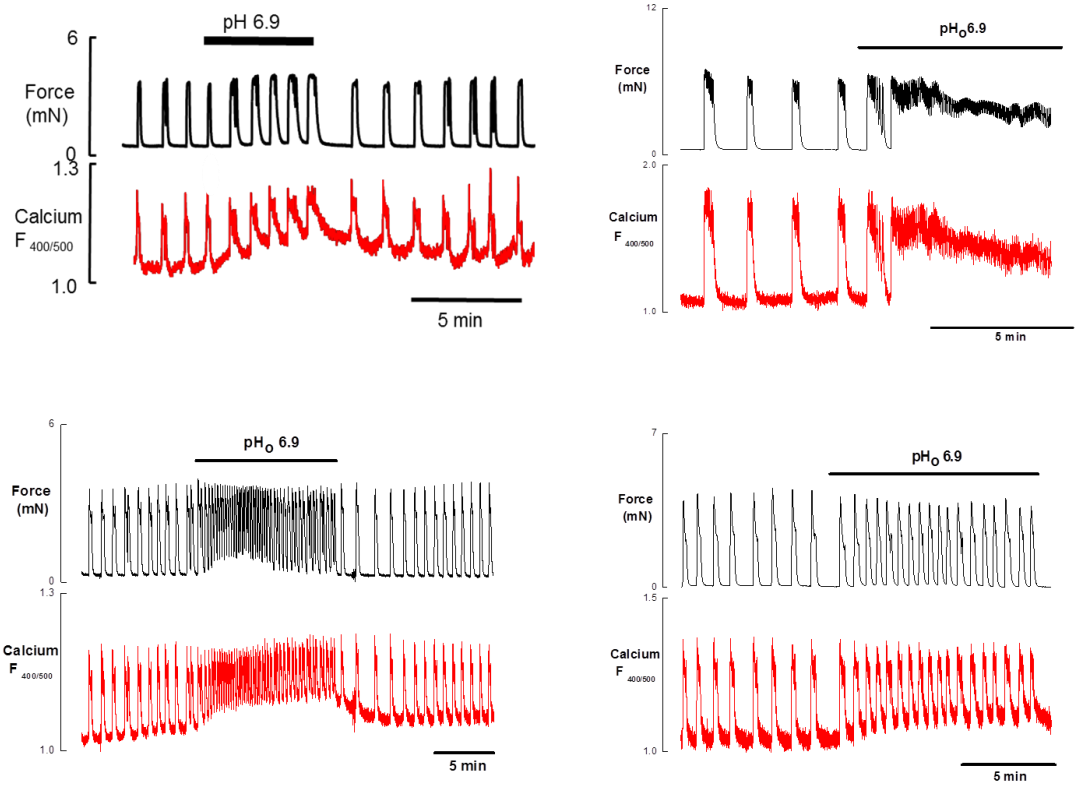

Figure 4.13 Effects of external acidification on force and intracellular calcium $[Ca^{2+}]_i$ in spontaneously contracting term pregnant mouse myometrium previously loaded with Indo 1-AM. The tissue was excited at 340nm and the ratio of emission signals at 400nm and 500nm was used to measure changes in $[Ca^{2+}]_i$. (These Figures were taken from Dr. Karen Noble).

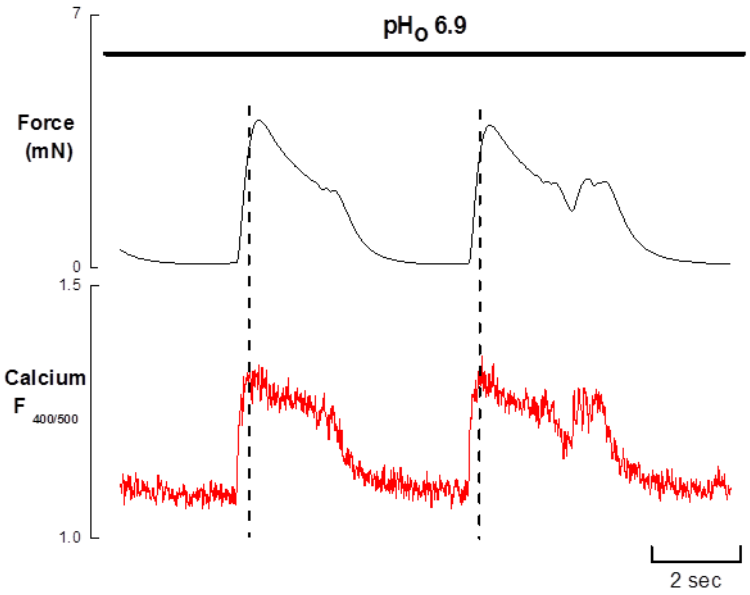

Figure 4.14 A close up view to show that the increase in $[Ca^{2+}]_i$ always precedes contraction which may indicate that acidic pH_o enhances the increase in $[Ca^{2+}]_i$ leading to the force increase. (This Figures was taken from Dr. Karen Noble and modified by me)

4.5 Discussion

In the previous chapter, I showed that extracellular acidification significantly increased uterine contractility in pregnant mouse. This remarkable effect might play an important role in either physiological or pathophysiological events in labour. In this chapter, I showed the effect of altering intracellular pH (pH_i) on uterine contractility and the relation between acidifying extracellular pH (pH_o) and intracellular pH and Ca^{2+}.

The main findings in this chapter are: a) intracellular acidification increases, while intracellular alkalinization decreases, uterine contraction in pregnant mouse; b) intracellular acidification did not affect uterine contraction in non-pregnant mouse; c) gestation changes the effects of intracellular acidification; d) the initial effect of pH_o is not mediated by intracellular pH changes, but is related to changes in intracellular Ca^{2+}.

Relation between extracellular and intracellular pH

In all tissues, including smooth muscle, the plasma membrane does not present an impermeable barrier to protons. Thus when their concentration rises, so this rise will be transmitted to the cytoplasm, and ultimately a new resting pH_i will be established. The changed pH will cause changes to many processes within the cell. The particular relation between extracellular and intracellular pH depends upon a number of factors, but experimental investigations have shown that the induced change in pH_i does vary between tissues and probably between smooth muscles. In mesenteric artery, acidifying the extracellular space resulted in acidification of the intracellular space (Austin and Wray, 1993b). However, in rat aorta changes in pH_o are not completely reflected in pH_i changes (Capellini et al., 2013).

In smooth muscle it is not clear whether the effects of acidic extracellular pH will be the same as acidic intracellular pH. For example in systemic blood vessels, acidification has long been known to produce relaxation. Although it is known that acidification is associated with relaxation of vascular smooth muscle, the relative roles of pH_i and pH_o in this is still discussed (Capellini et al., 2013, El-Galaly et al., 2014), and vessel size, bed and experimental protocols can all influence the outcomes on force. Heaton *et al* found that the response of the uterus, from pregnant rat, to external alkalinization was opposite to the response of the same tissue to intracellular alkalinization (Heaton et al., 1992).

Measurement of intracellular pH

Carboxy-SNARF-1 is a frequently used florescent pH indicator used to measure intracellular pH. It is a useful indicator due to its brightness and retention in the cells. During my experiments which had to last for around two hours, there was only a small decrease in the signal to noise ratio, as judged by noise at the beginning and end of the recording period. Others have calculated in keratinocytes and polymorphonuclear leukocytes that there is only a 10% leakage of indicator in two hours (van Erp et al., 1991). On the other hand, experiments using SNARF were difficult to perform due to the delicate balance needed upon dissecting the samples in order to achieve a myometrial layer thick enough to contract and transparent enough to allow good signalling. Also, the need to avoid photobleaching means that dark conditions around the set up are required.

Loading the pregnant mouse myometrial tissue with carboxy-SNARF-1 allowed measurements of pH_i to be recorded for up to two hours, and did not alter the contractile activity of the tissue as reported previously in human (Parratt et al., 1995b) and rat (Taggart and Wray, 1993a). Regulation of pH_i in response to changes in pH_o in the uterus was first documented by Wray (Wray, 1988a). Resting pH_i in term-pregnant mouse myometrium (7.18 ± 0.02) is similar to that documented in pregnant human and rat tissue (Parratt et al., 1995b, Wray, 1990) which may suggest that there is little or no species differences in acid loaders, pH regulation mechanisms and extracellular pH in myometrial cells. Intracellular pH was found to decrease 29% in response to pH_o decreasing by one pH point (7.4 to 6.4) in pregnant rat uterine tissue using ^{31}P-NMR spectroscopy (Wray, 1988a). My measurements in pregnant mouse using carboxy-SNARF-1 showed a drop of 9% in response to 0.5 pH unit decrease (7.4 to 6.9), and presumably 18% in response to 1 pH unit. These differences are most likely to be due to experimental techniques and conditions.

The functional effects of intracellular acidification on mouse myometrium

Term-pregnant mouse uterus responded in a similar way to intracellular or extracellular pH change. Both ways of acidification increased the contraction while both ways of alkalinization decreased it. Changes in extracellular and intracellular pH do not always produce the same functional results on smooth muscle. For example, in human myometrium, extracellular acidification caused decrease in amplitude but increase in frequency while addition of 40mM sodium butyrate caused abolition of the contraction

(Pierce et al., 2003). In rat as well, extracellular alkalinisation inhibited force but intracellularly it stimulated it (Heaton et al., 1992). Also, in ureteric smooth muscle from guinea-pig, addition of 40mM sodium butyrate caused an increase, while pH_o 6.8 caused a small decrease, in the amplitude (Burdyga et al., 1996).

Mechanisms by which changes in intracellular pH modify uterine contraction are still under investigation (Hanley et al., 2015), Old studies showed that changes in force produced by changes in intracellular pH manipulate Ca^{2+} entry and not the release from intracellular Ca^{2+} stores i.e. sarcoplasmic reticulum (Taggart et al., 1996). Also, intracellular acidification produced by butyric acid did not affect K^+ efflux from the rat myometrium (Heaton et al., 1992, Shmigol et al., 1995).

As with extracellular acidification, non-pregnant mouse uterus did not show a functional response to intracellular acidification. This suggests that the effect of intracellular acidification in the mouse is dependent on gestational state, as mid- and term pregnant myometrium were stimulated by intracellular acidification with butyrate. Uterine contractile activity in the non-pregnant mice long known to be dependent on the stage of oestrus cycle (Cruz and Rudolph, 1986). Uterine mechanical response to both electrical and chemical stimuli and receptors expression change according to the oestrus cycle (Naghashpour and Dahl, 2000, Dodds et al., 2015, Osaghae et al., 2019). This may be due to the different levels and different actions of sex hormones (Naderali et al., 1997). More work is needed to investigate the role of oestrogen and progesterone in the effect of acidification.

In the term pregnant myometrium with both butyrate and NH_4Cl, it was clear in the force records, that their effects were transient as force started to return to control in the continued presence of the weak acid or base. Experiments by others have shown that this correlates with the onset of pH_i regulation (Parratt et al., 1995b, Naderali and Wray, 1999, Duquette and Wray, 2001). Thus as the pH_i excursion starts to decrease, so too does its effect on force. Due to active pH regulation, removal of weak base or acid, produces a transient over- or under- shoot of force, and pH_i, until levels are restored to control. Rebound intracellular acidification after removal of the weak base (Heaton et al., 1992, Taggart and Wray, 1993a, Parratt et al., 1995b, Taggart et al., 1996, Naderali and Wray, 1999, Pierce et al., 2003) and rebound intracellular alkalinization upon withdrawal of weak acid (Heaton et al., 1993, Taggart and Wray, 1993a, Parratt et al., 1994, Pierce et al., 2003, Hanley et al., 2015) in uterus were noticed previously. In my

work, the instant force decrease after removal of butyrate indicates rebound intracellular alkalinization in the uterus of pregnant mouse. This is consistent with my data when I produced intracellular alkalinisation with NH_4Cl i.e. force falls. The opposite effects led to an increase in force upon the removal of the NH_4Cl. Thus two different methods of producing an intracellular acidification, application of weak acid or rebound acidification resulted from NH_4Cl withdrawal, both stimulated force, increasing confidence in my conclusions.

The contribution of intracellular pH to the functional changes produced by extracellular pH change

My work shows that both intracellular and extracellular acidification increase force in pregnant mouse myometrium. I have also measured pH_i and shown that pH_o will change pH_i. The question therefore to be addressed is, are the functional effects in the myometrium due the induced changes in pH_i or is there a stimulation produced by external pH independent of any change in pH_i? My data supports the latter suggestion. This is based on two sets of experimental data; the time course of the pH_i changes, and secondly, the pH_i null experiments performed with NH_4Cl. I will discuss these in turn.

The most compelling argument that pH_o affects force is that it stimulated force, especially frequency, as soon as it reaches the uterus. Clear and significant increases in contractility occur. Measurements of pH_i show that there is little change in the first few minutes of altering pH_o. Intracellular pH started only slowly to decrease in response to extracellular acidification, and it continued to decrease throughout the perfusion of PH_o 6.9 with a maximal drop by ($\approx$ 0.1 pH unit). Thus pH_o is stimulating force before pH_i has changed. These conclusions are consistent with data in pregnant rats, where the effect of pH_o alteration was not explained by the induced changes in intracellular pH (Taggart et al., 1996).

These findings were supported by testing the effect of extracellular acidification while preventing the effect of pH_i by alkalinizing the cytoplasm. There is clear initial stimulation of force, which can only be explained by the extracellular acidification. Interestingly, as shown in the results, the first third of the test period showed stimulation in response to the extracellular acidification. The middle third of the test period showed the force going back to control levels and the third period a further decrease. Having shown that NH_4Cl decreases mouse myometrial force, this suggests that 20mM NH_4Cl, with pH_o 6.9, was not just nulling the induced acidification, but was also producing an

alkalinisation. My data therefore suggests that pH_i alteration in response to acidic pH_o may boost the stimulatory effect of the extracellular acidification but does not explain the initial stimulation of force.

Heaton *et al* also have data that supports my conclusions. In rat uterus, extracellular alkalinisation inhibited force but intracellularly it stimulated force (Heaton et al., 1992). They tested the effect of simultaneous application of NH_4Cl (30mM) and pH_o 8.0 in order to increase both internal and external pH. Interestingly, they found that the effect of extracellular alkalinization (i.e. inhibition of uterine contraction) predominated over the effect of intracellular alkalinization (i.e. enhancement of uterine contraction). This again points to indicate distinct effect on force produced by altering external pH. This effect must be occurring at the level of actions at the cell membrane, where uterine excitability is initiated.

Relation between pH_o and intracellular Ca^{2+}

The changes in force produced by pH_o alteration may be expected to be accompanied by changes of intracellular Ca^{2+} concentration. Pierce *et al* studied the effect of changing pH_o and pH_i on the myometrial activity from pregnant women and its relation to the changes in $[Ca^{2+}]_i$. In their work, application of 40mM butyrate increased intracellular calcium baseline significantly (Pierce et al., 2003). A whole cell patch clamp on single cells from pregnant rats showed that intracellular pH alteration decreases inward Ca^{2+} current and extracellular pH changes affected the calcium current in the same direction as those produced by changes of intracellular pH (Shmigol et al., 1995). Similarly, pH_o was found to affect L-type Ca^{2+} channel current in human mesenteric arterial cells (Smirnov et al., 2000) and in bovine pial and porcine coronary arteries (Klockner and Isenberg, 1994) and in the myocardium as well (Kohlhardt et al., 1976).

I performed some experiments to measure changes in intracellular Ca^{2+} concentration in response to extracellular acidification in the mice, but my trials were not successful mainly due to technical limitations. And due to the limited time allowed for this work, similar unpublished work previously conducted by a collaborator Dr. Karen Noble, was used. The phasic myometrial contractions, are produced by rises and falls in intracellular Ca^{2+} and the increase in $[Ca^{2+}]_i$ always precedes contraction as seen in Figure 4.14. Extracellular acidification was accompanied by an increase in the baseline of $[Ca^{2+}]_i$ which went back to control values with the removal of acidic pH_o. It

is clear that the changes in force produced by external acidification are mirrored by changes in intracellular Ca^{2+}. The increase in intracellular [Ca^{2+}] in response to extracellular acidification is most likely due to Ca^{2+} entry from extracellular space more than its release from sarcoplasmic reticulum as noticed previously (Taggart et al., 1996) (Taggart 1996).

Conclusion

I showed in this chapter that intracellular pH alteration behaved similarly to extracellular pH changes, i.e. intracellular acidification increased, while intracellular alkalinization decreased, uterine contractility force in pregnant mouse. Gestation affects the response of the uterus to pH changes. Extracellular acidification increases uterine contraction mainly due to the increase $[Ca^{2+}]_i$ and partially because of the corresponding changes in pH_i. Mechanisms leading to increase $[Ca^{2+}]_i$ were investigated in Chapters 5 and 6.

Chapter Five

An Investigation into the Mechanisms involved in the Stimulatory Effect of Extracellular Acidification on Mouse Uterine Contraction

Chapter-5

An Investigation into the Mechanisms involved in the Stimulatory Effect of Extracellular Acidification on Mouse Uterine Contraction

5.1 Abstract

Introduction: My work in Chapter 3 showed that extracellular acidification enhanced uterine contractility in pregnant mouse and my data presented in Chapter 4 suggested that the induced change in pH_i following extracellular acidification can explain some, but not all, of the effects of low pH_o. The aim of this chapter was to investigate other possible mechanisms that could underlie the increase in contractility, when pH_o is reduced.

Methods: Term-pregnant C57BL/6J mice were used. Changes in pH_o to 6.9 were made by adding HCl to the PSS. High potassium (high-K^+) solution was prepared using isosmotic replacement of NaCl with 40 mM of KCl. Other drugs and agents used were nifidepine (L-type Ca^{2+} blocker, 1nM), niflumic acid (calcium-activated chloride channel blocker, 30µM), ATPγS (non-hydrolysable ATP, 100µM), A-438079 (P_{2X7} receptor antagonist, 5µM).

Results: Acidic pH_o did not stimulate uterine contractions when they were abolished by nifedipine or in depolarized tissue by high-K^+. Alternatively, extracellular acidification remained capable of increasing contractility in the presence of niflumic acid and A-438079.

Discussion: The absence of stimulatory effect of acidic pH_o when nifedipine caused block of L-VGCCs and when the maximal contraction was achieved by depolarization may suggest that acidic pH_o requires depolarization and L-type Ca^{2+} entry to stimulate uterine contractions. Calcium-activated chloride channels and P_{2X7} receptors may not be involved in the pathway by which extracellular acidification increased the uterine contractions in the pregnant mouse. Taken with my data in the previous chapters, I suggest acidic external pH in the pregnant mouse uterus stimulates contractility by depolarization and Ca^{2+} entry and the effects are sustained by induced changes in intracellular pH.

5.2 Introduction

My work in the previous chapters showed that extracelluar acidification increases force in pregnant uterus. The work to measure intracellular calcium, presented previously in Chapter 4, showed an increase in $[Ca^{2+}]_i$ in response to the acidic pH_o but the mechanisms of this increase in calcium in relation of pH_o has not been investigated.

L-type calcium channels and pH

I discussed in Chapter 4 that the stimulatory effect of extracellular acidification may be through increasing calcium influx. As mentioned earlier, L-type calcium channels are the main route for external calcium entry in myometrium and the main stimulus to contraction (Wray, 1993) (Wray 1993). L-type calcium channels, and subsequently calcium currents, were found to be modulated by the changes in extracellular pH in different smooth muscle (Klockner and Isenberg, 1994, Niu et al., 2014, Zhang et al., 2018). Nifedipine is a selective and potent blocker of the L-type calcium channels, and decreases the force of both spontaneous and agonist-induced contractions in human (Parkington et al., 1999), rats (Granger et al., 1986) and mouse (Brown et al., 2007). Here, I investigated the involvement of L-type calcium channels in the stimulatory effect of extracellular acidification by using nifedipine.

Membrane potential and pH

Alteration of pH changes the charge and therefore the activity of proteins in the cell membrane; e.g. receptors and channels (Austin and Wray, 2000). In vascular smooth muscle (VSM), pH_o was found to alter the conductance and gating of L-type calcium channels. They hyperpolarize during acidification (Klockner and Isenberg, 1994) and depolarize during alkalinization (G Siegel, 1981). In the uterus however, the relation between proton concentration and membrane potential is not well-established. High-K^+ depolarization of the pregnant and non-pregnant rat uterus produces a prolonged inward calcium current and hence, a sustained contraction. Also, it causes changes in metabolites; i.e. a marked decrease in phosphocreatine, a slight decrease in ATP, a slight increase in inorganic phosphate and a marked intracellular acidification (Wray, 1990). Testing the effects of acidification or alkalinization on a high-K^+ depolarized uterine tissue revealed some conflicting findings. For instance, both intracellular acidification and extracellular alkalinization increased force and intracellular calcium concentration in depolarized myometrial strips from non-pregnant rats (Taggart et al.,

1996, Naderali et al., 1997). However, the same tissue showed no change under the same experimental settings in the studies done by Heaton *et al* and Taggart *et al* (Heaton et al., 1992, Taggart et al., 1997b). Interestingly, intracellular alkalinization decreased the force of contraction produced by depolarized myometrium (Heaton et al., 1992) (Heaton 1992). It was suggested that the effect of cellular pH alteration on uterine contractility might be at the level of cross-bridging due to the presence of dissociation between force and Ca^{2+} as seen in (Pierce et al., 2003). High-K^+ was found to depolarize mouse uterus on a concentration-dependant manner, e.g. 20mM KCl depolarized the membrane from -70 mV to -45 mV (Hong et al., 2013). Hence, I examined if the stimulatory effect of extracellular acidification remains present in high-K^+-depolarized uterine strips. If external acidification requires depolarization for its mechanism of action, then in pre-depolarized tissue, its effect should be blunted or abolished.

ATP and pH

Extracellular adenosine triphosphate (ATP) has a powerful influence on muscle contraction. It stimulates uterine contraction in different species (Watts, 1953, Ninomiya and Suzuki, 1983, Suzuki, 1991, Piper and Hollingsworth, 1996, Hutchings et al., 2009, Burnstock, 2014, Alotaibi, 2017). Studies on the mechanisms underlying ATP stimulation of uterine contraction are still ongoing. Measurements of the electrical responses of the myometrial cells membrane, to ATP, from pregnant and non-pregnant mice were recorded using glass capillary microelectrodes. Those recordings found that ATP changed membrane potential producing initial brief hyperpolarization followed by depolarization separate from any hormonal influences (Ninomiya and Suzuki, 1983). Same study found that external ATP enhances uterine contraction in mouse by increasing Na^+ conductance. Extracellular ATP increased uterine contraction in pregnant and non-pregnant rats (Alotaibi, 2018) and uterine samples from pregnant women (Ziganshin et al., 2005, Hutchings et al., 2009) and that effect was abolished by removing extracellular calcium (Zafrah and Alotaibi, 2017, Alotaibi, 2018). ATP was found to enhance the effect of prostaglandin F2α on uterine contraction (Ziganshin et al., 2005). In a study using low and high affinity calcium indicators; mag-fluo-4 and Fura-2, respectively to simultaneously measure both SR intraluminal and cytosolic calcium levels in response to extracellular ATP in freshly isolated myocytes from pregnant rats. This study found that ATP increased Ca^{2+} transient and SR- Ca^{2+} release (Shmygol and Wray, 2005). Moreover, ATP-induced ion currents were detected in freshly isolated uterine myocytes from pregnant rats (Miyoshi et al., 2010) (Miyoshi

2010). Later, same group found that these currents go through P_{2x7} purinergic channels (Miyoshi et al., 2012).

Purinoceptors ($P_{2x7)}$ are ligand-gated ion channel receptors, widely expressed in many living cells. They are also expressed in the myometrium of 18-days pregnant mouse (Miyoshi et al., 2016). Recently, it was found that the stimulatory effect of ATP was abolished in the presence of A-438079, a selective P_{2x7} receptor antagonist, which may indicate their involvement in the ATP activation of uterine contraction (Miyoshi et al., 2010, Alotaibi, 2018). However, it did not affect uterine contraction in the absence of extracellular ATP (Miyoshi et al., 2010, Alotaibi et al., 2015). This in myometrium, the available data indicates that purinergic signalling is via p_{2x7} receptors.

To date, the relation between the ATP and external protons is not well established. The hydrolysis of ATP can produce intracellular acidification (Gevers, 1977), but these small changes are likely to be buffered and not relevant to my studies of extracellular ATP. Likewise, decreasing the external pH to 6.4 made no changes to the uterine ATP levels (Wray, 1988a). Extracellular protons effects on ATP-gated channels were found variant. In general, acidification inhibits P_{2x} receptors (Stojilkovic et al., 2014) although Li *et al* found that protons potentiate their response to ATP by interacting with the ATP receptor-ion channels complex (Li et al., 1996). In pulmonary artery, extracellular acidification (pH_o 6.8) abolished the effect of repeat applications of extracellular ATP through modulating the desensitization-resensitisation features in its receptors (Dubuis et al., 2004), however in the uterus, Ca^{2+} sensitization does not play a significant role. Although there has been relatively little research on the relation between extracellular acidification and $P_{2x7,}$ extracellular protons were found to inhibit the P_{2x7} receptors in different cell types (Virginio et al., 1997, Liu et al., 2009, Sekar et al., 2018). External protons act as allosteric modulators as they decrease P_{2x7} current amplitude without affecting their agonist sensitivity in HEK293 cells (Liu et al., 2009). However, other study reported that protons change the P_{2x7} affinity to bind to the external ATP in HEK293 cells but they are not involved in permeation path (Virginio et al., 1997). Extracellular protons caused different actions on P_{2X7} in microglial cells based on the duration of the acidification. Short period of acidification blocked their ion currents and the following mitochondrial functions. Alternatively, longer acidification activated P_{2X7} and initiated intracellular stress response (Sekar et al., 2018). No similar studies appear to have been performed in any smooth muscle cells.

As nothing yet known about the relation between external protons and P_{2x7} in the myometrial smooth muscle cells, I assumed that extracellular acidification may activate P_{2x7} which may, in turn, enhance calcium influx into the cells and that will lead to the increase in contractility. Therefore, I investigated in this chapter the effect of extracellular ATP and the role of P_{2x7} in relation to extracellular acidification.

Calcium-activated chloride channels and pH

Calcium-activated chloride channels (CaCCs) are expressed in mouse uterus in around one third of the myometrial cells and found to have functional significance (Jones et al., 2004, Bernstein et al., 2014, Dodds et al., 2015). It was proposed to have a role in membrane potential and pacemaker activation (Jones et al., 2004, Song et al., 2009). Jones et.al found that when CaCCs were blocked by niflumic acid, there were major reduction of frequency in spontaneously and oxytocin-induced contracting myometrium without affecting calcium entry but they have no effect on high-K-induced contractions (Jones et al., 2004). This might imply the role of these channels on managing action potential but some studies reported decrease in amplitude too (Yarar et al., 2001). It is documented that CaCCs are activated by calcium entry and depolarization as they are voltage-sensitive (Large and Wang, 1996, Sanborn, 2000, Jones et al., 2004). Role of CaCCs in mediating the effect of extracellular acidification on mouse myometrium is not yet studied, it was compelling to check that using the most potent blocker of CaCCs in smooth muscle; niflumic acid. Here, I assumed that CaCCs might be activated by extracellular protons which may enhance depolarization and therefore more calcium influx which enhance contractility.

In summary the experiments in this chapter were designed to investigate how acidic external pH contributes to depolarization and Ca^{2+} entry, and thereby stimulated contractility in the mouse myometrium.

Aims

In this chapter, I investigated mechanisms which might be involved in the stimulatory effect of extracellular acidification on mouse uterine contraction (Figure 5.1). I aimed to:

- investigate the effect of extracellular acidification in the presence of high K^+ solution.
- investigate the effect of extracellular acidification in the presence of L-type calcium channels blocker (nifedipine).
- investigate the effect of extracellular acidification in the presence of calcium-activated chloride channel blocker (niflumic acid)
- determine the effects of ATP on mouse myometrium (using ATPγS)
- investigate the effect of extracellular acidification in the presence of A-438079, a selective P_{2x7} blocker.

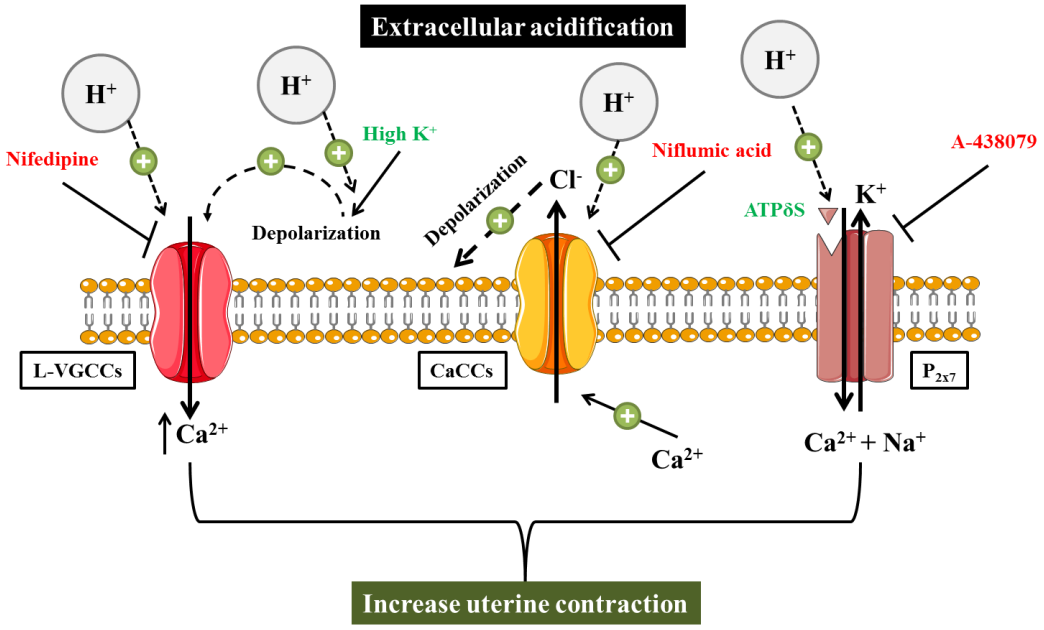

Figure 5.1 Schematic diagram showing mechanisms I investigated in relation to extracellular acidification in this chapter

I examined the effect of pH_o 6.9 in relation to L-type calcium channels (L-VGCCs), calcium-activated chloride channel (CaCCs), P_{2x7} and fully-depolarized tissue by High-K^+.

5.3 Methods

5.3.1 Tissue

C57BL/6J mice were used on day 18-19 of gestation. They were humanely killed using CO_2 anaesthesia and cervical dislocation, in accordance with UK Home Office regulations for Schedule 1 killing. The uterus was removed, cleaned and full-thickness uterine strips (10mm X 2mm) dissected. Individual strips were mounted between a fixed hook and force transducer using surgical silk threads in a 10ml bath and were continuously superfused with Physiological saline solution (PSS) (pH 7.4) at a rate of 5mL/min and maintained at 37 ºC and 100% O_2.

5.3.2 Experimental protocol

After spontaneous contraction reached a steady state, PSS at pH_o 6.9 was applied for 10 minutes. The solution was then returned to pH 7.4 for 20-30 minutes to allow recovery. Then, the solution containing the drug of interest (DOI) at pH 7.4 was added to the uterine strips for 30-60 minutes to ensure it had acted and a new steady state reached. Next, the same solution but at pH 6.9 (test solution) was added for 10 minutes. Later, PSS at pH 7.4 was resumed and pH 6.9 was added again for 10 to ensure its effect before ending the experiment. The test period is the 10 minutes application of the test solution at pH 6.9 while the control period is the 10 minutes preceding test period (Figure 5.2). If different protocol was used, it will be discussed separately in the related results section.

5.3.3 Solutions

Buffered physiological saline solution (PSS) was composed of (mM): NaCl 154, KCl 5.6, MgSO4 1.2, HEPES 10.9, $CaCl_2$ 2 and Glucose 8. All chemicals were obtained from Sigma-Aldrich, Dorset, unless otherwise stated. Changes in pH_o to 6.9 were made by adding HCl to PSS. High potassium (High K^+) solution was prepared using isosmotic replacement of NaCl with 40 mM of KCl. Other drugs used were nifidepine (L-type Ca^{2+} blocker, 1nM), ATPγS (non-hydrolysable ATP agonist, 100µM), 3-[5-(2,3-dichlorophenyl)-1 H-tetrazol-1-yl]methyl pyridine (A-438079, a selective P_{2X7} receptor antagonist, 5µM), niflumic acid (calcium-activated chloride channel blocker, 30µM). All materials were purchased from Sigma-Aldrich unless stated otherwise.

5.3.4 Analysis and statistics

Data was recorded and analysed as mentioned previously in Chapter 2. Statistical differences were tested using Student's t-test. Significance was taken as $P<0.05$

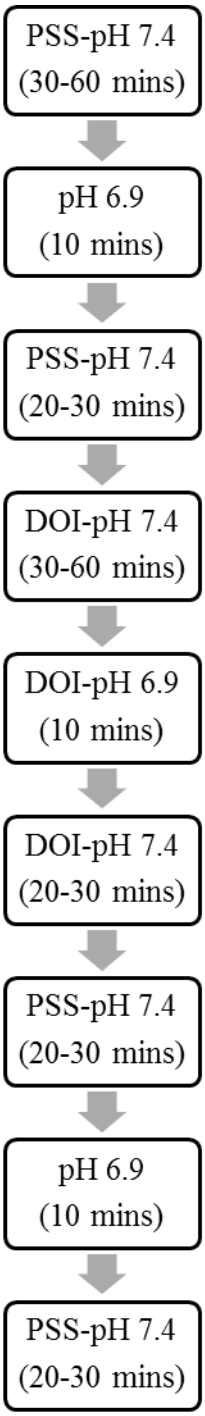

Figure 5.2

A schematic diagram showing the experimental protocol for most of the experiments performed in this chapter.

PSS, Physiological Saline Solution; DOI, Drug of Interest; mins, minutes

5.4 Results

5.4.1 Effect of Extracellular Acidification on Uterine Contractility in the presence of L-Type Calcium Channels Blocker (Nifedipine)

As L-type calcium entry is crucial for spontaneous uterine contractions, finding a concentration of nifidepine that clearly reduces contractility but does not rapidly abolish it can be challenging. I found that pregnant mouse uterine strips were very sensitive to nifedipine, and a concentration of 1nM could abolish the contractions, in some preparations, in a reversible and time-dependent manner (Figure 5.3). In other preparations, this concentration of nifedipine produced a clear decrease in contractions, 60% ($p=0.02$) decrease in AUC when first 10 minutes of the nifedipine was compared to the control 10 minutes preceding the nifedipine application.. This was therefore convenient to separate the effects of acidic pH when contractions were inhibited and when they were abolished.

A test application of pH_o 6.9 solution was made before and after nifedipine application. The acidic solution (pH_o 6.9) was applied twice for 10 minutes, to ensure its effect, in the presence of nifedipine (Figure 5.4). Adding pH_o 6.9 solution stimulated contractility each time as long as uterine contractions were still present (Table 5.1). However, pH_o 6.9 was not able to induce changes in contractility when the contractions had been abolished by nifedipine. This would suggest the need for L-type calcium entry for uterine response to pH_o 6.9.

Contractile parameters where measured from the first application of pH_o 6.9 in the presence of nifidepine which showed response (n=4, first 2 rows in Table 5.1). These values were compared to the preceding 10 minutes under the effect of nifedipine alone (top part of Table 5.2). When the contractile parameters from the first application of pH_o 6.9 in the presence of nifidepine were compared to the effect of pH_o 6.9 on spontaneous contraction, no difference was noticed (bottom part of Table 5.2).

Table 5.1 The effect of pH_o 6.9 in the presence of nifedipine (n=5).

n=	Figure (5.4)	First application of pH_o6.9	Second application of pH_o6.9	Abolition of the force
2	A	Increased force	Increased force	No
2	B	Increased force	No effect	Yes
1	C	No effect	No effect	Yes

Table 5.2 Mean data (100% of the control), standard errors (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification (pH_o 6.9) in the presence of nifedipine. Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
pH_o6.9 + Nifedipine Vs control	Mean (n=4)	122.1%	153.1%	98.5%	165.5%
	SEM	6.6	16.8	13.9	20.7
	P-value	0.03	0.04	0.6	0.04
pH_o6.9 + Nifedipine Vs pH_o6.9 alone	P-value	0.7	0.8	0.3	0.5

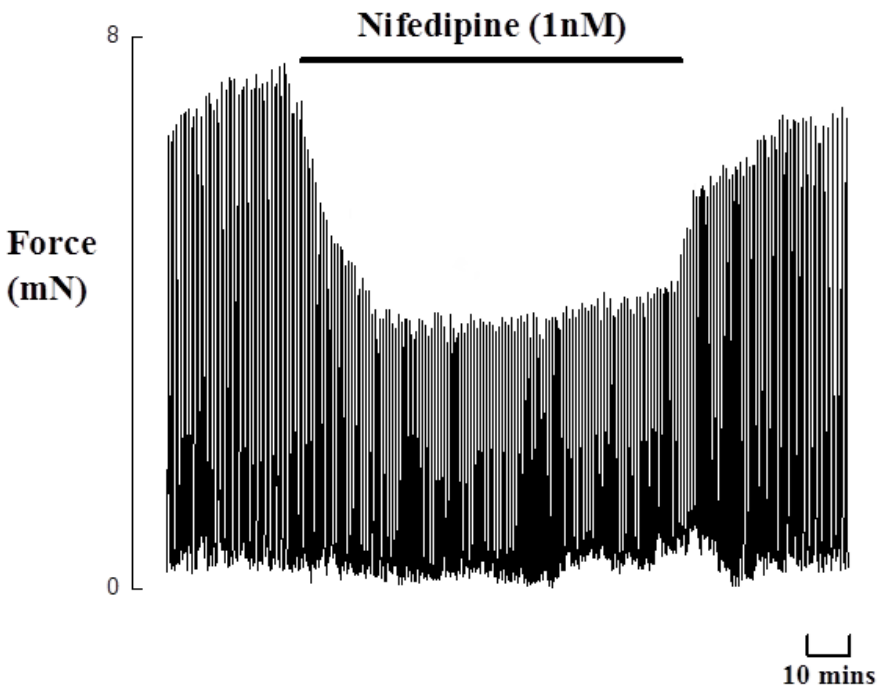

Figure 5.3

Control trace showing the effect of the L-Type Calcium Channels Blocker (Nifedipine, 1nM) on the term-pregnant mouse uterus.

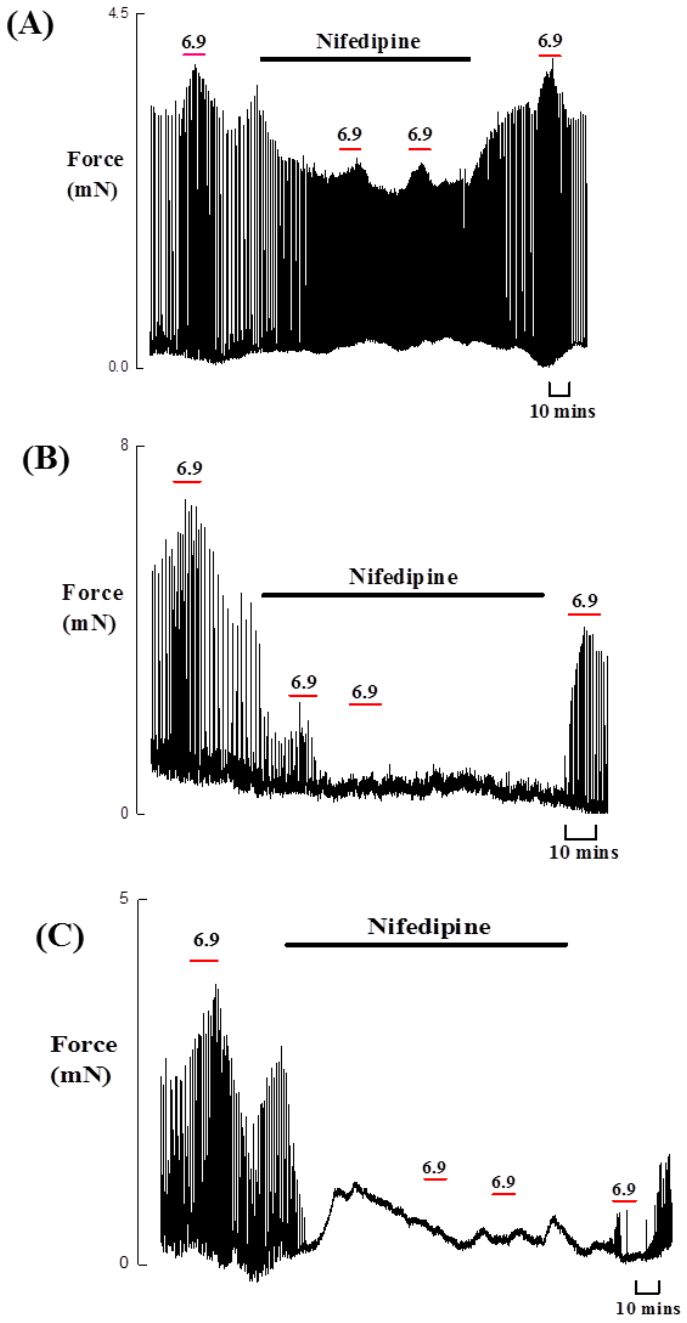

Figure 5.4 Isometric force recordings showing the effects of extracellular acidification in the presence of the L-Type calcium channels blocker (Nifedipine)

As can be seen in all traces (A-C), nifedipine clearly decreased contractions. The traces showing different responses of the pregnant uterine tissue in mouse to nifedipine (1nM), and consequently the effect of pH_o 6.9 (n=5)

5.4.2 Effect of Extracellular Acidification on KCl-Induced Uterine Contractility

Changing the perfusate from PSS to high-K^+ solution, to depolarize the uterus without stimulating any agonist pathways, produced a sustained contraction which lasted as long as the high K^+ solution was present. The effect of high K^+ started by an initial increase in frequency and amplitude of the contractions, followed by a relaxation of force, to a new, sustained, plateau level. This sustained contraction, which in some preparations had some oscillations in its amplitude, as L-type calcium channels switch between open and close (Noble 2002). Perfusing the tissue with PSS again relaxed the muscle and phasic contractions returned (Figure 5.5-A).

Applying pH_o 6.9 for 10 minutes to the depolarised tissue did not cause consistent changes (Figure 5.5-B). Measurement of amplitude during the application of pH_o 6.9 on depolarised uterine tissue was determined by measuring the amplitude halfway through the application of pH_o 6.9 on KCl-induced and comparing it to the value of the amplitude halfway through 10 minutes of the contraction before the application of pH_o 6.9. Analysis of the area under the curve (AUC) was taken for the whole 10 minutes application of the acidic solution and comparing it to the value of AUC for the 10 minutes before the application of pH_o 6. No differences were found between the two applications of pH_o 6.9 in the depolarized preparations – pH 6.9 did not stimulate force. The mean data are given in Table 5.3.

Table 5.3 Mean values, standard errors (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification (pH_o 6.9) on depolarised uterine tissue. Paired Student's t-test was used here.

		Amplitude		**AUC**	
		1st application	2nd application	1st application	2nd application
pH_o6.9 on depolarized uterine strips vs control	Mean (n=10)	97.1%	99.2%	101.7%	103.5%
	SEM	3.3	3.8	3.8	4.3
	P-value	**0.4**	**0.8**	**0.7**	**0.4**
1st application vs 2nd application	**P-value**	**0.5**		**0.6**	

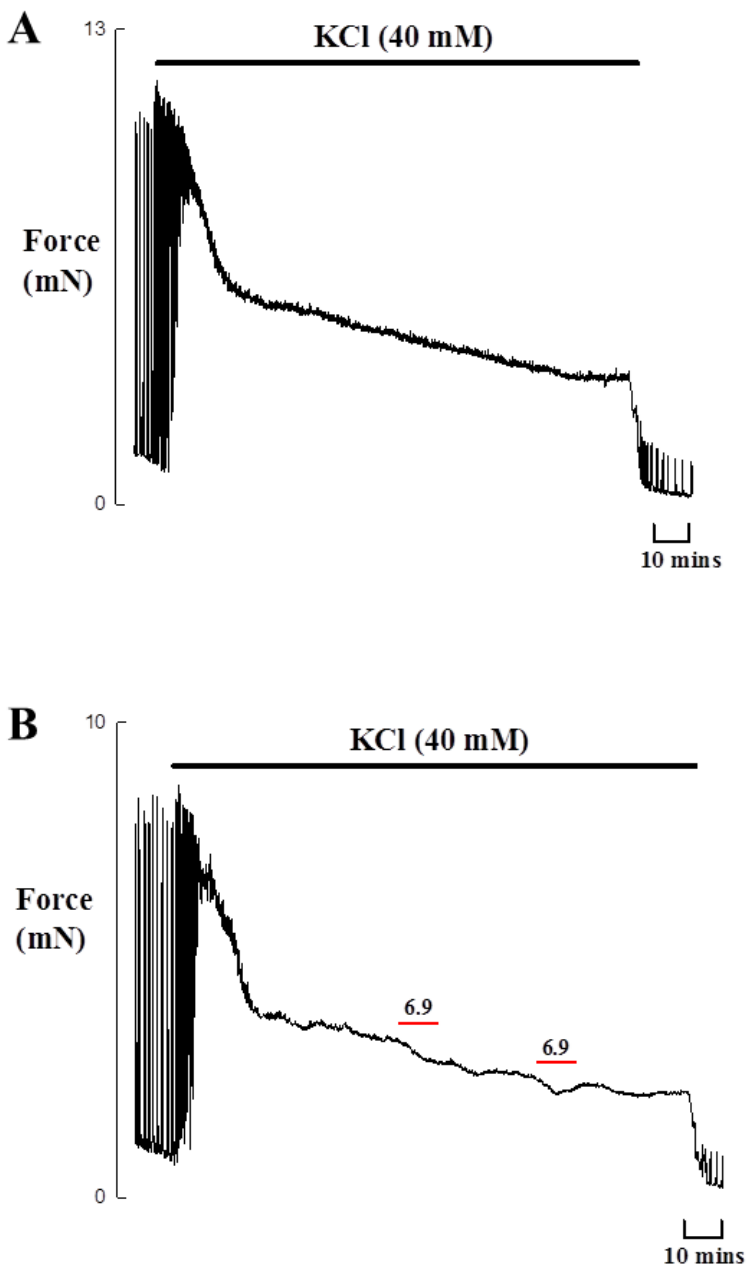

Figure 5.5 Effect of extracellular acidification on KCl-induced uterine contractility in mouse

Top trace is typical for the long lasting effect of high K^+ on mouse pregnant uterus (A). Bottom trace (B) shows the effect of 10 minutes applications of acidic solution (pH_o 6.9) under the effect of high KCl.

5.4.3 Effect of Extracellular Acidification on Uterine Contractility in the presence of purinergic agonist (ATPγS)

As there is limited data on the effects of purinergic receptors P_2 on mouse uterus, initially experiments were performed to investigate these effects using ATPγS. Application of ATPγS (100μM) was applied alone to some traces to test its effect (n = 6). It stimulated the contractions by increasing the amplitude and frequency significantly with no change in the duration (typical trace is shown in Figure 5.6-A). ATPγS significantly increased frequency and AUC in comparison to pH_o 6.9 (p=0.0001 and p=0.003, respectively). The mean data are given in Table 5.4

On paired uterine strips, the pH_o 6.9 solution containing ATPγS (100μM), referred to as 'both' in Table 5.4, was applied. This solution also increased uterine contractility but the frequency and AUC were significantly less than those under the effect of ATPγS alone (p=0.04 and p=0.01, respectively)**.** Figure 5.6-B is typical of 6 other traces.

The mean data are given in Table 5.4. Control used was always the 10 minutes of spontaneous contraction preceding the application of any test solution.

Table 5.4 Mean value (100% of the control), standard errors (SEM) and p-values of the contractile parameters of the effect of application of pH_o6.9, ATPγS and both. Paired Student's t-test was used here.

		Amplitude	Frequency	Duration	AUC
pH_o 6.9	Mean (n=12)	126.9%	155.3%	101.9%	178.5%
	SEM	7.2	10.9	14.1	22.1
	P-value	**0.003**	**0.0003**	0.9	**0.004**
ATPγS	Mean (n=6)	136.7%	353.0%	113.4%	298.1%
	SEM	7.5	45.9	11.0	16.9
	P-value	**0.001**	**0.0006**	0.7	**< 0.0001**
Both	Mean (n=7)	131.4%	287.1%	104.7%	224.5%
	SEM	7.9	18.9	9.3	11.8
	P-value	**0.002**	**< 0.0001**	0.4	**< 0.0001**
pH_o 6.9 vs ATPγS	P-value	0.4	**0.0001**	0.3	**0.003**
pH_o 6.9 vs Both	P-value	0.6	0.2	0.2	0.7
ATPγS vs Both	P-value	0.7	**0.04**	0.1	**0.01**

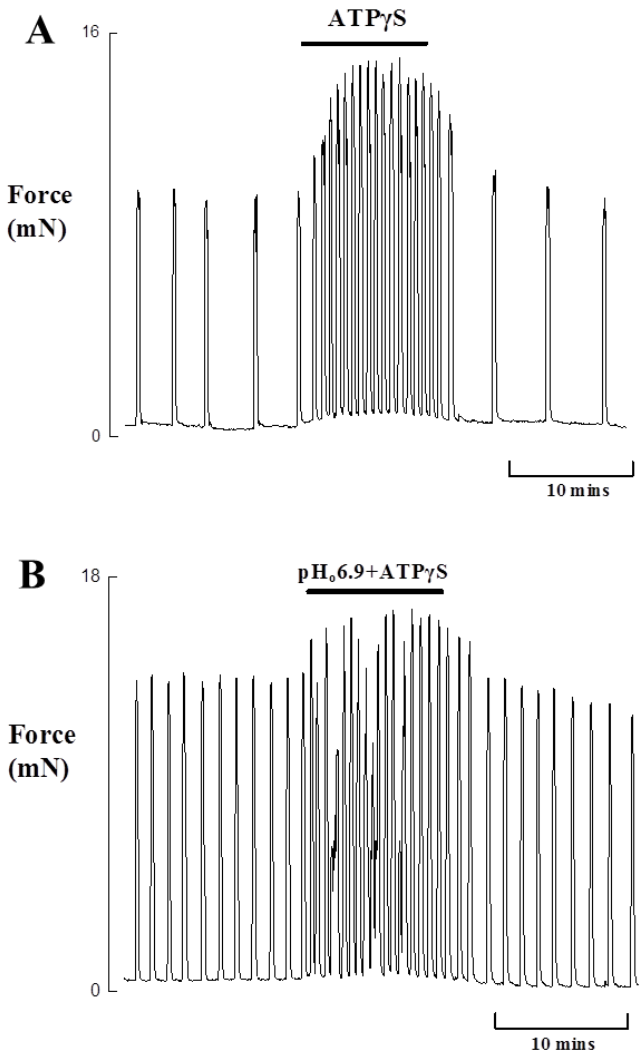

Figure 5.6 Isometric recording of the uterine contraction in term-pregnant mouse showing the effect of (A) ATPγS alone (100µM, n=6) and (B) in association with pH_o 6.9 (n=7).

5.4.4 Effect of Extracellular Acidification on Uterine Contractility in the presence of Selective P_{2X7} Purinoceptor Antagonist (A-438079).

I tested if P_{2X7} purinoceptors are involved in the stimulatory effect of extracellular acidification, by using the blocker, A-438079 (5μM). The A-438079, in the absence of extracellular ATP, did not affect uterine contraction (AUC=102.4%, p=0.6, n=4, Figure 5.7). Blocking P_{2X7} receptors did not affect the stimulatory effect of pH_o 6.9 on uterine contraction (Figure 5.7, n=4). Contractile parameters of 10 minutes application of pH_o 6.9 in the presence of A-438079 were compared to the previous 10 minutes, which represents the effect of A-438079 alone, which was used as the control. The mean data are given in Table 5.5.

Table 5.5 Mean values (100% of the control), standard errors (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification pH_o 6.9 in the presence of A-438079 (5µM). Paired Student's t-test was used here.

		Amplitude	Frequency	Duration	AUC
pH_o6.9 + A-438079 Vs Control	Mean (n=4)	124.3%	135.8%	100.1%	129.7%
	SEM	3.7	10.0	15.6	9.0
	P-value	**0.007**	**0.03**	**0.9**	**0.04**
pH_o6.9 + A-438079 Vs pH_o6.9 alone	**P-value**	**0.1**	**0.3**	**0.9**	**0.1**

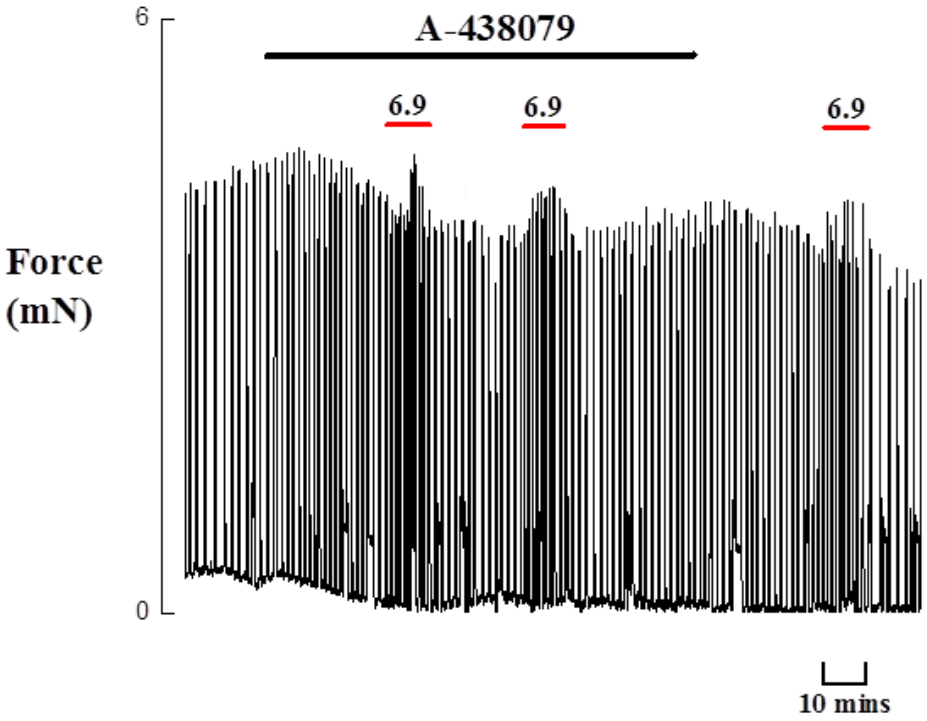

Figure 5.7 Isometric recording of the uterine contraction in term-pregnant mouse shows the effect of 10 minutes application of pHo6.9 under the effect of the selective P_{2X7} purinoceptor antagonist (A-438079). (n=4).

5.4.5 Effect of Extracellular Acidification on Uterine Contractility in the presence of Calcium-Activated Chloride Channel Blocker (Niflumic Acid).

Niflumic acid (30μM) was used to inhibit calcium-activated chloride channel. It immediately decreased both amplitude and frequency of spontaneously contracting myometrium without abolition of the force (Figure 5.8). Spontaneous contraptions started to increase once the niflumic acid was replace by PSS. A test application of pH_o 6.9 solution was made before and after the application of niflumic acid. Acidic solution (pH_o 6.9) was applied twice for 10 minutes, to ensure its effect, in the presence of niflumic acid. Adding pH_o 6.9 solution increased the contraction in each application (Figure 5.8). Table 5.6 gives the mean values, standard error of the mean and p-values of comparing the contractile parameters of the 10 minutes application of extracellular acidification pH_o 6.9 in the presence of niflumic acid to the 10 minutes of contraction under the effect of niflumic acid preceding the application of pH_o 6.9. Comparing the effect of pHo 6.9 in the presence of niflumic acid to the effect of pHo 6.9 alone, revealed no difference (Table 5.6).

Table 5.6 Mean values (100% of the control), standard errors (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification pH_o 6.9 in the presence of niflumic acid (30µM) Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
pH_o6.9 + Niflumic acid	Mean (n=4)	124.7%	115.6%	88%	117.8%
	SEM	18.6	6.3	9.1	11.6
	P-value	**0.03**	**0.01**	**0.3**	**0.02**
pH_o6.9 + Niflumic acid Vs pH_o6.9 alone	**P-value**	**0.8**	**0.1**	**0.09**	**0.2**

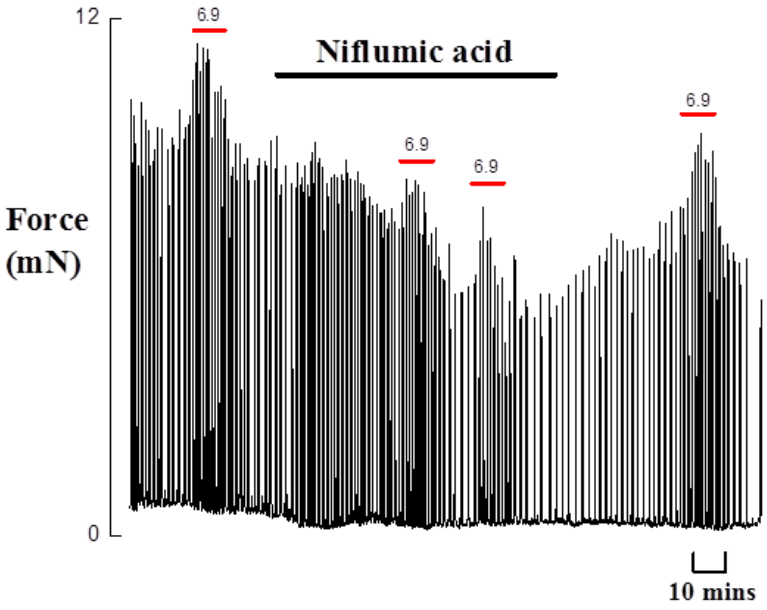

Figure 5.8 Effect of extracellular acidification in the presence of the niflumic acid

Isometric recording of the spontaneous contraction of uterus from term-pregnant mouse shows the effect of 10 minutes application of pH_o 6.9 with and without niflumic acid (n=4)

5.5 Discussion

In previous chapters, I showed that extracellular acidification significantly increased contractility in pregnant mouse myometrium. That increase in force was due to the increase in intracellular calcium concentration and partially because of the following decrease in pH_i. Stimulation of the force caused by extracellular acidification was prevented by oxytocin. Mechanisms involved in pH modulation of uterine smooth muscle are poorly understood and have been little investigated. In this chapter, I studied other possible mechanisms might be involved in the stimulatory effect of extracellular acidification on murine uterine contractility.

The main findings in this chapter are: a) opened L-type calcium channels are required for the stimulatory effect of extracellular acidification; b) extracellular acidification did not change the contraction of the depolarized tissue; c) extracellular acidification effect does not involve CaCCs or P_{2X7} purinoceptor.

Opened L-type calcium channels are required for the stimulatory effect of extracellular acidification

It is well-documented that L-type calcium channels (L-VGCCs) are the main entrance gate for the calcium into myometrium cells in response to different uterine stimuli (Crichton et al., 1993, Shmigol et al., 1998, Shmigol et al., 2001, Wray et al., 2003, Wray et al., 2015). In the rat, extracellular calcium was found essential for the effect of pH_i to be made in portal vein (Taggart et al., 1995). I found that when the nifedipine abolished uterine contraction, suggesting total block of L-VGCCs, pH_o 6.9 had no effect on the contractility. However, pH_o 6.9 was still able to increase the contractility when there was no total block of the L-VGCCs. That may suggest that acidic pH_o could depolarize the membrane further and open more L-VGCCs and caused further Ca^{2+} influx. Similar findings were found in the rat aorta, where acidic pH-induced aortic contraction was reduced by L-VGCC blockers, verapamil and nifedipine (Rohra et al., 2002a). My data therefore suggest that extracellular acidification needs L-type calcium entry in order to stimulate uterine contraction. The rise in Ca^{2+} transients with acidic pH_o was demonstrated in the previous chapter.

Extracellular acidification did not change the contraction in depolarized tissue

To determine if the action of extracellular acidification lies beyond the excitation of cell membrane, extracellular acidification was applied to a fully depolarized pregnant mouse myometrium. High-K^+ solution used at a concentration of 40mM to depolarize the myometrial cell membrane and to ensure opening of L-type Ca^{2+} channels. This concentration was able to maintain tonic contraction in all tissue.

Uterine smooth muscle is a myogenic tissue which means it can contract without hormonal or neural stimulus (Wray, 1993). In order for the spontaneous uterine contraction to happen, it needs depolarization of the cell membrane and subsequent external calcium influx into myometrial cells (Wray, 1993). Depolarization by high-K^+ was documented to enhance smooth muscle contractility mainly by increasing intracellular calcium concentration through Ca^{2+} entry through its channels without affecting its sensitivity, i.e. the effect of high K^+ is dependent on external calcium availability (Himpens et al., 1988, Itoh et al., 1991, Karaki et al., 1992, Austin and Wray, 1995, Kupittayanant et al., 2001, Pierce et al., 2003). High-K^+ depolarization of the pregnant and non-pregnant rat uterus produces inward calcium current, hence sustained contracture. Also, it causes changes in the metabolites and marked intracellular acidification (Wray, 1990, Taggart and Wray, 1993a, Shmigol et al., 1995, Smith et al., 2002a).

In the uterus, relation between extracellular pH and depolarization is not well studied. To the best of my knowledge, this is the first work investigating the effect of extracellular acidification on depolarized uterine strips from pregnant mouse. My work showed that extracellular acidification could not produce the same effect found in spontaneously contracting uteri, i.e. pH_o6.9 did not increase the contraction in the uterine strips depolarized by high-K^+ (40mM). Since membrane depolarization opens all membrane bound calcium channels, acidic pH_o may not be able to induce any further Ca^{2+} entry into the cell. Therefore, its usual effect will not be seen in depolarized myometrium. Nevertheless, more detailed studies are needed to study the direct relation between acidic pH_o, pH_i, and $[Ca^{2+}]_i$ in the depolarized uterine tissue from mouse, and measurements of membrane potential would be useful.

Extracellular acidification effect does not involve P_{2X7} purinoceptor

The relation between ATP and protons is not clear. ATP synthesis by glycolysis and ATP hydrolysis needed during muscle contraction both produce protons (Gevers, 1977, Owicki and Parce, 1992) while dropping pH_o to 6.4 made no changes to the uterine metabolites including ATP (Wray, 1988a). ATP significantly drops during spontaneous myometrial contractions in vivo (Larcombe-McDouall et al., 1999). This has been correlated with occlusion of uterine vessels (Harrison et al., 1994).

The effect of ATPγS, a non-hydrolysable ATP agonist, appears not to have been previously tested on the mouse uterine tissue. In this work, ATPγS was used to test the effect of extracellular ATP alone and in association with extracellular acidification. Extracellular ATP increased the force significantly more than pH_o6.9 alone and the combination of both. Interestingly, combination of both produced an increase in contraction similar to that of pH_o6.9 but significantly less than the stimulation produced by ATP alone. That may be referred to the inhibitory action of the extracellular acidification on P_{2X7} (Liu et al., 2009, Sekar et al., 2018).

On the other hand, blocking P_{2X7} by A-438079 in the absence of extracellular ATP did not affect spontaneous contraction in mouse similar to what was seen previously in the rat (Miyoshi et al., 2010, Alotaibi et al., 2015). When acidic pH_o was applied during P_{2X7} blocking, it was still able to enhance the contractility force. This may indicate that P_{2X7} was not involved in the stimulatory effect of extracellular acidification.

In summary, extracellular acidification decreased the stimulatory effect of extracellular ATP while its effect was not affected by the inhibition of $P_{2X7.}$ Further investigations in the complicated relation between extracellular acidification and ATP are required.

Extracellular acidification effect does not involve calcium-activated chloride channels.

Calcium-activated chloride channels (CaCCs), specifically anoctamine 1 (TMEM16A) and anoctamine 2 (ANO 1 and ANO 2, respectively), CLCA3 and CLCA4, are expressed strongly in mouse uterus (Jeong et al., 2006, Verkman and Galietta, 2009, Bernstein et al., 2014, Dodds et al., 2015) and found to have some functional significance (Song et al., 2009, Wray et al., 2015). They were proposed to

have a role in membrane depolarization and pacemaker activation (Osa and Yamane, 1977, Large and Wang, 1996, Jones et al., 2004). However, work on non-pregnant mouse concluded that ANO1 and CLCA4 are unlikely to be involved in the spontaneous contraction due to their distribution (Dodds et al., 2015). Interestingly, nifedipine and niflumic acid both block noradrenaline-induced contraction (Criddle et al., 1996). Jones et.al found that when CaCCs were blocked by niflumic acid, there were major reduction in frequency in spontaneously and oxytocin-induced contracting myometrium without affecting calcium entry but they have no effect on high-K-induced contractions (Jones et al., 2004). This might imply the role of these channels on managing action potential but some studies reported decrease in amplitude too (Yarar et al., 2001). It is documented that CaCCs are activated by calcium entry and depolarization as they are voltage-sensitive (Large and Wang, 1996, Sanborn, 2000, Jones et al., 2004).

In most studies involved CaCCs in smooth muscles, niflumic acid was used as their potent blocker (Knauf and Mann, 1984, Jones et al., 2004, Oriowo, 2004, Yang et al., 2006, Chu and Adaikan, 2008, Chung et al., 2009, Forrest et al., 2010, Dodds et al., 2015). Niflumic acid was found also to block other channels at different concentrations. It was reported to activate potassium current in rabbit portal vein (Toma et al., 1996) and inhibit Ca^{2+}-dependent activation of the contractile process in pulmonary artery (Kato et al., 1999). The ability of extracellular acidification to stimulate uterine contraction in the presence of niflumic acid indicates that CaCCs are not involved in that effect.

Conclusion

The data, presented in this chapter, may suggest the need for L-type calcium entry in order for the extracellular acidification to stimulate uterine contraction. That is supported by; first, the absence of stimulatory effect of acidic pH_o when nifedipine caused total blockage of L-VGCCs. Second, inability of extracellular acidification to enhance the force when the maximal contraction was achieved by depolarization which suggest that no more Ca^{2+} entry can be added by acidic pH_o. In addition, measurements of intracellular Ca^{2+} showed that it increased with external acidification (Chapter-4) Moreover, my data suggest that calcium-activated chloride channels and P_{2X7} receptors are not involved in the pathway by which extracellular acidification increased the uterine contractions in the pregnant mouse (Figure 5.9).

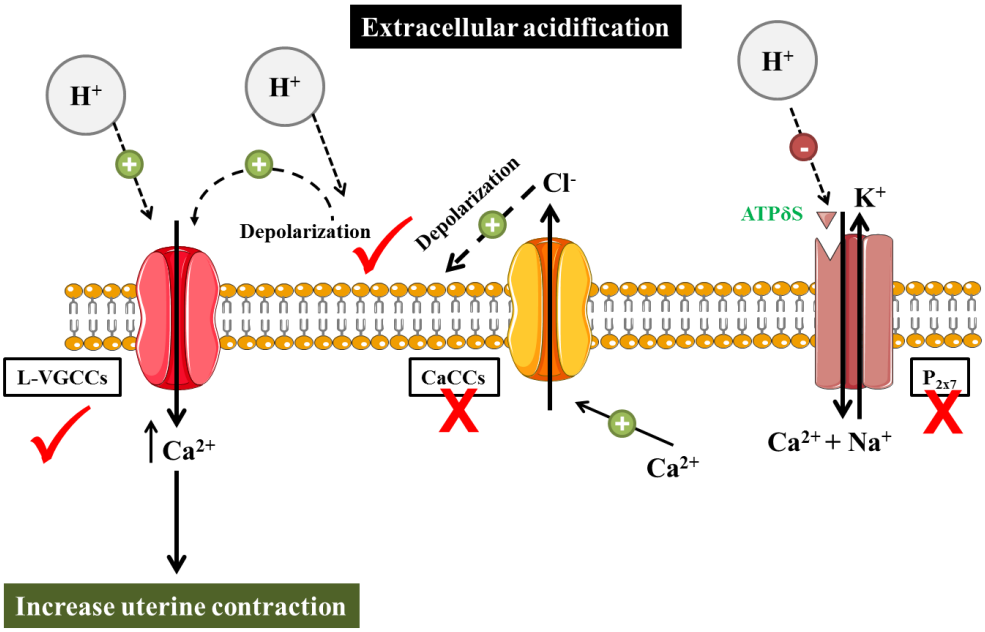

Figure 5.9 Schematic diagram showing the effects of external proton on the channels tested in this chapter. Extracellular acidification needs opened L-type Ca^{2+} channels (L-VGCCs) to show its effect. Calcium-activated chloride channels (CaCCs) and P_{2X7} receptors do not mediate the stimulatory effect of acidic pH_o.

Chapter Six

The Role of Acid-Sensing Ion Channels in the Effect of Extracellular Acidification on Mouse Uterine Contraction

Chapter 6

The Role of Acid-Sensing Ion Channels in the Effect of Extracellular Acidification on Mouse Uterine Contraction

6.1 Abstract

Strong uterine contractions during labour cause brief ischemia due to occlusion of the blood vessels. These bouts of ischemia lead to extracellular acidification in the uterus. My investigations revealed that extracellular acidification increased intracellular calcium concentration and therefore enhanced uterine contraction in the pregnant uterus of the mouse. The source of this increase in $[Ca^{2+}]_i$ is still under investigation. Ion channels sensitive to external acid and conducting inward current (ASICs) have been reported. Immunohistochemistry and western blotting were used to probe the expression of ASICs in the uterus of pregnant mouse and their function was studied using amiloride as an inhibitor. ASIC2a and 3 were found expressed in both myometrium and endometrium. ASIC1 was found expressed in the endometrium only. I found that acidic external pH was still able to enhance uterine contraction despite the inhibition of the ASICs. Use of specific inhibitors will help further investigation of their roles in uterine contractility, but my data suggest that ASICs are not functionally important in bringing about the effects of pH_o 6.9 in mouse myometrium.

6.2 Introduction

I showed previously how extracellular acidification can be a powerful modulator of uterine contraction which might be reflected either positively or otherwise on the parturition. Acidification is associated with many painful, inflammatory and ischaemic conditions, therefore its role in labour cannot be neglected. Almost four decades ago, ‘’proton receptors’’ were described in the nerve cell membrane by Krishtal and Pidoplichko (Krishtal and Pidoplichko, 1981). Later in the nineties, proton receptors were sequenced by different researchers (Waldmann et al., 1995, Price et al., 1996, Bassilana et al., 1997, Garcia-Anoveros et al., 1997), cloned and categorised as proton-gated cation channels by Waldmann et al., 1995. Currently, these receptors are known as acid sensing ion channels or ASICs. They are voltage-insensitive, amiloride-sensitive proteins belong to Na^+ channel/degenerin family (epithelial Na^+ channels, ENaC/Deg) of ion channels (Waldmann et al., 1997b). They are activated by extracellular protons producing fast, but transient, inward current (Waldmann et al., 1997a, Horisberger, 1998, Waldmann et al., 1999). ASICs are permeable to different cations with higher preference to Na^+ as the permeability ratios are as follows pNa^+/Ca^{2+} is 2.5 and pNa^+/H^+ is 0.8, respectively and ASIC1a has more permeability to Ca^{2+} than others (Waldmann et al., 1997b, Chu et al., 2002, Yermolaieva et al., 2004, Xiong et al., 2006). ASICs provide Ca^{2+} influx in the absence of extracellular Na^+ and Ca^{2+} inhibits Na^+ influx through them (Waldmann et al., 1997a). They are activated by external pH between 6.0 and 7.0 and inhibited reversibly by amiloride (Waldmann et al., 1997b, Holzer, 2003).

To date, there are five genes (ASIC1 to 5) encoding seven subunits; ASIC1a, ASIC1b (Waldmann et al., 1997a), ASIC2a, ASIC2b, (Price et al., 1996, Waldmann et al., 1996, Lingueglia et al., 1997), ASIC3 (Waldmann et al., 1997b, de Weille et al., 1998, Babinski et al., 1999), ASIC4 (Akopian et al., 2000, Grunder et al., 2000) and ASIC5 (Sakai et al., 1999). ASICs in neurons were significantly enhanced under ischemic conditions and their desensitization was reduced (Xiong et al., 2004). Functionally, they have been linked to many higher cerebral and sensory functions like pain sensation, fear, learning and mechanosensation and taste transduction (Boscardin et al., 2016, Cheng et al., 2018).

As expected from their identified functional roles, ASICs are highly expressed in sensory neurons, neurons of the central nervous system (brain and dorsal root ganglia DRG) (Waldmann et al., 1997a). Little is known about their presence and function in

non-neuronal tissue. There are however suggestions that they are expressed in smooth muscles. Recently, they were found expressed in cerebral artery smooth muscle (Chung et al., 2010, Chung et al., 2011). In addition, ASIC1 and ASIC2 and ASIC3 are expressed in the detrusor smooth muscle in mouse and rats (Kobayashi et al., 2009, Corrow et al., 2010). However, ASICs' functions in these tissues are not yet to be clearly determined. It has however been shown that ASIC1a, expressed in pulmonary arterial smooth muscle, mediate calcium influx and vasoconstriction in these small vessels (Jernigan et al., 2009). Moreover, ASIC1 may contribute to store operated calcium entry (SOCE). ASIC1 null mice showed decreased SOCE in freshly isolated pulmonary artery smooth muscle cells (Nitta et al., 2014, Plomaritas et al., 2014). ASICs are fairly new area of research and there are still under a lot of investigations to clearly describe their biophysical and functional properties. Amiloride is the classic blocker of the ASICs with IC_{50} 10-100 µM (Kellenberger and Grutter, 2015). Specific inhibitors are still under development, and currently unavailable (Vullo and Kellenberger, 2019).

Aims

In this chapter, I proposed that ASICs might have a role in the mechanism by which extracellular acidification stimulate uterine contraction in pregnant mouse. Then I tested their expression, specifically ASICs1, 2a and 3 in the pregnant uterus and tested their function during extracellular acidification using classical contractility experiments. In this chapter, I aimed to:

- determine the expression of ASICs1, 2a and 3 in the pregnant myometrium.
- investigate the effect of extracellular acidification in the presence of ASICs pan-inhibitor (amiloride)

6.3 Methods

6.3.1 Tissue

C57BL/6J mice were used on day 18-19 of gestation as term. They were humanely killed using CO_2 anaesthesia and cervical dislocation, in accordance with UK Home Office rules for Schedule 1 killing. The uterus was removed, cleaned and full-thickness myometrial strips (10mm X 2mm) dissected. Individual strips were mounted between a fixed hook and force transducer using surgical silk threads in a 10ml bath and were continuously superfused with Physiological saline solution (PSS) (pH 7.4) at a rate of 5mL/min and maintained at 37 ^oC and 100% O_2. If the tissue was to be used for protein extraction and western blotting, uterine chunks were either kept as the whole thickness or dissected to be myometrium only. Then they were weighed and snap frozen in liquid nitrogen. They were stored at -80^oC until enough samples were collected to commence extraction. For Immunohistochemistry (IHC), the tissue was kept in neutral buffered formalin in the fridge for further processing.

6.3.2 Solutions

Buffered physiological saline solution (PSS) composed of (mM): NaCl 154, KCl 5.6, $MgSO_4$ 1.2, HEPES 10.9, $CaCl_2$ 2 and Glucose 8. All chemicals were obtained from Sigma-Aldrich, Dorset, unless otherwise stated. Changes in pH_o to 6.9 were made by adding HCl to PSS. Amiloride (100µM) was used as a pan-ASICs inhibitor.

6.3.3 Western Blot

I here used immunoblotting to determine the ASICs (1, 2a, and 3) expression in the mouse term uterus. In this work, proteins were separated on SDS-PAGE gels (MINI-PROTEAN TGX 10%, Cat# 456-1045, BIORAD, UK) and transferred to a PVDF membrane (Immobilon-P-Cat# IPVH00010-Pore size 0.45µm) through wet transfer method. Samples used were term mouse uterus (both whole uterus and myometrium only). Same animal's brain and urinary bladder were used as control. β-Actin antibody (Abcam ab8224) was used as the loading control. Polyclonal primary antibodies (Anti-ASIC 1, 2α and 3) were used

Tissue samples were lysed on ice in extraction buffer containing phosphate buffer saline (PBS), IGEPAL (membrane protein solubiliser), Sodium deoxycholate (detergent to disrupt protein interactions), RIPA buffer and phosphatase and protease inhibitors. The homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was separated and stored at −80 °C until use. Protein concentration was determined using Biorad modified-Lowry based protein assay kit (Bio-Rad Laboratories, Watford, UK). Protein samples were separated by 10 % SDS-polyacrylamide precast gel and then transferred to PVDF membranes. After blocking with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween-20 (TBST) for 2 h at room temperature, transferred membranes were incubated overnight at 4 °C with rabbit polyclonal antibodies for ASIC1, ASIC2α and ASIC3 (Alomone Labs) were used as a primary antibody at a dilution of 1:200. Following three washes with TBST, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) in TBST with 5 % nonfat milk for 1 h at room temperature. After repeated washes, membranes were reacted with enhanced chemiluminescence reagents (ThermoFisher Scientific) for 5 min and visualized with Hyperfilm ECL (SigmaAldrich)

6.3.4 Immunohistochemistry

Immunohistochemistry (IHC) was used here to localize ASICs. Frozen tissue was sectioned as per protocol and dried by onto microscope slides and stored in room temperature for 24 hours then till used for IHC. On the day of experiment, sections were dried at 60°C for 60 min then rehydrated prior to heat mediated antigen retrieval in citric buffer. Endogenous peroxidase activity and nonspecific staining were blocked with 0.3% hydrogen peroxidase. Non-specific binding sites were blocked by incubating slides in 2.5% Normal Horse Serum. Rabbit polyclonal antibodies for ASIC1, ASIC2α and ASIC3 were used as a primary antibody at a dilution of 1: 250 (for ASIC1) and 1:100 (for ASIC2a and 3) overnight at 4°C in a humidified chamber. Next day, sections were washed and incubated with horse anti-rabbit secondary antibody for 30 min at room temperature in the humidified chamber. Then, DAB solution was applied for colour development. Sections without incubation with primary antibody served as negative control while mouse urinary bladder was used as positive control. Both the negative and positive controls were included in each staining run.

6.3.5 Analysis and statistics

Data was recorded and analysed as mentioned previously in Chapter 2. Statistical differences were tested using Student's t-test. Significance was taken as $P<0.05$

6.4 Results

6.4.1. ASICs1, 2a and 3 are Present in the Uterus of Pregnant Mouse

The expression of ASIC 1, 2a and 3 proteins in pregnant uterus and myometrium of pregnant mouse was examined using western blot analysis. Figure 6.1 shows that ASIC2a and 3 were expressed in the whole pregnant uterine tissue including the myometrium. On the other hand, ASIC1 was expressed only in the whole uterus but not in the myometrium alone. Mouse brain and urinary bladder were used as positive control and β-Actin was used as a loading control.

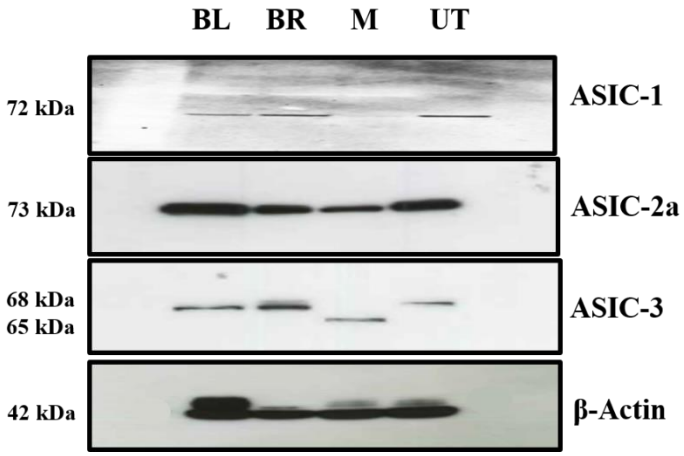

Figure 6.1 Western blot showing the expression of ASICs 1,2a and 3 Western blot analysis showed expression of ASIC 2a and 3 in the whole pregnant uterine tissue of the mouse and myometrium while ASIC1 was expressed only in the whole uterine tissue. Brain and urinary bladder were used as positive control and ß-actin was used as a loading control.

6.4.2 Location of the ASICs in the Uterus

Due to ubiquitous expression of the ASICs in the neurons and despite isolating myometrium from the rest of the uterine tissue, we still needed to localize the ASICs in relation to the myocytes and/or neurons. Hence, we analysed pregnant uterus again using immunohistochemistry (IHC). Figure 6.2 shows Hematoxylin & Eosin (H&E) staining of a cross section of the murine term-pregnant uterus. Endometrium, myometrium and endometrial glands can be seen clearly. Negative and positive controls for ASICs are shown in figures 6.3 and 6.4, respectively. Purple colour represent DAB staining while brown colour represents ASICs. Immunohistochemistry staining confirmed the expression of ASIC1 in the endometrium but not the myometrium (Figure 6.5). However, ASIC2a (Figure 6.6) and ASIC3 (Figure 6.7) can be seen expressed in both endometrium and myometrium.

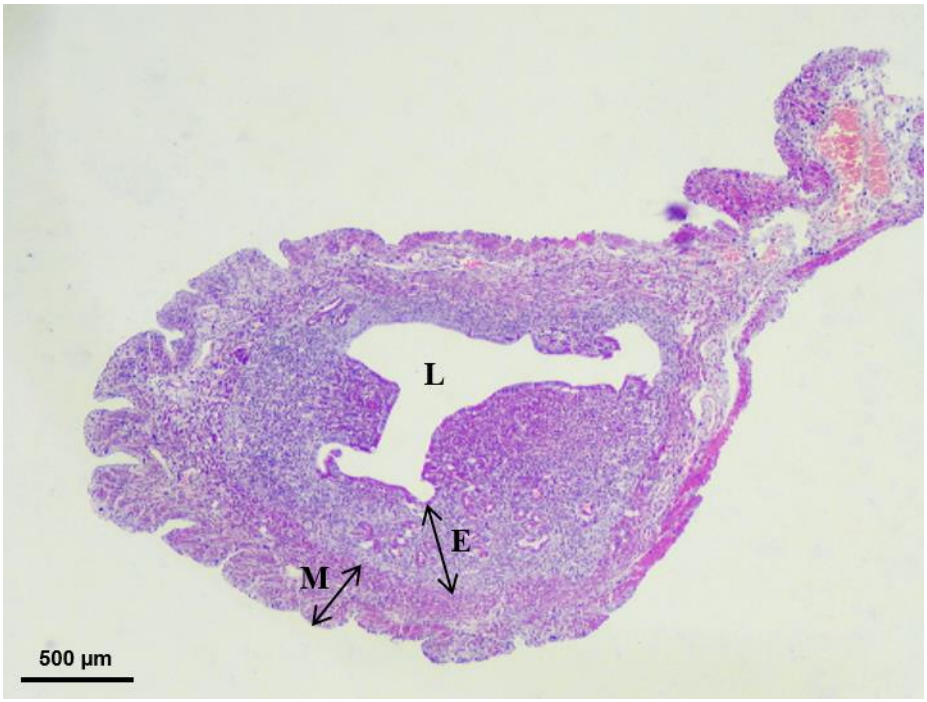

Figure 6.2

H&E staining of a cross section of uterus from term-pregnant mouse showing the normal histology (4x).

L=Lumen, E=Endometrium, M=Myometrium

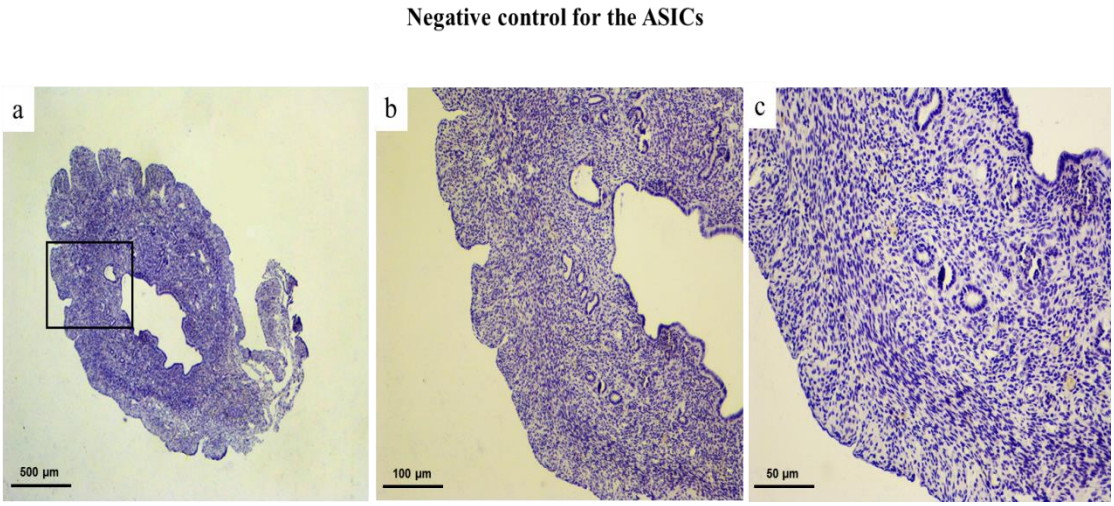

Figure 6.3 Negative control for ASICs

Representative section from term-pregnant mouse uterus used as negative control (no primary antibody was added). Negative control was included in each staining run (No brown staining can be noticed).

Magnifications a=4x, b=20x and c=40x

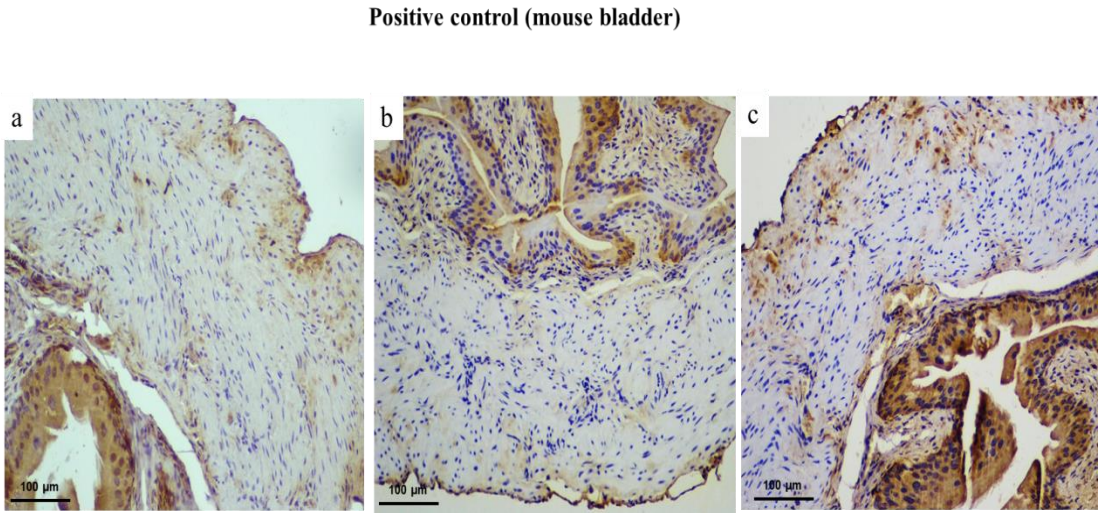

Figure 6.4 Positive controls for ASICs

Representative sections from mouse bladder used as positive controls. Positive control was included in each staining run.

a=ASIC1 1:250, b=ASIC2a 1:100, c=ASIC3 1:100 (brown staining)

20x magnification in all sections

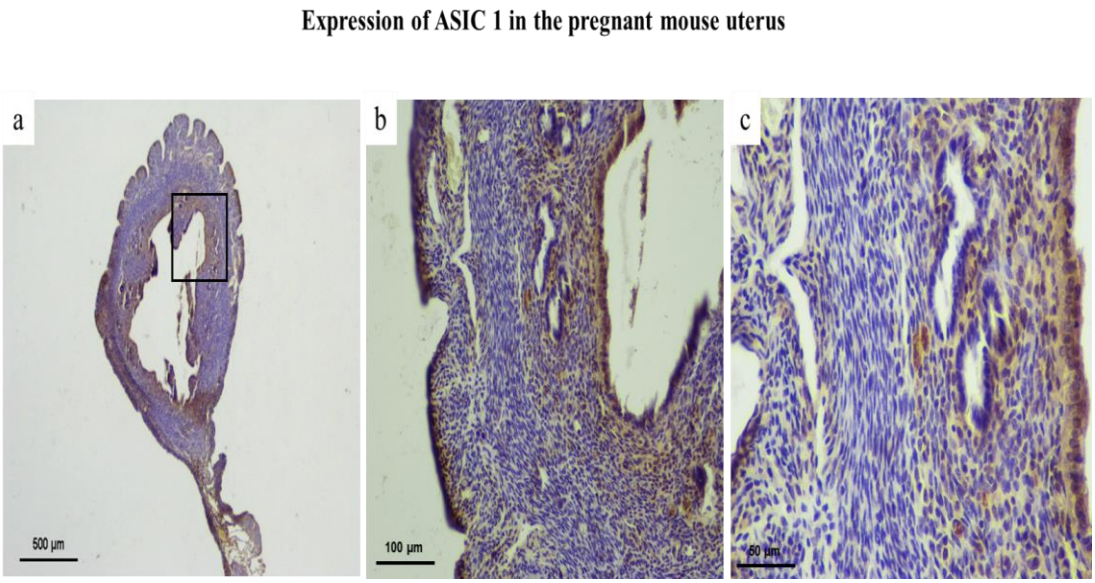

Figure 6.5 Expression of ASIC1 in the pregnant mouse uterus

Representative sections showing the expression of ASIC1 (1:250) in murine term-pregnant uterine tissue is more restricted to the endometrium (brown staining).

Magnifications a=4x, b=20x and c=40x

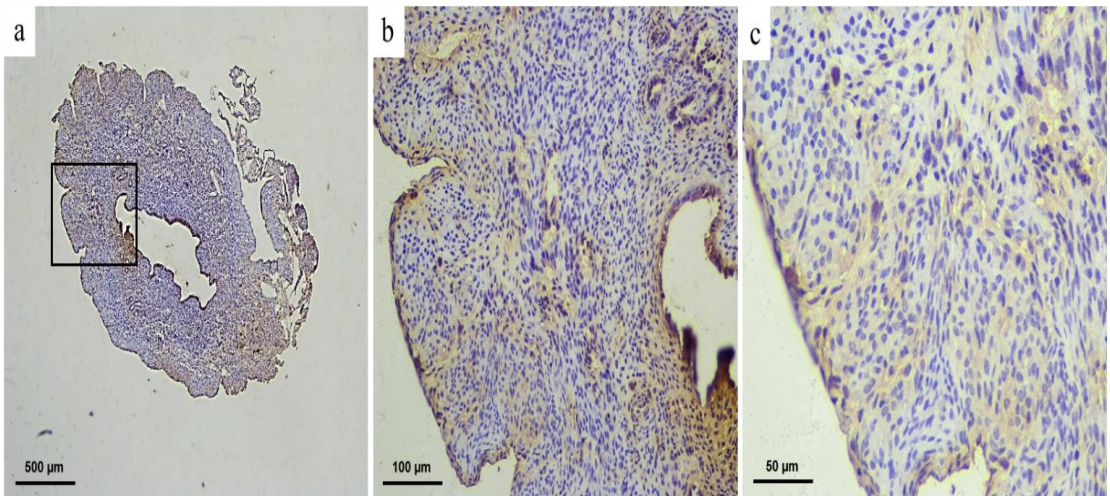

Figure 6.6 Expression of ASIC2a in the pregnant mouse uterus

Representative sections showing ASIC2a (1:100) expression in murine term-pregnant uterine tissue in both endometrium and myometrium (brown staining).

Magnifications a=4x, b=20x and c=40x

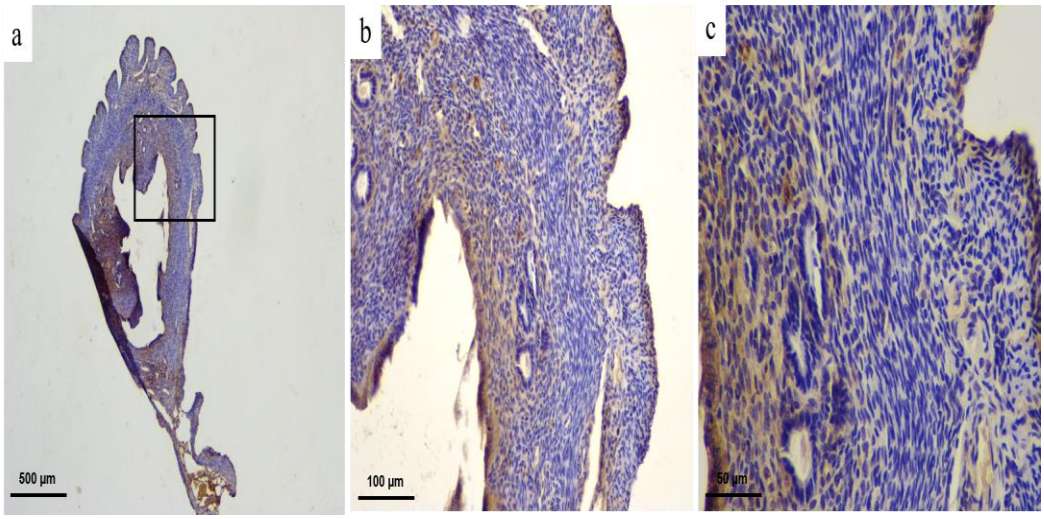

Figure 6.7 Expression of ASIC3 in the pregnant mouse uterus

Representative sections showing ASIC3 (1:100) expression in murine term-pregnant uterine tissue in both endometrium and myometrium (brown staining).

Magnifications a=4x, b=20x and c=40x

6.4.3 Effect of Extracellular Acidification on Uterine Contractility in the Presence of Pan-ASICs inhibitor (Amiloride)

Adding Amiloride (100µM) to spontaneously contracting pregnant uterus caused a time-dependent, marked reduction in the force. Figure 6.6 is representative of 7 others. Amplitude decreased under the effect of amiloride by 66.7% ($p<0.05$). The frequency also decreased by 83.3% ($p<0.05$). Therefore, the overall force under the effect of amiloride decreased, the mean was by 55.5% ($p<0.05$) with no change in duration. Applying acidic pH_o (6.9) in the presence of amiloride resulted in its usual stimulatory effect (Figure 6.8). Mean data are given in Table 6.1.

Table 6.1 Mean values (normalised to 100% control), standard error (SEM) and p-values of the contractile parameters of the 10 minutes application of extracellular acidification (pH_o 6.9) under the effect of amiloride. Paired Student's t-test was used here.

		Amplitude	**Frequency**	**Duration**	**AUC**
Amiloride + pH_o 6.9 vs Amiloride alone	Mean (n=8)	130.4%	190.5%	103%	223.3%
	SEM	6.8	11.7	12.9	17.3
	P-value	**0.009**	**0.03**	**0.9**	**0.04**
Amiloride + pH_o 6.9 vs pH_o 6.9 alone	**P-value**	**0.5**	**0.2**	**0.3**	**0.2**

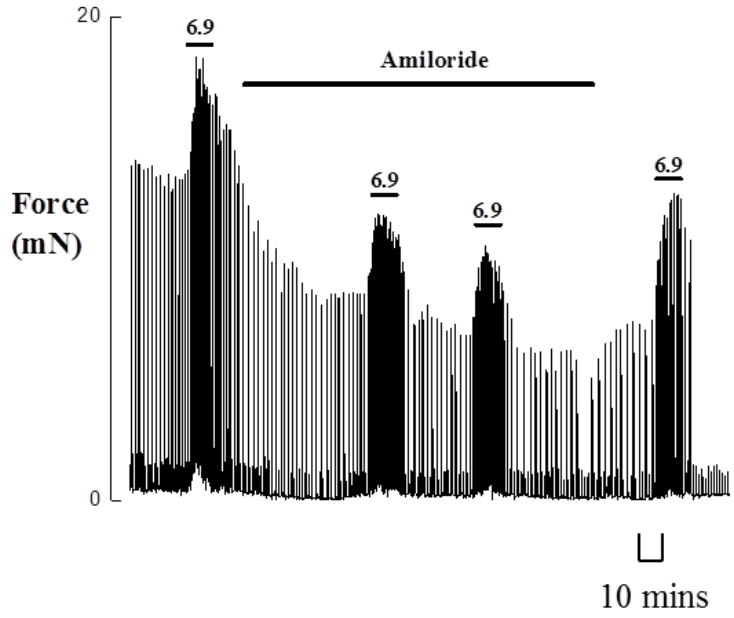

Figure 6.8 Effect of extracellular acidification in the presence of amiloride

Isometric recording of the spontaneously contracting uterus from pregnant mouse showing the effect of pH_o 6.9 with and without the presence of amiloride. Acidic pH_o caused clear stimulation of contraction in both conditions (n=8).

6.5 Discussion

In the previous chapters, I showed that extracellular acidification enhanced uterine contraction and it increased $[Ca^{2+}]_i$. I hypothesized that acidic pH_o increases Ca^{2+} entry either through L-type calcium channels or another Ca^{2+} permeable channels. A group of interesting channels are acid-sensing ion channels, which are referred to as ASICs. ASICs have been intensively studied in the nervous system and they were related to neurological functions. Very little is known about their expression or function in smooth muscles. Therefore, it was interesting to test their expression in the uterus specially the myometrium and their role in relation to the effect of extracellular acidification on uterine contraction since they are activated by protons and permeable for calcium ions.

Stimulatory effect of extracellular acidification does not involve ASICs

To the best of my knowledge, this is the first work where ASIC's presence and function have been examined in the uterus. Protocols were optimized to produce clear bands in western blotting and clear expression in the IHC as much as possible (the final protocols used are given in Chapter-2). Slides were showing clear cross sections from term-pregnant mouse uterus with the endometrium, myometrium and endometrial glands can be seen.

I examined the expression of ASIC proteins; specifically 1, 2a and 3 in the term-pregnant mouse uterus. ASIC2a and 3 were expressed in both endometrium and myometrium while ASIC1 was found only in the endometrium. There is little nervous tissue in the myometrium, which presumably explains why no nerve endings or their staining could not be achieved by standard immunohistochemistry.

Amiloride is widely used non-specific inhibitor of the ASICs with IC_{50} 10-100 µM (Bassilana et al., 1997, Champigny et al., 1998, Kellenberger and Grutter, 2015). The downside of its use that it is not selective for ASICs as it can also block epithelial sodium channel (ENaC) thereby inhibiting sodium reabsorption; Na^+/H^+ exchangers which prevents Na^+ reabsorption and H^+ secretion; Na^+/Ca^{2+} exchanger which modulate calcium ions excursion and T-type calcium channels (Besterman et al., 1985, Zavecz et al., 1991, Chen et al., 2010). Blocking these channels may explain the decreased myometrial contraction effect produced by amiloride. Despite the decrease in myometrial contraction produced by amiloride, pH_o 6.9 was still able to markedly enhance the contraction.

Other ASICs, namely ASIC2b, 4 and 5, were not examined in this work because they are not proton sensitive (Akopian et al., 2000, Lingueglia et al., 1997, Sakai et al., 1999). Lack of specific inhibitors for each ASIC is a known limitation and it will be interesting to tackle that in the future work, if such inhibitors become available. My conclusion is that some ASICs are expressed in the myometrium but there functional significance needs to be better understood, and I cannot conclude that they are involved in the stimulatory effects of pHo 6.9.

Chapter Seven

Final Discussion

Chapter-7

Final Discussion

7.1 Summary of Thesis Results

Due to the interesting and important role of extracellular acidification in smooth muscle contraction, and the limited work done to investigate this in the uterus, and the possibility of its involvement in parturition, I focused this work to investigate the effect of external acidification on mouse uterine contractility, to study some possible mechanisms by which it occurs and to find if there are any gestational differences.

From my thesis, I extract these main novel findings:

1. Extracellular acidification increases uterine contraction in pregnant mouse.
2. Extracellular alkalinization decreases uterine contraction in pregnant mouse.
3. Intracellular pH alteration behaves like extracellular pH alteration in affecting the uterine contraction.
4. Neither extracellular, nor intracellular acidification affects uterine contraction in non-pregnant mouse.
5. pH regulation is active in the mouse uterus.
6. Extracellular acidification causes an increase in $[Ca^{2+}]_i$ and a slow decrease in pH_i.
7. Oxytocin abolishes the stimulatory effect of extracellular acidification.
8. Extracellular acidification may require L-type Ca^{2+} entry in order to increase uterine contraction.
9. ASICs 1, 2a and 3 are expressed in the uterus.
10. CaCCs, P_{2X7} receptors and ASICs are not involved in the pathway by which extracellular acidification increases the uterine contractions in the pregnant mouse.

Extracellular acidification has been found to affect contraction in cardiac, skeletal and smooth muscles. However, only small number of studies investigated the effects of extracellular acidification on uterine contraction and lesser number investigated the mechanisms (Table 1.2 summarised the main findings of these studies). Therefore, my work is novel in studying the gestational differences and testing some of the mechanisms in relation to its effect on uterine contractility. I used C57BL/6J mouse uterus which is more available than human samples. It allows testing at different gestational age and easy access to transgenic models if it is needed. Mouse uterus was used before as a model for human uterus (Matthew et al., 2004, Matsuki et al., 2017). All experiments in this thesis were performed in an *in-vitro* setting.

My work showed that extracellular acidification significantly increases amplitude and frequency of the term- and mid-pregnant uterus while extracellular alkalinization has the opposite effect, i.e. decreases contractility. Likewise, intracellular acidification increases, while intracellular alkalinization decreases, the contraction of the pregnant uterus. However, acidification did not affect contractility in non-pregnant uterus which indicates that pregnancy modifies the mechanisms by which proton affects contractility. It is interesting to find that both ways of acidification produced the same effect on contractility in pregnant uterus while in most smooth muscles both ways of pH alteration produced different effects. For example, in human myometrium, extracellular acidification increased frequency while addition of sodium butyrate caused abolition of the contraction (Pierce et al., 2003). In rat as well, extracellular alkalinisation inhibited force but intracellularly it stimulated it (Heaton et al., 1992). Also, in ureteric smooth muscle from guinea-pig, addition of sodium butyrate caused an increase, while pH_o 6.8 caused a small decrease, in the amplitude of contractions (Burdyga et al., 1996). Having both extra- and intracellular acidification produce the same effect on contractility, suggests a synergistic effect of combining them in physiological or pathological myometrial settings. The effect on contractility produced by altering the intracellular pH was transient as force started to return to control in the continued presence of the weak acid or base. This was correlated with the onset of pH_i regulation in other studies, and this is assumed to also explain my findings (Parratt et al., 1995b, Naderali and Wray, 1999, Duquette and Wray, 2001). Due to active pH regulation, removal of weak base or acid, produces a transient over- or under- shoot of force, and pH_i, until levels are restored to control. Rebound intracellular acidification after removal of the weak base and rebound intracellular alkalinization upon withdrawal of weak acid in uterus were

noted previously. These rebound pH changes also act as another way to verify the effects of intracellular pH on force, as the same effect was found whether the acidification was produced directly with butyrate, or indirectly from weak base withdrawal.

Given the variety of functional responses to extracellular pH change in smooth muscles, it is perhaps not surprising that the responses of pH_i and $[Ca^{2+}]_i$ to extracellular acidification in smooth muscles are also inconsistent. For example, in vascular smooth muscle cells freshly isolated from rat cerebral arterioles, changes in pH_i in response to changes in pH_o were minimal, and thus indicated that the motor response of the cells to external pH was not due to corresponding changes in pH_i. The same study, found that external alkalinization increased the contractility because of increased Ca^{2+} influx and not because of increased Ca^{2+} release from SR (Nazarov et al., 2000). The same conclusions were found in the uterus of pregnant rat as the effect of pH_o alteration was not explained by the induced changes in intracellular pH (Taggart et al., 1997b). Measurements of pH_i and $[Ca^{2+}]_i$ in intact myometrium in this project showed that extracellular acidification increases uterine contraction mainly due to the increase $[Ca^{2+}]_i$ and partially because of the corresponding changes in pH_i. These findings were supported by testing the effect of extracellular acidification while preventing the effect of pH_i by alkalinizing the cytoplasm. There is clear initial stimulation of force, which can only be explained by the extracellular acidification. Interestingly, as shown in Chapter 4, the first third of the test period showed stimulation in response to the extracellular acidification. The middle third of the test period showed the force going back to control levels and a further decrease was seen in the third period. Having shown that NH_4Cl decreases mouse myometrial force, this suggests that NH_4Cl, with pH_o 6.9, was not just nulling the induced acidification, but was also producing an alkalinisation. As acidic intracellular increases force, my data therefore suggests that pH_i alteration in response to acidic pH_o may boost the stimulatory effect of the extracellular acidification. My data shows that the initial stimulation of force produced by acidic external pH can be explained by the immediate increase in $[Ca^{2+}]_i$ showed in the same chapter (Figure 4.13).

My findings therefore led me to investigate the mechanisms of the increase in calcium in relation of pH_o. As explained in Chapter-1, in myometrium a full SR has been shown to limit contractions and an empty SR brings Ca^{2+} into the myometrial cell, via SOCE, and so a role for the SR is unlikely to explain my data. Also, I observed the

effects of acidic pH_o in the absence of agonists, making it further unlikely that the SR was involved as the source of Ca^{2+}. Hence, I focused my subsequent work to investigate if extracelluar acidification directly increases the Ca^{2+} influx through Ca^{2+} permeable channels or indirectly by affecting the depolarization which may open more of these channels. My work in Chapters 5 and 6 showed that extracellular acidification may require L-type Ca^{2+} entry in order to stimulate the contraction. This is supported by; first, the absence of stimulatory effect of acidic pH_o when nifedipine caused total block of L-type calcium channels, and secondly, the inability of extracellular acidification to enhance force when the maximal contraction was achieved by depolarization, which suggest that no more Ca^{2+} entry can be added by acidic pH_o. Extracellular acidification was also recently found to increase mouse myometrial contraction by inhibiting the two pore domain acid sensitive K^+ (TASK-2) channels but not K_{Ca} or K_V channels (Hong et al., 2013, Kyeong et al., 2016). Moreover, my data suggest that calcium-activated chloride channels (CaCCs), P_{2X7} receptors and ASICs are not involved in the pathway by which extracellular acidification increased the uterine contractions in the pregnant mouse. Putting this information together, I suggest the main targets affected by external protons are those effecting excitability of the membrane, and specifically L-type Ca channels and TASK-2 channels. It is these changes that lead to depolarization of the myometrial cell membrane and an increase in intracellular [Ca^{2+}] and then force, when protons are elevated outside the cell membrane.

As force was not increased in non-pregnant myometrium, my data suggests that there will be a gestational difference in the expression of either or both of TASK-2 or L-type calcium channels. There is evidence for this in the work of Lee *et al* who showed immunohistochemical expression of TASK-2 was significantly increased in pregnant uterus (Hong 2013, Keyong2016). This fits with acidic pH_o working through these channels. There is also evidence that L-type channels are much increased in pregnant uterus, which is also consistent with my findings (Collins et al., 2000).

In the physiological setting, oxytocin concentration and its receptor expression increase dramatically toward parturition (Risberg et al., 2009, Padol et al., 2017). Thus to increase the physiological and clinical relevance of my work, I needed to test the effect of extracellular acidification in the presence of oxytocin. Interestingly, oxytocin abolished the stimulatory effect of acidic pH_o on the pregnant uterus. The exact relation between oxytocin and hydrogen ions needs further investigation but given that oxytocin acts mainly by increasing Ca^{2+} entry, and I have suggested that this will be the main

way that external acidification increases force, it could be that both agents are using the same pathways. Thus if oxytocin has already stimulated force, pH_o 6.9 can produce no further increase.

Over the past years, our understanding of the physiology behind uterine contraction has advanced yet many pieces of the puzzle are still missing. Possible mechanisms to explain the effects of external acidic pH on mouse uterus are shown in Figure 7.1. Acidic external pH was found to increase uterine contraction in pregnant mouse mainly by increasing L-type Ca^{2+} entry and then, acidifying the cytoplasm leading to an increase in intracellular calcium concentration. Also, acidic pH_o was found to inhibit TASK-2 channels, inducing depolarization. Stimulatory effect of extracellular acidification was not affected by blocking calcium-activated chloride channels, ASICs or P_{2x7} receptors.

7.2 Physiological Significance and Clinical Relevance

It is too early to clearly define the physiological or pathological role of extracellular acidification on the parturition. Uterus is relatively quiescent during early pregnancy. Then, when the pregnancy reaches term, the uterus becomes more active producing strong, frequent and regular contractions until the delivery of the foetus and placenta. These contractions have been long connected to ischemia and/or hypoxia and consequently acidification of the uterus. A clinical study, which evaluated 1,433 pregnant women at parturition, found that frequent, strong uterine contractions during labour were accompanied by foetal acidosis at birth (Bakker et al., 2007). Surprising results were found in a study done by Quenby *et al* (Quenby et al., 2011) where they examined the quality of uterine contractions and calcium transients in uterine strips taken from 120 women underwent caesarean sections. Stronger contractions and increased amplitude of calcium transients were found in uterine strips from women with foetal distress/acidosis than the cases without foetal distress/acidosis. Recently, an interesting study found that transient and frequent application of hypoxia resulted in continuous and maintained increase in uterine contractility in labouring rat myometrium and that phenomenon was named hypoxia-induced force increase or HIFI (Mohammed 2015). Strong uterine contractions cause occlusion of blood vessels which then causes ischemia and hypoxia. Hence, decreasing the external pH which in turn increases Ca^{2+} entry then, decreasing intracellular pH causing more increase in the intracellular $[Ca^{2+}]$

and hence stronger contractions and so on (Figure 7.2). This repetitive event causes repetitive hypoxia and then acidification and hence my findings might also be related to the HIFI. The absence of extracellular acidification stimulatory effect in the presence of oxytocin might reveal a physiological protective measure to prevent a synergistic effect and therefore uterine hyperstimulation which can lead to foetal acidosis and distress. Investigating the effects of extracellular acidification on labouring uterus is an acknowledged limitation of my work and it will be highly considered in the future.

7.3 Future Work

This research hold a lot of promise for further development and the findings will be rewarding for this project area. It is very appealing to:

- investigate the effects of extracellular acidification on labouring uterus.
- investigate the effects of extracellular acidification in presence of oxytocin inhibitors like atosiban.
- investigate the effects of extracellular acidification in relation to sex hormones.
- simultaneously measure the force, pH_i and $[Ca^{2+}]_i$ by loading the myometrial strips with both the Ca^{2+} and pH indicators however this may be technically challenging.
- perform measurements of membrane potential during the acidification of the extracellular space.

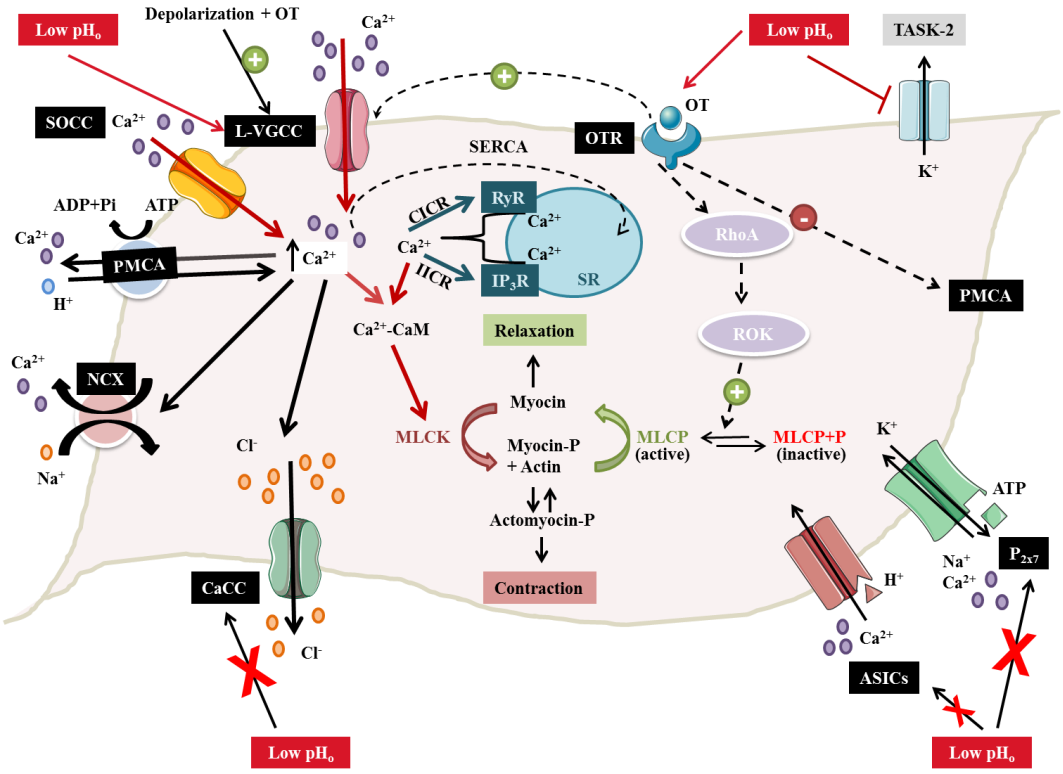

Figure 7.1 Summary of the mechanisms found to underlie the stimulatory effect of extracellular acidification on uterine contraction from pregnant mouse.

L-VGCCs=L-type voltage-gated calcium channels, SOCC=store-operated calcium channels, PMCA=plasma membrane Ca^{2+}-ATPase, NCX=Na^+/Ca^{2+} exchanger, CaCC=Ca-activated chloride channel, ASICs=acid-sensing ion channels, TASK-2= two pore domain acid sensitive K^+ channel, OT= oxytocin, OTR=oxytocin receptor, SR=sarcoplasmic reticulum, SERCA= sarcoplasmic reticulum Ca^{2+}-ATPase, CICR=Ca^{2+}-induced Ca^{2+} release, IICR= IP_3-induced calcium release, MLCK=myocin light chain kinase, MLCP= myocin light chain phosphatase. ROK=RhoA-associated kinase

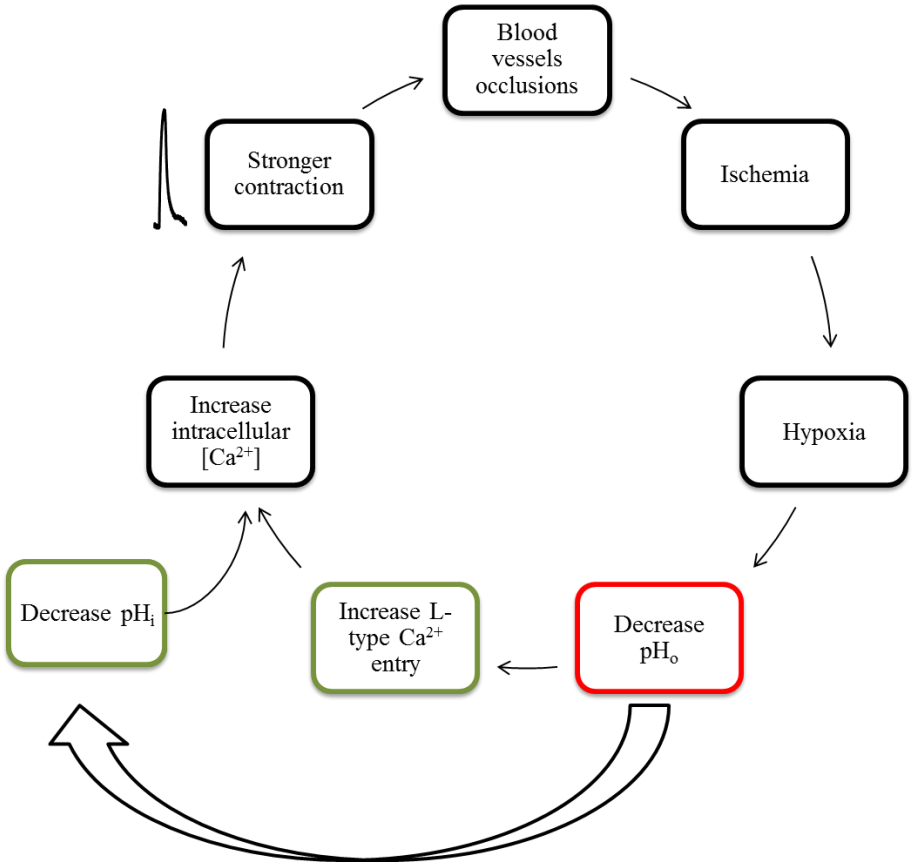

Figure 7.2 Positive feedback showing relation between extracelluar acidification and contraction in mouse myometrium

Stronger uterine contractions lead to occlusion of blood vessels which then causes ischemia and hypoxia decreasing the external pH which in turn increases Ca^{2+} entry mainly through voltage-gated L-type calcium channels. Then it decreases the pH in the cytoplasm which causes more increase in the intracellular Ca^{2+} concentration and hence stronger contraction.

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