An investigation of the effects and mechanism of adenosine triphosphate (ATP) on labouring and non-labouring human myometrial contractility

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

By

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Women & Children's Health / Life Course & Medical Sciences

Declaration

I hereby certify that work presented in this thesis is all my own product, and appropriate credit is given where I have used the expressions or writings of others.

I declare that the work has describes original work that has not previously been presented for any other degree.

Hind Zafrah (Candidate)

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Presentations

Oral presentation

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-An investigation of the effects and mechanism of ATP on human myometrial contractility. Virtual Future Physiology conference, UK, 2020

Poster presentations

-The Effect of ATP and its Analogues on Myometrial Contractility in Non-Labouring Women. Future Physiology conference. Aberdeen, UK, 2019.

-E-Poster, An investigation of the effects and mechanism of ATP on human myometrial contractility. Virtual Future Physiology conference, UK, 2020.

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Awards

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List of Abbreviations

Aa	Amino acid
ANOVA	Analysis of variance
A-438079	3-(5-(2,3-dichlorophenyl)-1h tetrazol-1-yl) methyl pyridine hydrochloride hydrate
A-740003	Methyl amino}-2,2-dimethylpropyl)-2-(3,4 dimethoxyphenyl) acetamide
ADP	Adenosine 5'-diphosphate
α, β-MeATP	α, β- methylene adenosine 5'-triphosphate
ATP	Adenosine triphosphate
ATPγS	Adenosine 5′-[γ-thio] triphosphate tetralithium salt
AMP	Adenosine 5'-monophosphate
AUC	Area under the curve
BCA	Bicinchoninic acid
BMI	Body mass index
BK_{Ca}	Large conductance calcium-activated potassium channel
BzATP	2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate
Ca-CaM	Calcium calmodulin complex
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CS	Caesarean section delivery
CTG	Cardiotocography
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
EC_{50}	The molar concentration of agonist that produces 50% of maximal effect
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IC_{50}	The molar concentration of antagonist that produces 50% of maximal effect
IHC	Immunohistochemistry
IP_3	Inositol 1, 4, 5-trisphosphate
IP_3R	Inositol 1, 4, 5-trisphosphate receptor
K_{ATP}	ATP sensitive potassium channel
K_{Cl}	Potassium chloride
Mins	Minutes (time)
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
n	Number of samples from different women
NCX	Na^+/Ca^{2+} exchanger
OT	Oxytocin
OTR	Oxytocin receptor
P1	Adenosine receptor
P2	Purinergic ATP receptor
PG	Prostaglandin

PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PIP2	Phosphatidylinositol 4, 5 biphosphate
PLA2	Phospholipase A2
PLC	Phospholipase C
PMCA	Plasma membrane Ca^{2+}-ATPase
PPADS	Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate
P-value	Probability value
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
ROCC	Receptor operated calcium channel
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
SOCC	Store-operated calcium channel
SR	Sarcoplasmic reticulum
TM	Transmembrane domain
UTP	Uridine 5'-triphosphate
VGCC	Voltage-gated calcium channel
WB	Western blot
$[Ca^{2+}]_i$	Intracellular calcium concentration

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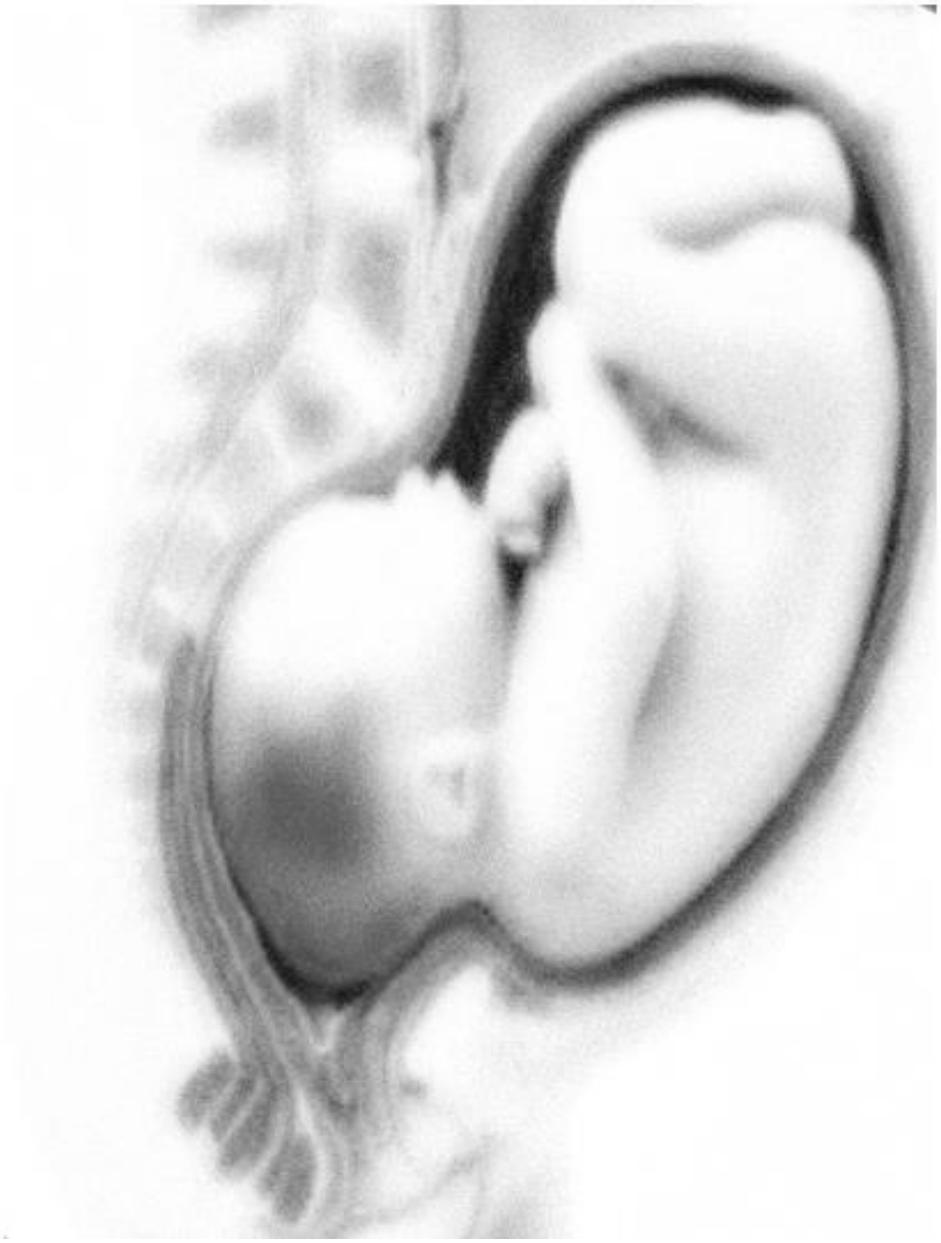
Thesis Abstract

Dysfunctional labour is defined as uncoordinated myometrial contractions associated with a lack of cervical dilatation. Currently, oxytocin is the only treatment available, and approximately 50% of women who have dysfunctional labour do not respond to oxytocin administration. This results in a large proportion of women requiring an emergency caesarean delivery which comes with associated risks. Adenosine triphosphate (ATP) has been shown to increase rat uterine contractions via purinergic receptors (P), mainly P2X1 or P2X7 receptors. Therefore, this study aimed to investigate the effects of ATP on human myometrial contractile activity, and its potential role in dysfunctional labour, with the hypothesis being that expression of P2X1 and/or P2X7 receptors in women's myometrial tissues is up-regulated at term, and ATP binding to one of these receptors would potentiate myometrial contraction during the onset of labour. On the other hand, the depletion of one or both receptors would lead to dysfunctional labour. Quantitative immunoblotting studies measured the expression level of P2X1Rs and P2X7Rs and determined their location in myometrial tissue obtained from 1) non-labouring term pregnant, 2) normally labouring, 3) dysfunctionally labouring and 4) non-pregnant women. The effect of ATP and its analogues, ATPγS (a non-hydrolyzing form of ATP) and BzATP (a more potent agonist at the P2X7R), on labouring and non-labouring human myometrial contractility was also measured in organ bath assays. To further determine the role of P2X7R in mediating this action, selective antagonists, A-438079 and A-740003, were used. Immunoblotting studies demonstrated that P2X1Rs were expressed in human myometrium, but that the pattern and quantity of expression did not differ between groups. P2X7R expression was also detected in human myometrium and interestingly, was significantly greater in the normal labouring group (32845±8655%, n=3, P= 0.0025) than non-pregnant (4911±751%, n=3), term pregnant non-labouring (12256± 2118%, n=10), and dysfunctionally labouring (15599±3146%, n=5) groups. In non-labouring tissues, ATP produced a significant increase in contractile frequency (230±38.7%, n=7). All three agonists increased the frequency of spontaneous contractions significantly with the rank order of ATPγS> BzATP> ATP (656±102.4% (n=6) > 250.2±38.8%(n=9) > 230±38.7%(n=7)), respectively. Selective P2X7R antagonists, A-438079 and A-740003, were unable to inhibit the stimulatory effects of ATP. Furthermore, there were no significant differences in the effect of ATP on myometrial activity between non-labouring and dysfunctionally labouring women. It is concluded that the increased P2X7R expression observed in the normal labouring group could potentially be implicated in

the myometrial contractility response to ATP and help in the augmentation of contraction during the onset of labour, although no normally-labouring tissues were available to confirm this in the contractility studies. The data do not support a major role for P2X7R in human myometrial responses to purinergic stimulation, at least in non-labouring women; however, comparisons still need to be made with normal labouring myometrial tissue.

CHAPTER 1



Chapter 1: Introduction

This thesis focuses on increasing understanding of P2X signalling in the myometrium to apply this knowledge to help develop a treatment for dysfunctional labour. Therefore, the role of purinergic signalling in smooth muscle, particularly in the myometrium, will be described.

1.1 Uterus structure

1.1.1 Anatomy of the Uterus

The uterus, also known as the womb, is the stretchable pear-shaped muscular sac in the female reproductive system. It is a major female hormone-responsive secondary sex organ of the reproductive system in most mammals. This organ has the capability of expansion to accommodate a growing fetus during pregnancy.

The uterus is located in the female pelvis between the bladder and rectum. In the human, the uterus is composed of two main parts: the uterine body and the uterine cervix. The cervix, the cylindrical distal portion of the uterus, opens into the vagina. The uterine body occupies the upper two-thirds of the uterus and consists of the fundus, united to the fallopian tubes (**Figure 1.1**). The uterus weight increases from 50gm in the non-pregnant state to 1000gm at term (Jain et al., 2020).

The anatomical structure of the uterus can be divided into the following 3 tissue layers: The inner layer, known as endometrium, consists of an epithelial layer and mucus-secreting membrane that rich with blood vessels, glands, and nerve endings, providing them with a source of many hormones and metabolites, which both, directly and indirectly, can affect myometrial contractility. The middle layer, myometrium, is a bulky layer composed of longitudinal and circular smooth muscle cells, where the excitation and contraction processes take place (Wray, 1993). The outer layer of the uterus, the serosa, is a thin layer of tissue that envelop the uterus to protect adjacent structures during free movement in pelvic or abdominal areas. However, ligaments such as broad ligament, round ligament, uterosacral ligament, and suspensory ligament stabilise the uterus in position and limit its range of movement (Kaniewska et al., 2018), (**Figure 1.1).**

Uterine arteries supply blood to the uterus. These arteries derivative from one branch of the internal iliac artery, and ultimately anastomosing with the ovarian artery. The latter arises from the abdominal aorta inferior to the renal arteries. The blood supply to the uterus penetrates deep through the myometrium to give the arcuate arteries and terminates as radial

arteries, which gives further branches to the endometrial layer as the basal and spiral arteries, which supply the decidua and the placenta throughout gestation (Gossman et al., 2019). The uterus is closely connected to provide consistent blood flow (**Figure 1. 2**).

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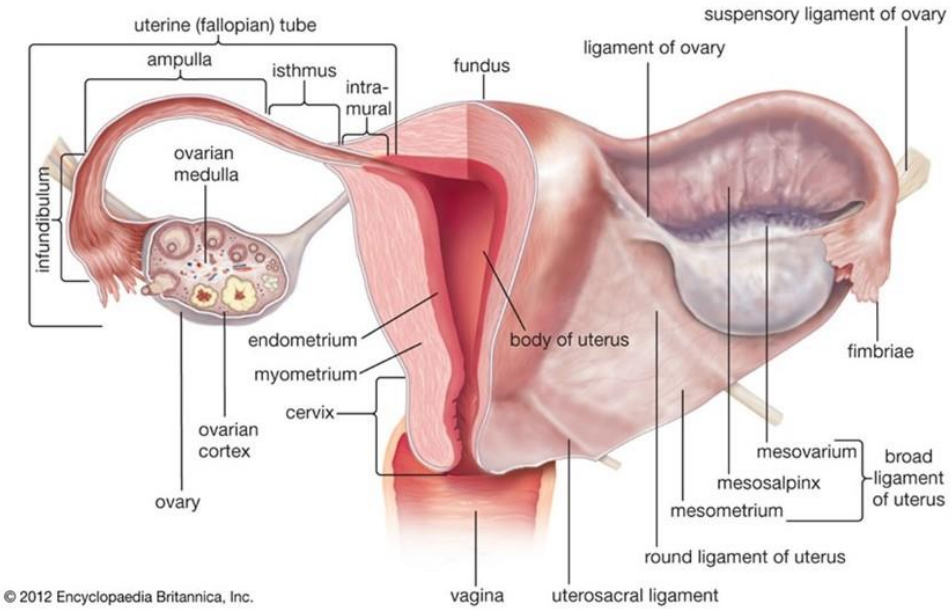


Figure 1. 1: The anatomical structure of the human uterus.

Adapted from Encyclopaedia Britannica, (2012).

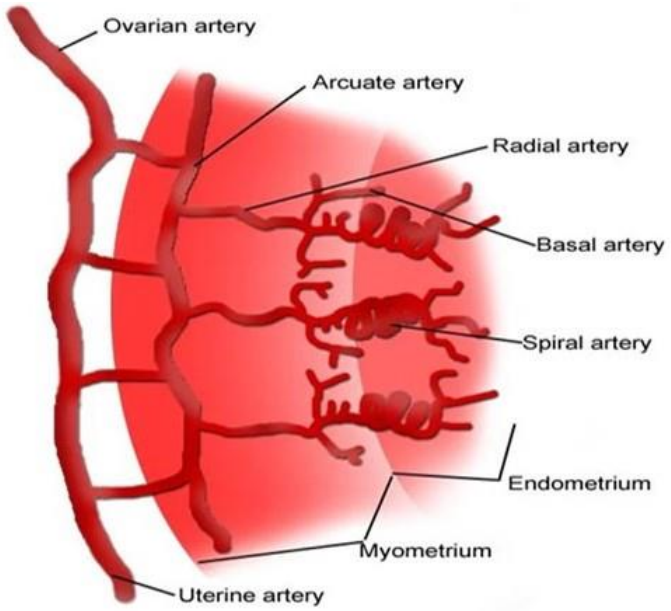


Figure 1. 2: Blood supply of the human uterus.

A cross-section view shows the extensive blood supply to the human uterus, and its branches penetrate through the myometrium. Adapted from Snell, Clinical Anatomy by regions, 8th edition.

1.1.2 The uterine myocytes

Myometrial cells, known as myocytes, are the main cell structure of the myometrium. They are also the leading cell type within the uterus (Young, 2007). Myocytes are spindle-shaped cells tapered at the ends with a centered oval nucleus (Dawson and Wray, 1985). These cells are bundled together and enclosed in a connective tissue matrix. Important proteins are embedded in this matrix, such as elastin, collagen, glycoproteins and proteoglycans, which assist in transmitting contraction force throughout the uterine wall (Yu and Bernal, 1998). The cell membrane of the myocyte contains ion channels and receptors that play a critical role in transmitting signals between cells. Myocyte cytoplasm contains calmodulin, a calcium regulatory protein, initiating the activation of contraction after binding with free cytoplasmic Ca^{+2} (Aguilar and Mitchell, 2010). In the cell cytoplasm, long bundles are distributed throughout the cell, known as the contractile machinery proteins, which include actin thin myofilaments, myosin thick myofilaments, intermediate filaments, and dense bodies and dense bands (Young, 2007); (Wray, 1993), (**Figure 1.3**).

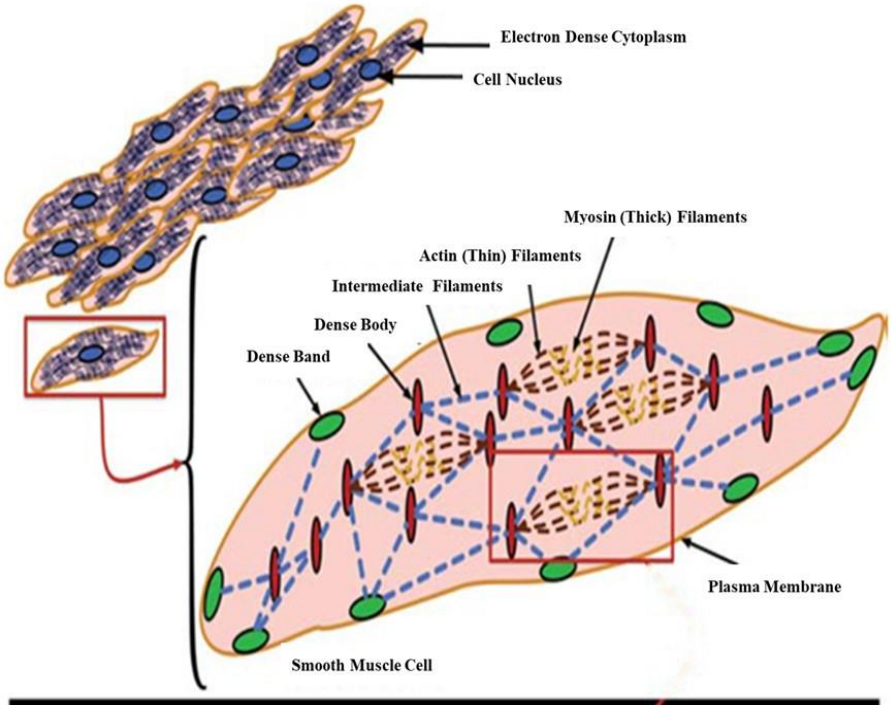


Figure 1.3: **Smooth muscle contractile machinery.**

The cytoplasm of smooth muscle cells (SMCs) is extremely dense with contractile machinery components (thick myosin and thin actin filaments) and other structural elements (dense bodies, dense bands, intermediate filaments). Adapted from (Aguilar and Mitchell, 2010).

1.2 The role of the uterus in pregnancy

The uterus is the main reproductive organ that serves various functions and is involved in fertility and childbirth. The vital function of the uterus is to accommodate the fetus and provide them with the required nourishment and protection throughout the pregnancy period. It undergoes multiple changes in order to provide the secure environment required for the increased metabolic process, blood supply and biochemical support of the growing fetus and eventually parturition (Davis, 1967); (Habiba et al., 2021). After parturition has been taken place, these changes are gradual, within 6 weeks, reversed in a process known as involution due to subsides in endocrine hormones and the removal of mechanical stretch (Wray, 1982).

The normal average length of a human pregnancy is 38-40 wks, starting from the 1st day of the female's last menstrual period (LMP). Delivery before 37 weeks is commonly categorised as preterm labour, and delivery after 41 weeks is categorised as post-term labour. When pregnancy approaches term, the uterus undertakes multiple preparatory processes for the onset of labour contractions. The uterine changes throughout pregnancy include changes in cell size, blood flow, and channel and receptor expressions:

1.2.1 Cell size

In the early weeks of pregnancy (12 weeks), hyperplasia occurs in myometrial cells, which is accompanied by an increase in anti-apoptotic proteins due to the action of pregnancy-related endocrine change. (Jaffer et al., 2009). While in the mid-pregnancy onwards, myometrial hypertrophic growth is encouraged by mechanical stretch caused by the growing foetus and the effects of sex hormones such as oestrogen and progesterone (Shynlova et al., 2010); (Douglas et al., 1988). Furthermore, changes in the extracellular matrix with the ability of myocytes to synthesise collagen are occurred (Shynlova et al., 2010).

1.2.2 Blood flow

The uterus is a rich blood supply organ. This high volume of blood flow needs to maintain nourishment and oxygenation during the labour process, where repetitive and intense myometrial contractions are needed to deliver the fetus. Different hemodynamic alterations, such as high blood volume and cardiac output with lower vascular resistance and blood viscosity, supported this local increase in blood flow (Burton et al., 2009).

1.2.3 Channel and receptor expression

The different ion channels, including calcium, potassium, chloride and sodium, are expressed on the myometrial cell membrane and control uterine excitability. Their expression is changed throughout pregnancy, suggesting their critical role in controlling the contractility of the myometrium. L-type calcium channel affinity to their agonists increases during pregnancy, and also, their expression is dramatically up-regulated towards term pregnancy in several species (Tezuka et al., 1995); (Collins et al., 2000). BKca channels were suggested to hyperpolarise the uterus during pregnancy and maintain its quiescent state in the mouse (Benkusky et al., 2002). They are downregulated during labour in human uterine smooth muscle cells (Matharoo-Ball et al., 2003). Similarly the density of K_{ATP} channels was identified to be less in non-pregnant uteri than in pregnant uteri, which are up-regulated throughout mid-pregnancy and then down-regulated during labour (Curley et al., 2002);(Sawada et al., 2005). Interestingly, myometrial smooth muscle cells isolated from pregnant rats were found to have non-selective cation channels (NSCC) (Miyoshi et al., 2004). Later, these channels were characterised in rat myometrial smooth muscle cells, with the properties of a P2X7 channel (Miyoshi et al., 2010). So, the P2X7R was identified as one of the myometrial non-selective cation channels in the rat myometrial cell (Miyoshi et al., 2012). For other ion channels present in the myometrium, see (Wray and Prendergast, 2019). In addition to the alteration in channel expression, the concentration of uterine hormone receptors changes toward the end of pregnancy. For example, there is a modification in the expression of prostaglandin receptors during parturition. Prostaglandin receptors (PGI_2), which may contribute to keeping the pregnant uterus relaxed, were shown to be expressed more in mid-pregnancy compared to labour and postpartum in rats (Brodt-Eppley and Myatt, 1998). On the other hand, the expression of ($PGF_{2\alpha}$) receptors, which possess a contractile effect, was dramatically increased during labour (Brodt-Eppley and Myatt, 1998). Myometrial oxytocin receptors are elevated throughout pregnancy and after parturition, according to a comprehensive investigation of myometrial oxytocin receptors (Jurek and Neumann, 2018). Purinergic receptor expression has also been shown to progressively increases throughout pregnancy, peaking during labour, and its activation has been found to cause muscular contractions, indicating that it plays a role in a successful labour (Alotaibi, 2018). Regulation of hormone receptor expression and implications for pregnancy and labour are discussed in more detail in sections 1.6 and 1.7.

1.2.4 Myometrium

The myometrium is known for its rhythmic contractions, which result in Braxton Hicks contractions during pregnancy which occur unpredictably. These contractions are variable in length, frequency, and intensity (McEvoy and Sabir, 2021). These contractions are converted to painful and regular contractions associated with cervical dilation during the actual onset of labour. The reason is that the myometrium has rhythmic contractions because it is an excitable tissue, which means the action potentials are generated within the smooth muscle cells. These action potentials occur spontaneously and reflect the electrical activity state of membrane potention (Wray et al., 2015). Ca^{2+} and Na^{+} influx and Cl^{-} efflux cause membrane depolarisation and enhance cellular excitability, while K^{+} efflux inhibits membrane excitability and causes membrane repolarisation (Kao and Carsten, 1997). It is worth noting that no one knows where these spontaneous activities come from. It has been speculated that specific cells may act as pacemaker cells (Sanders et al., 2014). In other smooth muscle cells such as the gastrointestinal tract, interstitial cells of Cajal (ICC) or ICC-like cells (ILC) act as pacemakers which give rise to the rhythmical activity (Zheng et al., 2014). However, these pacemaker cells in the myometrium are not anatomically defined (Young, 2018), and further work is needed to determine how electrical activity is initiated and what kind of cells form uterine pacemakers.

The resting membrane potential (RMP) in the uterine smooth muscles has been recorded between -35 and -80 mV (Aguilar and Mitchell, 2010). As pregnancy approaches its term stage, the resting membrane potential becomes less negative, bringing it closer to the threshold for firing action potentials (threshold potential) and facilitating Ca^{2+} influx through voltage-operated Ca^{2+} channels. Parkington and his team conducted a study to measure the RMP of myocytes in the 3rd trimester of a human pregnancy. They reported that the RMP has changed from an average of −70 mV at 29 weeks to −55 mV at labour and is accompanied by an increase in the frequency of myometrial contractions (Parkington et al., 1999). In addition, as parturition progresses, the conducting system between the adjacent cells is developed in the form of gap junctions that help transfer this activity rapidly between myometrial cells and finally produce coordinated and synchronised contractions (Sheldon et al., 2014).

1.3The stages of labour

It is known that the parturition process is not just one essential event but rather a series of interconnected, complicated alterations that might trigger labour. Modifications in ion channels, which impact action potentials, are part of these continual changes. The increased number of gap junctions and their conduction aids in the quick transmission of excitability to muscle bundles and the coordination of contractions. The endocrine environment has changed, with increased oxytocin release and receptors, increased prostaglandin synthesis, and reduced progesterone efficacy being the most notable changes. Hyperplasia and hypertrophy that are associated with uterine growth throughout pregnancy; all together contribute to the powerful contractions required for labour (Wray and Prendergast, 2019).

Labour is the physiological process of delivering the baby, followed by the placenta, membranes, and umbilical cord, from the uterus to the vagina to the external world. True labour is defined as regular and painful uterine contractions leading to cervical effacement, accompanied by descent and ultimately by pushing the foetus. Normal labour progress in nulliparous women might be slower than in multiparous (Ashwal et al., 2020).

Labour involves three main stages –the first, second and third stages of labour. These stages depend on the mechanical, biochemical and hormonal changes in the myometrium and cervix during labour (Irani and Foster, 2015). Physiological differences in the duration and progression of labour among women are taken into account clinically, particularly in early labour (Zhang et al., 2010); (Zhang and Duan, 2018).

First stage of labour

The 1st stage of labour is defined as the period from the beginning of uterine contractions to full cervical dilatation (0 to 10 cm). It is usually divided into 2 phases: latent and active. The latent phase is when there is a cervical dilatation to 3-4 cm associated with irregular uterine contractions. The next phase is the active phase, where the regular painful uterine contractions start and are accompanied by progress in widening the cervix until full dilatation. The latent phase lasts longer, even several hours, in contrast to the active phase, which is shorter and faster. The normal rate of cervical dilatation during the active phase is 1cm/hour (Pitkin, 2003). However, this might vary according to the population, parity, and foetal weight, whether spontaneous or induced labour (Juhasova et al., 2018).

Second stage of labour

The 2nd stage of labour is referred to be the time from the full dilatation of the cervix until the delivery of the baby. The length of this stage is also fluctuated depending on the parity. The second stage has two phases: the passive phase, which is initiated by the full dilatation of the cervix until the passive descent of the foetal head through birth pelvis. Then the active (expulsive) phase will start as contractions become intense, and the pushing actively begins.

Third stage of labour

The 3rd stage of labour starts at the time of full newborn delivery and lasts until full placental and membrane delivery. It usually lasts between 5 and 15 minutes when manually controlled and up to 60 minutes when spontaneous. When the placenta is delivered, it is accompanied with intense contractions, umbilical cord lengthening and vaginal bleeding as the placenta separates from the myometrial wall. Unfortunately, there is a lack of knowledge on the fundamental physiology of the third stage of labour. Postpartum haemorrhage is the commonest complication occurring during this stage. It can be controlled by using proper clamping and cutting of the umbilical cord and administration of uterotonic agents, such as oxytocin (Güngördük et al., 2018).

1.4 The mechanism of uterine contraction and relaxation

The uterus is in a quiescent state throughout pregnancy. It then becomes highly excitable and contractile during labour to expel the fetus and placenta to the external world through the vagina. The contractile force increases as the RMP of the myometrial cells becomes more depolarised. This can be explained by a reduction in the level of negativity of the RMP, which would allow Ca^{2+} influx through voltage-gated Ca^{2+} channels and increase contractility. The uterus is a myogenic organ; the smooth muscle contained within it can generate contractions autonomously (Wray and Prendergast, 2019). The contraction of its smooth muscles is preceded by a series of action potentials, this process known as excitation-contraction coupling. It is predominantly regulated by intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ (Pehlivanoğlu et al., 2013). This might occur via 2 mechanisms which are electrochemical or pharmacomechanical coupling (Somlyo and Somlyo, 1994a).

In electrochemical coupling: when the myometrial cell membrane is excited and depolarised from -55mV to -40mV, Ca^{2+} influx via voltage-gated Ca^{2+} channels occurs. It then binds to calmodulin (CAM), a calcium-modulated protein, forming a calcium- calmodulin complex that activates the myosin light chain kinase (MLCK) (Shmigol et al., 1998). This enzyme is

responsible for phosphorylation of serine 19 on the regulatory light chains of myosin, enabling actomyosin cross-bridge cycling, breakdown of ATP and the generation of force (Taggart et al., 1997) (**Figure1.4**).

When it comes to pharmacomechanical coupling, uterine stimulants augment contraction by binding to their particular receptor on the cell surface and allow small monomeric G-proteins to bind GTP and trigger phospholipase C (PLC) activity. As a result, phosphatidylinositol biphosphate (PIP_2) hydrolysis takes place, and inositol triphosphate (IP_3) and diacylglycerol (DAG) are generated (Wray and Prendergast, 2019). IP_3, after that, binds to its particular receptor in the membrane of SR, causing release of Ca^{2+} and $[Ca^{2+}]_i$ to rise (Somlyo and Somlyo, 1994b) (**Figure1.4**).

Relaxation of myometrial cells follows an opposite direction of the Ca^{2+}–calmodulin–MLCK pathway. The MLC is dephosphorylated by myosin light chain phosphatase (MLCP), and as such, $[Ca^{2+}]_i$ decreases because L-type Ca^{2+} channels close and Ca^{2+} outward mechanisms are triggered. As a result, Ca^{2+} dissociates from calmodulin, and MLCK is inactivated. Calcium leaves the cell by moving against its concentration gradients across the cell membrane, via plasma membrane Ca^{2+}-ATPase (PMCA) and a Na^+–Ca^{2+} exchanger that allows 1 Ca^{2+} to leave the cells in exchange with 3 Na^+ or sequestration of Ca^{2+} into the SR by SERCA pumps (Wray and Prendergast, 2019) (**Figure1.4**).

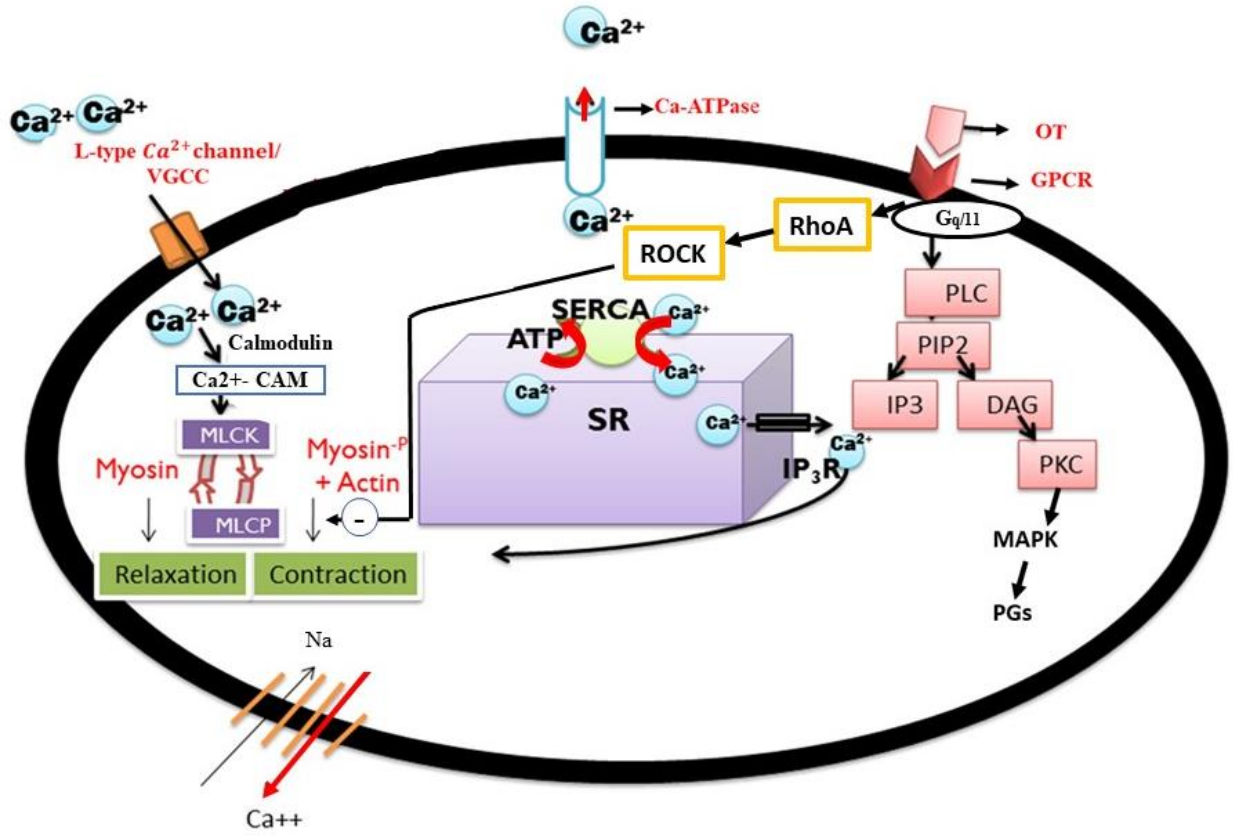


Figure 1.4: Schematic of uterine contraction/relaxation pathways, and mechanisms of increasing the contraction by agonists such as oxytocin. VGCC= voltage gated Ca^{2+} channels, Ca^{2+}-CAM= Ca-calmodulin complex, MLCK= myosin light chain kinase, P= phosphorylation, MLCP= myosin light chain phosphatase, Ca^{2+}-ATPase= Ca^{2+}-ATPase pump, SERCA= sarcoendoplasmic reticulum Ca pumps, OT= oxytocin hormone, GPCR= G-protein coupled receptors, PLC= phospholipase-C, PIP2= phosphatidylinositol biphosphate, IP_3= inositol triphosphate, DAG= diacylglycerol, IP_3R= inositol triphosphate receptor, ROCCs= receptor operator Ca channel, DAG= diacylglycerol, PKC= protein kinase C, MAPK= mitogen-activated protein kinase, PGs= prostaglandins, ROCK= RhoA-associated protein kinase. Created by PowerPoint tools.

1.5 Modulation of contraction

1.5.1 Neuronal effect

Although the uterus is innervated by parasympathetic and sympathetic innervation, uterine contraction is not dependent on either branch of the autonomic nervous system (Morizaki et al., 1989). The uterus does not have any motor innervation, and it is a myogenic organ, which may contract without being stimulated by nerves or hormones. As a result, the myometrium lacks synapses between axonal terminals and myocytes (Kota et al., 2013). Moreover, it has been claimed that functional degeneration results in denervation of the uterus toward term pregnancy in all species (Wikland et al., 1984); (Zupko et al., 2005); (Elmer et al., 1980).

1.5.2 Hormonal effect

Hormones are the strongest modulators for uterine contraction, and evidence points towards sustained changes in their function and expression to control pregnancy and parturition (Wray and Arrowsmith, 2021). Progesterone is suggested to play a crucial role in maintaining uterine quiescence throughout gestation as its receptor's expression is up-regulated in the myometrium (Arrowsmith et al., 2010). The main source of progesterone at the beginning of pregnancy in humans is the corpus luteum, while the placenta takes over progesterone production in the early few weeks of pregnancy (Chwalisz and Garfield, 1994). Progesterone depletion in maternal circulation has been suggested as a way to trigger labour in animals (Veronesi et al., 2002). This theory could not be applied to humans because circulating progesterone stays high during labour (Mitchell and Taggart, 2009). It has been found that progesterone inhibits spontaneous and oxytocin-induced contractions, and it is suggested to be through preventing Ca^{2+} entry and SR release or blocking oxytocin receptors (Arrowsmith et al., 2016). On the other hand, estrogen concentration and receptor activity increases close to the term, influencing excitability and increasing uterine contraction (Renthal et al., 2015).

Prostaglandins (PGs)

Prostaglandin is a family of fatty acids synthesised from arachidonic acid (AA) in the mammalian plasma membrane via cyclooxygenase. This enzyme mediates the synthesis of PGs, prostacyclin, and thromboxane and is the target of anti-inflammatory agents such as indomethacin. Mechanical stretch, oxytocin, and adenosine triphosphate (ATP) influenced PGs production considerably during labour (Patel and Challis, 2001); (Ziganshin et al., 2005). Myometrial contraction is influenced by the structure of PGs. When $PGF_{2\alpha}$ and PGE_2 are stimulated, for example, there is an increase in $[Ca^{2+}]_i$, which enhances myometrial

contraction during term pregnancy (Brown et al., 1998). PGI_2 stimulation, on the other hand, causes relaxation by inhibiting inflammation by activating adenylate cyclase, which in turn maintains myometrium quiescence (Hertelendy and Zakár, 2004).

Oxytocin (OT)

Oxytocin is the most-studied among the hormones which affect excitability. It is a nonapeptide hormone synthesised in the hypothalamus and secreted by the posterior pituitary gland, then released into the systemic circulation. It has a molecular structure similar to antidiuretic hormone (ADH), allowing it to attach to OTR and function as an agonist. Oxytocin is a powerful myometrial stimulant commonly utilised as a uterotonic to induce parturition (Blanks and Thornton, 2003).

Oxytocin binds to G-protein coupled receptors (GPCR) in the cell membrane, which are expressed in high density at term in human myometrium (Kimura et al., 1992). This triggers the $G_{q/11}$ family of the G-proteins, and then the cascades of the GPCR pathway take place (Arrowsmith and Wray, 2014); (Wray and Prendergast, 2019), as shown in **(Figure 1.4).** The DAG stimulates protein kinase C (PKC), which in turn activates the mitogen-activated protein kinase (MAPK) pathway and leads to prostaglandin (PGs) synthesis and eventually enhances contractile activity (Molnár et al., 1999). Extra investigation of the oxytocin mechanism of action has illustrated its ability to activate the RhoA/ROCK signalling pathway and increase the machinery contractile activity (Somlyo et al., 1999) (**Figure 1.4**). Moreover, oxytocin can inhibit Ca^{2+} extrusion through the PMCA; these additional mechanisms confirm that oxytocin activation can occur without extracellular calcium (Monga et al., 1999). Oxytocin receptors could be occupied by progesterone which inhibits their activation and relaxes the uterus.

1.6 Regulation of hormone receptor expression in pregnancy

The exact mechanisms of pregnancy preservation and the initiation of onset of labour are not clearly understood. However, evidence suggests that hormones play a crucial role in controlling gestation and parturition. Oestrogen enhances uterine contractility during labour, whereas progesterone promotes myometrial relaxation and serves to maintain the pregnancy continuing (Kota et al., 2013). Progesterone and oestrogen control myometrial contractility essentially by regulating the expression of genes that encode contraction-associated proteins (CAPs). These CAPs include the OT receptor (OTR, prostaglandin (PG)-F2α (PGF2α) receptor, the gap-junction protein connexin-43 (Cx-43), and ion channels such as L-type voltage-gated Ca^{2+}channels (VGCCs) (Mesiano and Welsh, 2007);(Tezuka et al., 1995);(Ilicic et al., 2020). In term pregnancy, an increase in the oestrogen concentration was shown to stimulate the expression of oxytocin receptor OT mRNA in the uterus and increased uterine sensitivity to oxytocin, enhancing myometrial excitability and increasing uterine contraction during labour (Murata et al., 2003); (Veiga et al., 2015). Progesterone, on the other hand, was found to reduce myometrial OTR levels indirectly by suppressing oestrogen-induced OTR expression (ADACHI and OKU, 1995); (Fang et al., 1997). Cx43 expression is reported to be up-regulated toward term pregnancy by oestrogen, boosting cell-cell communication to synchronise myometrial contractions (Garfield and Hayashi, 1981); (Di et al., 2001) and inhibited by progesterone (Zhao et al., 1996);(Ilicic et al., 2020). In preparation for labour, progesterone withdrawal has been suggested to be responsible for modulating mRNA levels for L-type voltage-gated Ca^{2+} channels in the rat myometrium resulting in an enhanced influx of calcium ions into myocytes and prompting the uterine muscle to produce high-intensity contractions (Tezuka et al., 1995);(Ilicic et al., 2020). Furthermore, evidence suggests that uterine inflammation and mechanical distention of the uterus during the late stage of pregnancy also promotes OTR expression and prostaglandin synthesis that enhances the uterine contraction during labour (FANG et al., 2000);(Terzidou et al., 2011);(Terzidou et al., 2005);(Sooranna et al., 2005);(Sooranna et al., 2004).

As circulating hormone levels rise, the expression of their receptors on the cell increases, making the cell more responsive to the hormone and allowing for increased cellular activity. However, sustained hormone exposure causes a considerable reduction in the ability of variable receptors on myometrial cells to respond to the stimuli. This is referred to as receptor desensitisation (Shankaran et al., 2007). Desensitisation to hormones could be concentration

or time-dependent (Arrowsmith and Wray, 2014). The different mechanisms of receptor desensitisation could regulate the receptor's surface expression. For instance, in the down-regulation process, there is a net loss of receptors from a cell surface expression due to a decrease in receptor production or an increase in receptor degradation (Shankaran et al., 2007). Internalisation or sequestration of the receptors is another mechanism in which the receptor is endocytosed in vesicles and then degraded by lysosomes (Burke et al., 2001). Sometimes surface expression is not altered, but instead, a receptor conformational change occurs, leading to the uncoupling of the receptor from the downstream signalling pathway and thereby preventing the cell from over-responding to the hormone (Arrowsmith and Wray, 2014).

1.7 Purinergic receptors: Expression and Physiology

As briefly outlined in section 1.2.3, purinergic receptor expression has been shown to increase during pregnancy. The signalling pathway of these purinergic receptors was initially investigated fifty years ago (Burnstock, 1972), and major advances have been made in our understanding of several purinergic receptors. In 1978, two separate families of purinoceptors were suggested, P1 and P2, for adenosine and ATP, respectively (Burnstock et al., 1978). A pharmacological approach has been successfully used to recognise the two superfamilies of P2 receptors as either ionotropic P2X or metabotropic P2Y receptors (Burnstock, 2004).

Adenosine purinoceptors (P1 receptors) and P2Y receptors belong to G-coupled protein receptors, typically with seven hydrophobic transmembrane-spanning α-helical domains and extracellular N-terminal domain and intracellular C-terminal domain **Figure 1.5** (Gacasan et al., 2017). Four subtypes of P1 receptors have been reported (A1, A2A, A2B, and A3) (Fredholm et al., 2001). Among these four subtype receptors, the A1 subtype has been identified in human myometrium (Tsai et al., 1996), and its stimulation leads to the contraction of uterine smooth muscle. This subtype is coupled with inhibitory $G_{i/o}$ to decrease the synthesis of cyclic adenosine monophosphate (cAMP) (Sheth et al., 2014).

1.7.1 P2Y Receptors

P2Y receptors are present in cell membranes, and in humans, eight P2Y receptor subtypes (P2Y1, 2, 4, 6, 11, 12, 13 and 14) have been cloned and characterised (Webb et al., 1993). P2Y receptors can be triggered by a range of extracellular nucleotides (ATP, ADP, UTP and UDP), and the response mainly depends on the P2Y subtype (Jacobson et al., 2015). Previous studies have shown that P2YR are expressed in different tissues, including the uterus in the female reproductive system (Slater et al., 2002). For instance, in rat myometrium, P2Y2R's expression increases until attachment and implantation occur in early pregnancy. Ziganshinc and his team have examined the effect of different P2 agonists, including ATP, α,β-methylene-ATP, UTP, and ADP, on preparations of the isolated human pregnant uterus. They found the rank order potency of these nucleotides were α,β-methylene-ATP>UTP>ATP=ADP. The α,β-meATP is a recognised P2X1 and P2X3 receptor agonist. Furthermore, due to a comparatively high potency of UTP, they concluded that P2Y4R might contribute along with other purinergic receptors in contractile response induced by ATP in this tissue (Ziganshin et al., 2003). However, no further studies have been confirmed the expression of P2YR or determined its role in human myometrium contraction since then.

1.7.2 P2X Receptors

P2XRs are ligand-gated nonselective cation channels with a high affinity for ATP, much less to ADP, but not at all to AMP or adenosine (North, 2016). ATP binding induces a plasma membrane depolarisation by allowing the flow of cations such as Ca^{+2} and Na^{+} ions, leading to an increase in free cytosolic Ca^{2+} concentration **(Figure 1.5)**. This depolarization opens L-type Ca^{2+} channels and influences Ca^{2+}-sensitive processes, including muscle contraction (Bradley et al., 2011).

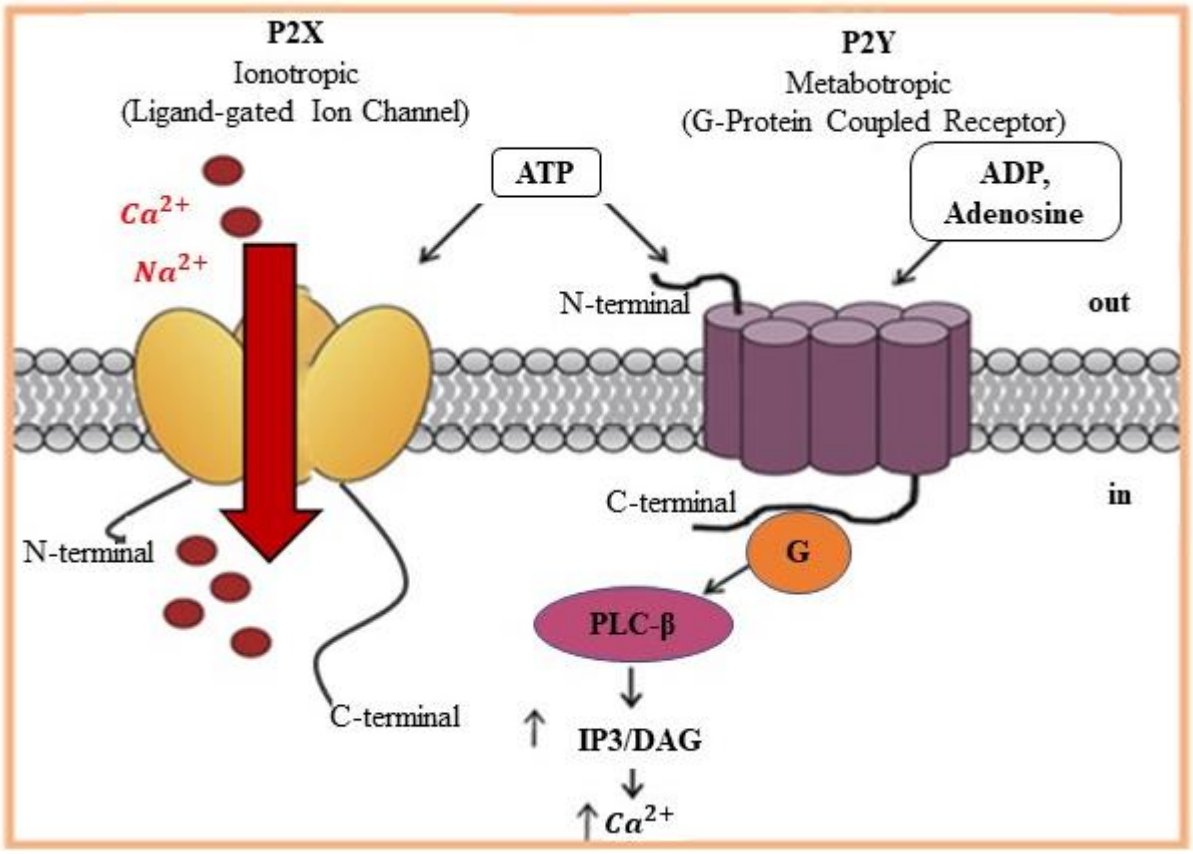


Figure 1.5: P2 purinergic receptors-mediated high intracellular calcium concentration.

Ionotropic P2X receptors are permeable to Na^{+} and Ca^{2+}. Their activation by extracellular ATP causes membrane depolarisation and cell excitation. The activation of metabotropic P2Y receptors by ATP or its metabolites breakdown results in coupling of Gq, and then the cascades of the GPCR pathway take place, which promotes intracellular mobilisation of Ca^{2+}.

In mammalians, seven P2X purinoceptors subtypes have been cloned to date (P2X1 – P2X7). Each subtype contains a large ectodomain containing ATP binding sites and 10 conserved cysteine residues forming an extracellular loop. The subunits also have two hydrophobic transmembrane domains, short intracellular N and variable size C termini (**Figure 1.6**) (Burnstock et al., 2010). The sequence length of P2X subunits varies from 384 amino acids for P2X4R to 595 amino acids for P2X7R (Coddou et al., 2011). Growing evidence has shown that the structure of P2X receptors involves three subunits, which form a trimer ion pore (Burnstock, 2006). These subunits form either a homomeric receptor as P2X7R or a heteromeric receptor as P2X2/3, P2X4/6, P2X1/5, P2X2/6, P2X1/4, and P2X1/2 receptors (Burnstock, 2006). A P2X6R is always expressed as a heterotrimeric receptor because its subunits are combined with other P2XR subunits (Kaczmarek-Hájek et al., 2012). The kinetics of desensitisation (in prolonged exposure to the physiological agonist, the receptor becomes temporarily unable to open even though the binding of the agonist persists) depends on the P2X receptor subtype. Studies have shown that the homomeric P2X1 and P2X3 receptors have fast desensitisation (Giniatullin and Nistri, 2013). In contrast, P2X2, P2X4, and P2X7 receptors desensitise slowly (Kaczmarek-Hájek et al., 2012). Ionotropic receptors' desensitisation may involve internalisation and degradation of the P2X receptor (Feng et al., 2005); (Khadra et al., 2013). It can also happen when membrane receptors undergo conformational changes, leaving them unable to affect signalling pathways further (Jarvis and Khakh, 2009). All P2XR subtypes require a low micromolar concentration of ATP to have a great effect, except P2X7R, which requires >100μM concentrations for stimulation (Coddou et al., 2011).

P2X subtypes such as P2X2, P2X3, and P2X4 are widely distributed in the central nervous system, and their role is determined in pain perception, age-related hearing loss, and some neurodegenerative diseases like Parkinson’s disease (Yan et al., 2013); (Wirkner et al., 2007);(Zhang et al., 2021). Studies have reported that the P2X5 subtype does not have a major role in human physiology (Kotnis et al., 2010). The P2X6 receptors expressed in the kidney (Jones et al., 2004) and their overexpression have been linked to renal cell carcinoma's poor prognosis in humans (Gong et al., 2019). P2X1 is predominantly found in smooth muscle tissues, such as the arteries and urinary bladder (Kennedy et al., 2007); (Mahaut Smith et al., 2019). Evidence has demonstrated that P2X7Rs expression is abundant in the urinary bladder (Svennersten et al., 2015) and saphenous vein smooth muscle (Cario-

Toumaniantz et al., 1998) and their stimulation have been identified to induce muscle contractions.

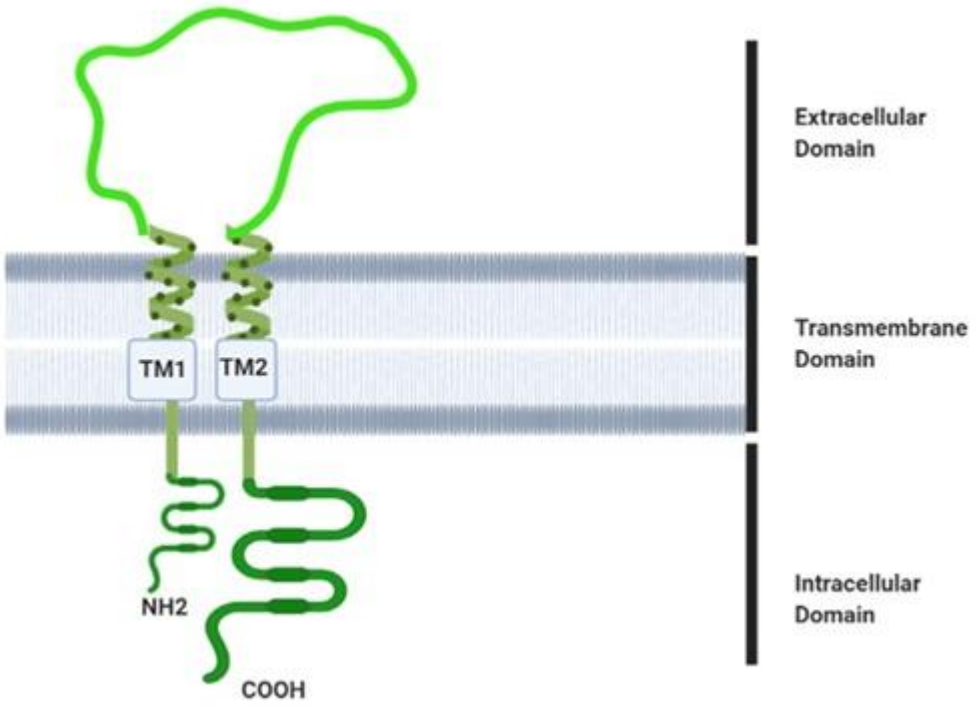


Figure 1.6 Structure of P2X receptor subunits.

P2X receptors contain two transmembrane domains (TMs), connected extracellular by a large ectodomain and from the cytosol by a short-sized N- terminus and a variable-sized C-terminus, ranging between 25 amino acids (P2X4) to 240 amino acids (P2X7).

Interestingly, P2X receptor subtypes have a functional significance and expression in myometrial smooth muscle. (**See Table 1.1**). Ziganshin et al. have studied the effect of α,β-meATP (known as P2X1 receptor agonist) on myometrial samples obtained from term pregnant and early pregnant women undergoing caesarean section using a pharmacological organ bath technique. Moreover, the effect of pyridoxalphosphate azophenyl-2',4'-disulphonic acid (PPADS), a P2 receptor antagonist, was also examined. They found that the force of myometrial contractions to α,β-meATP was stronger in term pregnancy than in earlier pregnancy, and the PPADS inhibited myometrial responses to α,β-meATP in both groups. Therefore, it has been suggested that the myometrial contraction induced by ATP in pregnant women was probably mediated through the stimulation of P2X1 receptors (Ziganshin et al., 2006). Furthermore, a study on pregnant rat myometrial contraction has suggested that the markedly peaked P2X1 receptor expression in the post-partum uterus might contribute to coordinating the uterine contractions during the involution period (Khanam and Burnstock, 2007). On the other hand, Urabe et al. have reported that P2X7 receptors were strongly expressed in pregnant rat myometrium and suggested that their potential expression is the leading cause of uterine contraction in a preterm delivery model (Urabe et al., 2009). Miyoshi and their team confirmed that the P2X7 receptor was the main channel involved in the ATP-induced ion currents in myometrial cells isolated from pregnant rats (Miyoshi et al., 2010); (Miyoshi et al., 2012). Alotaibi recently found that A-438079, a selective P2X7R antagonist, can abolish the stimulatory effect of ATP in labouring rats and concluded that P2X7Rs are functionally implicated in ATP stimulatory effect on uterine contraction (Alotaibi, 2018). Because the P2X1 and P2X7 receptors have been studied the most in the uterus due to their abundance and significant role in mediating smooth muscle contraction in a variety of species, it is critical to find out if they are expressed in human myometrium and conduct preliminary research to identify their functional significance, this in turn may reveal potential abnormal function playing a role in dysfunctional labour.

Table 1.1: summary of the P2X receptor subtypes expressed in the myometrium.

Immunohistochemistry =IHC, the quantitative real-time reverse transcriptase-polymerase chain reaction =RT-PCR, enzyme-linked immunosorbent assay =ELISA

P2 receptor subtypes	Tissue type	Methods of detection	Expression	Reference
P2X1	Early pregnant (6 days) rat	IHC	The level of P2X1R expression increased at the time of implantation	(Slater et al., 2000)
	Term pregnant & postpartum rat	Immunostaining & fluorescence and confocal microscopy	The level of P2X1R expression increased in postpartum>pregnant	(Khanam and Burnstock, 2007)
	Term pregnant mouse	IHC	The level of P2X1R has no change in the expression pattern during pregnancy.	(Vial and Evans, 2001)
P2X2	Non-pregnant rat	IHC	The P2X2R expressed in the uterus	(Bardini et al., 2000)
P2X3	Early pregnant (6 days) rat	IHC	The level of P2X3R expression increased at the time of implantation	(Slater et al., 2000)
P2X4	Term pregnant & Labouring rat	-RT-PCR -Western blot	Expression levels of mRNAs and proteins of P2X4 increased during the late stages of pregnancy & at the time of delivery	(Urabe et al., 2009)
P2X7	Early pregnant rat	IHC	Expression increased until attachment and implantation on Day 6	(Slater et al., 2002)
	Labouring & postpartum rat	- ELISA	- Expression significantly increased towards labour & postpartum period.	(Alotaibi, 2018)
	Term pregnant & Labouring rat	-RT-PCR -Western blot	Expression levels of mRNAs and proteins of P2X7 increased during the late stages of pregnancy & at the time of delivery	(Urabe et al., 2009)

1.8 Abnormalities in labour

It is known that the uterus is a dynamic organ. This active state is vital in many reproductive activities such as migration of sperm and embryos, menstruation, pregnancy and parturition (Wray, 1993). Improper or irregular uterine contraction may cause premature deliveries if initiated too early in pregnancy, while if uterine activities are too weak or incoordinate, an emergency caesarean section will required (Wray, 2007). The latter is called dysfunctional labour, and it will be briefly discussed below as it used in the current study

Dysfunctional labour

Parturition is a process which ends with normal vaginal birth and delivery of a healthy child. However, about 10 per cent will not if their contractions begin to weaken and become uncoordinated, which prevents cervical dilatation and slows or even arrests the labour. This condition is termed dysfunctional or dystocic, or obstructive labour. It is the leading cause of emergency C-section deliveries, particularly in nulliparous women (Wray and Prendergast, 2019). However, multiparous women may also experience dysfunctional labour (Nystedt and Hildingsson, 2014). Scientists have worked hard investigating what maternal factors might lead to dysfunctional labour. They found that parity, obesity, age, diabetes, fetal weight and position, pelvic size and shape and genetic cause play a role in labour length, but none are predictive or causal; in other words, older obese, diabetic women can have normal labours, (Wray and Arrowsmith, 2012);(Algovik et al., 2010). A differential gene expression study of women with delay in the first stage found some differences, but it was hard to link these to causing dystocia (Mittal et al., 2011).

The physiological cause of dysfunctional labour has been suggested to be due to the low pH level and increased lactate in myometrial capillary blood (Quenby et al., 2004). The metabolic requirements can be compensated in normal labour. In contrast, if labour is prolonged, myometrium will constantly consume its metabolic reserves of glycogen and ATP, which eventually leads to an increased acidic environment secondary to ATP depletion, anaerobic metabolism, and systemic ketoacidosis. According to research from several species, when myometrial lactic acidification develops, which is linked to a change in intracellular [Ca^{2+}], uterine contractility is reduced or even abolished (Taggart and Wray, 1995);(Taggart and Wray, 1993);(Hanley et al., 2015);(Pierce et al., 2003). Indeed, the pathological mechanism underlying dysfunctional labour is obscured, where the adaptive reactions are developed to respond to the effects of acidification. On the other hand,

purinergic signalling has been suggested to play an important role in uterine contraction, and the low expression of their receptors might lead to dysfunctional labour (Alotaibi, 2018); (Miyoshi et al., 2012). The increased expression of P2X7Rs in the uterus and the increased response to ATP during labour demonstrate the necessity of these purinergic receptors for successful labour. Furthermore, Miyoshi and his colleagues determined that P2X7R was significantly elevated in the rat myometrium of normally delivered as well as under LPS-induced inflammatory circumstances that simulated preterm birth, implying that P2X7R overexpression is linked to normal delivery and preterm birth (Urabe et al., 2009). Moreover, inhibiting P2X7R has been recommended as a clinically beneficial approach for preventing premature labour in a mouse model of intrauterine inflammation (Tsimis et al., 2017).

1.9 Treatments for dysfunctional labour

Oxytocin is extensively used for labour induction and augmentation. It causes a wide range of responses, but half of the women with difficulty labouring, do not respond to OT treatment and require CS. As a result, dysfunctional labour continues to be a key contributor to the non-elective CS rate, with OT having little impact (Arrowsmith et al., 2010). In addition, an intravenous fluid infusion is advised as a major component for correcting dehydration and electrolyte imbalance. However, it is unlikely that it entirely restores the metabolic acidosis associated with dysfunctional labour (Dawood et al., 2013). Interestingly, a recent randomised control trial found that oral bicarbonate ingestion was extremely encouraging to assist in neutralising the low pH and help to reduce threatened dysfunctional labour (Wiberg-Itzel et al., 2018). The study's sample size, on the other hand, was relatively small. Interestingly, in the case of primary dysfunctional labour, an intravenous infusion of Adenosine triphosphate (ATP) has been found to enhance the full cervical dilatation when combined with a prostaglandin infusion compared with prostaglandin infusion alone (Ziganshin et al., 2005), however, the sample size in the study is also quite small.

Despite all the prior treatment options, the dysfunctional labour has most frequently ended by Cesarean Section. According to Maternity Services Monthly Statistics in England (2021), around 35% of births were by caesarean sections. More than half of these emergency C-sections were due to emergency causes such as dysfunctional labour. Therefore, new approaches to improving uterine contractility are required.

1.10 Aims of the study

Dysfunctional labour is defined as a failure to progress in labour because of insufficient uterine activity. The currently available treatment for dysfunctional labour is limited, and women ultimately require CS (Arrowsmith et al., 2010). There is, therefore, a need for another stimulator as ATP could potentially be used for labour augmentation. There are few data so far to suggest that P2X1R or P2X7R are involved in mediating the contractile responses of the human myometrium to extracellular ATP. These findings are discussed in the light of work on animal myometrium and other P2X receptors. This study aimed to:

1- Investigate the effect of extracellular ATP on spontaneous uterine contractions in myometrial biopsies obtained from women who have elective caesarean sections (non-labouring women).

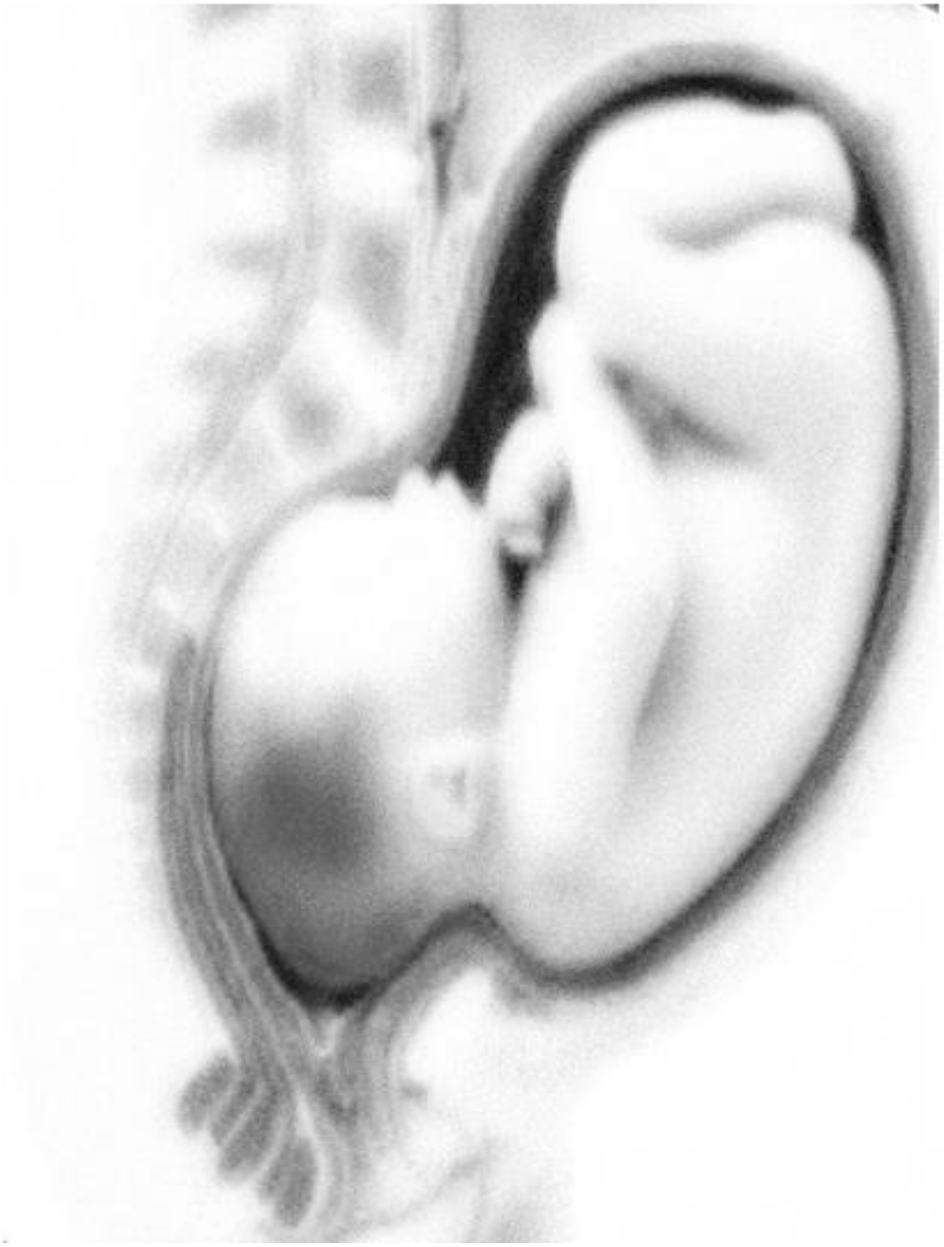
2- Investigate the effect of other ATP analogues, ATPγS and BzATP, on spontaneous uterine contractions in myometrial biopsies obtained from non-labouring women.

3- Investigate the effect of extracellular ATP on spontaneous uterine contractions in myometrial biopsies obtained from women who have emergency Caesarean sections (using ATPγS).

4- Determine if there is a role for P2X7Rs in contraction induced by ATP or BzATP via using selective P2X7 receptor blockers.

5- Investigate the presence of P2XR1 and P2X7R in the human myometrial tissue as their expression is expected to increase towards term pregnancy. ATP binding to one of these receptors could potentiate the myometrium contraction, which would help in contractile augmentation during the onset of labour. On the other hand, the depletion of one or both receptors would lead to dysfunctional labour.

CHAPTER 2



Chapter 2: General Materials and Methods

2.1 Contractility studies

2.1.1 Human Tissue Ethics and Regulation

Experiments were performed on human uterine tissues. Informed consent from patients undergoing caesarean sections (CS) at the Liverpool Women's Hospital was written to obtain labouring and non-labouring human myometrial biopsies. A further application was submitted and accepted for the use of tissue from the Myometrial Research Tissue Bank (MRTB) at Liverpool Women's Hospital.

2.1.2 Collection and Storage

A biopsy was taken after the baby had been delivered and before the administration of oxytocin, which is applied to prevent post-partum haemorrhage. The uterine biopsy measuring 1cm x 1cm was taken from the upper lip of the incision in the lower uterine segment, was immediately placed in a buffered physiological saline solution (PPS) that contained (mM) [NaCl 154, KCl 5.6, $MgSO_4 7H_2O$ 1.2, HEPES 10.9, Glucose 7.8, and $CaCl_2$ 2], pH 7.40. Immediately after taking the biopsy, part of the tissue biopsy was taken and fixed in neutral buffered formalin (NBF) for immunohistochemistry studies or snap-frozen by Liquid Nitrogen and stored at -80°C for future experiments such as western blotting studies. The rest of the biopsy was stored in the fridge at 4ºC overnight and used for contractility studies the following day. This did not change the contractile ability of the uterus, and there was no difference between tissues used immediately and those used the next morning (Kawarabayashi et al., 1989);(Popat and Crankshaw, 2001). Anonymised maternal demographic data (age, parity, gestation, reason for section, BMI, Medications, Diabetic or not, Gravidity) was obtained for each biopsy and is described in the appropriate results chapter.

2.1.3 Dissection

The uterine tissue biopsy was placed in a clean, shallow dissecting dish that contained PSS solution at room temperature under a stereomicroscope (Micro Instruments Ltd, Oxford) for fine dissection. As the majority of fibres run in the same direction, a bundle of fibre was pinched by forceps, and then a straight cut was done using fine scissors. This was done to reduce the trauma to the tissue. Small uterine strips measuring (2 mm in width ×10mm in

length) were dissected. Each strip was tied with surgical silk thread at both ends in preparation for mounting in an organ bath containing PSS solution at 37ºC for isometric force measurement (**Figure 2.1**).

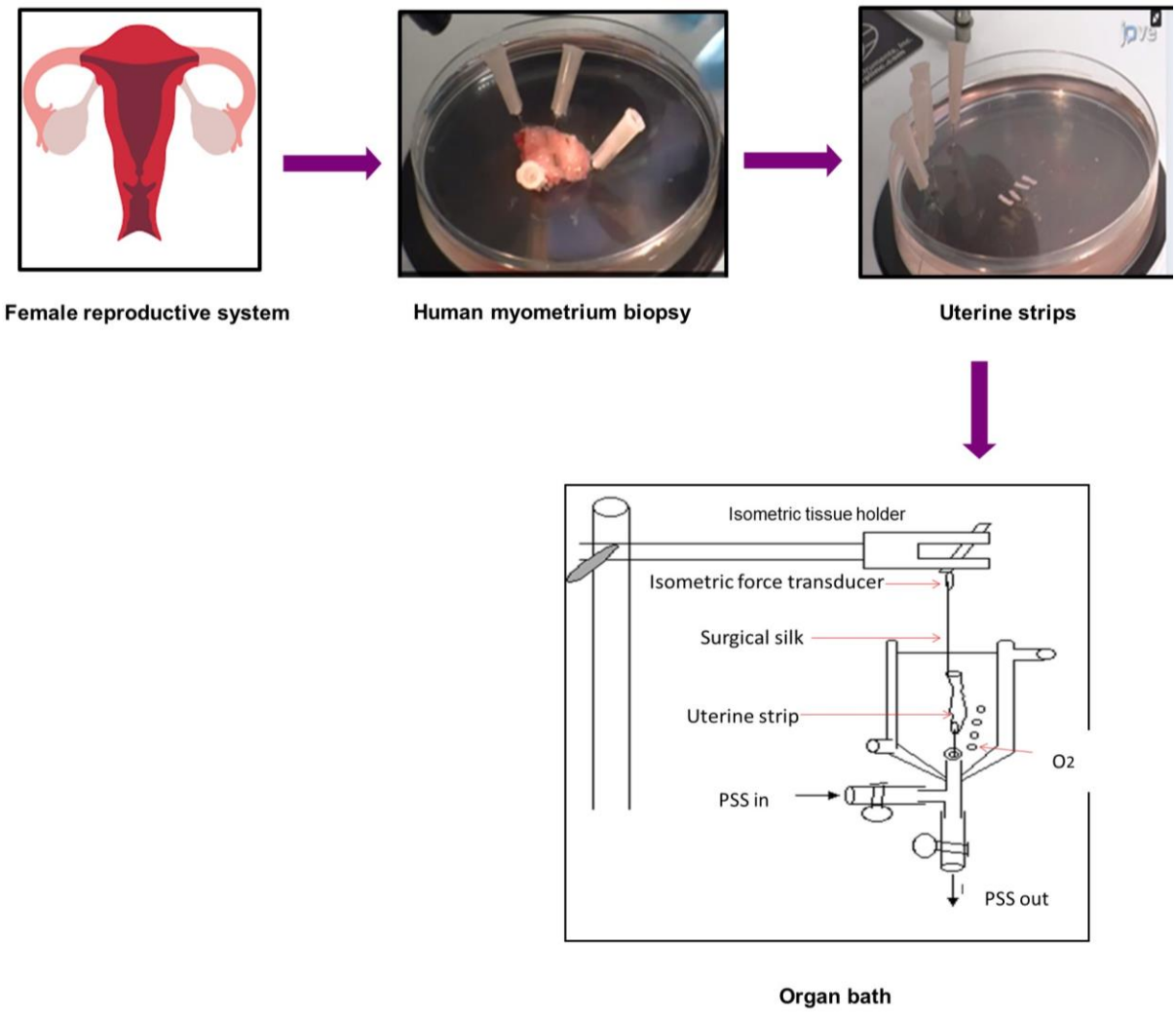


Figure 2.1 Recording of the human uterine contraction using an organ bath.

A diagram showing a small human myometrial biopsy was dissected into small strips, measuring 2mmx10mm. The strips are then tied up by surgical silk from both ends and mounted vertically in an organ bath setup, the bottom end attached to a fixed hook and the top end connected to a sensitive isometric force transducer, and continuously perfused with PSS at 37˚C and bubbled with 100% O2 for isometric force measurement.

2.1.4 Isometric Force Measurement in Organ Bath

The uterine strips were mounted vertically in a 10 mL organ bath (World Precision Instruments, UK), using surgical silk to attach the bottom end to a fixed hook and the top end to an isometric force transducer (**Figure 2.1**). The strips were constantly perfused with PSS (pH=7.4) at the rate of 1 ml/min, using a peristaltic pump (Peri-Star Pro, World Precision Instruments, UK), and the temperature was maintained at 37ºC throughout the entire experiment using a Thermostatic water bath (Grant Instruments Ltd, Cambridge) that circulated warm water through the organ bath jackets.

The strips were then placed under 2mN resting tension and were allowed to equilibrate in PSS bubbled with 100% O_2 for an average of 2hrs before the strips started to contract in vitro. Experiments weren't started until stable contractions were produced, meaning at least 3 contractions of equal amplitude were obtained.

2.1.4.1 Calibration of Force

Electrical signals from the force transducer are amplified and converted to a digital signal recorded on the computer using Lab scribe V.3 software (iWorx Systems, Inc, USA). Before mounting the tissue in the organ bath, these electrical signals need to be calibrated, allowing the measurement of responses as mN of force. To achieve this, a known weight (1mN) was suspended on the force transducer, and then the force was recorded using the Lab scribe software.

2.1.4.2 Exclusion Criteria for Experiments

For each experiment, three identical tissue strips from the same myometrial biopsy were mounted in three identical organ baths. The human myometrial strips develop spontaneous contractions 1-2 hours after mounting in an organ bath, and these tissues are capable of contracting regularly over many hours. Strips from 5 women that failed to spontaneously contract were excluded. These samples represented around 9% of the total percentage of the non-labouring group n=53. However, when it comes to the dysfunctional labouring women, 4 out of 5 samples contracted spontaneously, while the 5th one failed to contract and was challenged with ATP to see whether it could induce contractions or not, as it is hypothesized in this study that ATP might be a potential treatment for augmentation the contraction in those group, and it was successful. Therefore, no samples from the dysfunctional labouring group were excluded. However, these data were not included in the analysis (n=1).

2.1.5 Analysis of Contractile Parameters

Data were obtained from uterine strips that were allowed to equilibrate and generate stable contractions for at least 1 hour to establish a sufficient control period. Four parameters of contraction were analysed during control and treatment periods, force amplitude, duration, frequency, and area under the curve (AUC), and the analysis of these parameters was carried out in Origin Pro 2015 software (Origin Lab Corporation, USA). All those parameters were measured over a 20 mins treatment period and normalised against the 20 mins control period (set as 100% control). Addition of agonist caused a rise in the baseline of contraction in some experiments. This shifted baseline was measured and expressed as a mN increase compared to the baseline in the control period.

An illustration of how contractile parameters were measured and obtained is shown in (**Figure 2.2**). The force amplitude of uterine contractions was determined by measuring the value of the baseline of each contraction and subtracting it from the value of the peak of such contraction. The mean force amplitude was calculated for a 20 mins period before applying a drug or treatment; this was termed ‘control’. The control period was then compared to the mean force amplitude calculated during the 20 mins treatment period. Frequency is the number of contractions at a given time period. The frequency of contractions was measured over 20 mins time periods, during the last 20 mins control and the treatment period. The duration of contraction is represented by how long each contraction lasts (in minutes) and is measured at a half-maximal height of the contractions. It was measured during the last 20mins control and the treatment period application. The force integral or the AUC for a number of uterine contractions (the contractile integral of force over a fixed period of time) was measured over the last 20 mins of the control period and over the 20 mins treatment period application. The baseline shift is determined by measuring the difference between the shifted baseline in the treatment period and the control baseline (in mN). The baseline shift was measured during the last 20mins of the control and the treatment period.

2.1.6 Sample Size

The sample size is the number (n) of uterine biopsies collected from each woman. Prior animal and human research conducted in our department or elsewhere led us to determine the number of samples we required for our investigation, we also considered employing power analysis for the sample size calculation. In order to determine the minimal sample size, the effect size, type 1 Error, power of the study, direction of effect, and statistical test were determined. Investigators need to know the effect size in order to determine how much minimum variance between the means of 2 groups can be considered clinically significant. This can be obtained from prior studies, if possible, as well as the standard deviation in the case of quantitative studies. The type 1 Error is measured by significance level, which is usually fixed at the level of 5% (P = 0.05); however, it can be modified according to the study question. The power of the study is the likelihood of detecting the potential effect, which can range from 80 to 99% but is commonly kept at 80%. The direction of effect determines the type of test the investigator can apply while investigating the effect. If the effect is in the same or opposite direction, the investigator can use a one-tailed test; if the effect is in both directions, a two-tailed test can be used. It is necessary to understand the statistical tests that will be used in order to calculate the sample size. The calculation for simple statistical tests like the t-test is achievable using a formula, but for advanced statistical tests, for instance, the analysis of variance (ANOVA), proper software or statistician assistance is required.

The sample size (N) was calculated using the following equation:

$$\text{Sample size} = \frac{(\text{level of confidence})^2 * (\text{Standered deviation})^2}{(\text{Error rate})^2} \qquad N = \frac{(Z)^2 * (\sigma)^2}{(E)^2}$$

$$\text{Where } E = \frac{Z * \sigma}{\sqrt{n}}$$

Z= Confidence level (1.96 at 95%)

E= Margin of error

σ = Standard deviation, data from initial experiments (n=10) of the effect of ATP on uterine contractility showed a change in the frequency have standard deviation = 90.

$$N = \frac{(1.96)^2 * (90)^2}{(57)^2} = 9$$

As a result, power analysis suggests that the calculated sample size is at least 9 samples (confidence level of 95%, margin error of 5% (0.05) and significance level of 0.05).

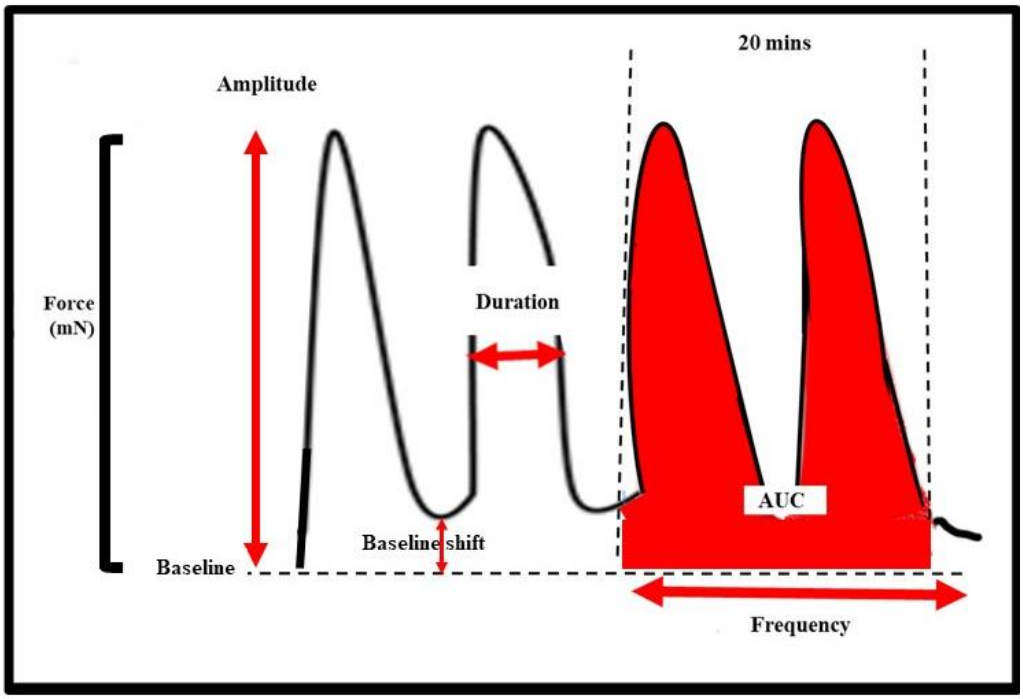


Figure 2.2: Measurement of uterine contractile Parameters.

Contraction amplitude is represented by the height or peak of the contraction for each contraction over 20 mins. The contractions frequency is represented by the number of contractions occurring over 20mins. The AUC was obtained by measuring the contractile integral of force over 20 mins (equivalent to a Red shaded area). Duration is measured at half-maximal amplitude (minutes) for each contraction over 20 mins. The shifted baseline is determined by subtracting the baseline in the control period from the shifted baseline in the treatment period, in mN.

2.2 Western blot

Western blot is a technique widely used in research to determine the relative protein levels between samples and establish the molecular weight of the target protein. To achieve this, western blot implements three steps: 1) separation by size, (2) transfer to a membrane, and (3) visualizing target protein using primary and secondary antibodies. In this study, immunoblotting was utilized to identify the P2X1R and P2X7R expression in the human myometrial tissue samples obtained from non-pregnant and term pregnant women who underwent elective CS deliveries secondary to previous CS and breech foetal presentation or emergency CS deliveries secondary to dysfunctional labour or foetal distress. In the current 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). Anti-P2X1 (Cat. #APR-022) and Anti-P2X7 (Cat. #APR-008) antibodies were used. These antibodies were purchased from (Alomone Labs, Israel) to detect the P2X1 and P2X7 receptors. A knockout validation of these antibodies in mouse vas deferens and spinal cord has determined their specificity to these proteins (Apolloni et al., 2013):(White et al., 2013). GAPDH was used as a loading control.

2.2.1 Protein extraction and quantification

Frozen human myometrial tissues were taken out from the -80°C freezer, defrosted on ice, weighed and minced into small pieces using a clean razor blade. Then these tissues were homogenized by using IKA Labortechnik Ultra Turrax T25 Basic S2 homogenizer in Radioimmunoprecipitation assay (RIPA) buffer, which was mixed with a protease inhibitors cocktail (The volume of buffer was made depending on the size of the tissue). Lysate next was transferred into Eppendorf tubes and incubated on ice for 30 mins. The lysate was then centrifuged at 4°C, 12,000 rpm for 5 min. and the supernatant was transferred to fresh tubes, and the pellets were discarded. The centrifugation step was repeated, and this time the supernatant was obtained and used to estimate the total protein content. The total protein amount was quantitatively assayed using bicinchoninic acid assay (BCA). The assay is based on the fact that proteins in a highly alkaline environment reduce Cu^{2+} ions to Cu^{1+}. BCA is a chromogenic reagent that chelates Cu^{1+} producing a purple complex with a significant absorption at 562 nm. The absorbance is directly proportional to the amount of protein present in the solution. Then a standard curve of absorbance was plotted, and the protein concentration was calculated. After that, the loading volume of the prepared sample for western blot was calculated and expressed as μg/μl.

2.2.2 P2X1 and P2X7 Western Blot procedure

Samples were prepared by mixing tissue lysate with 2x Laemmli loading buffer (Thermo Scientific, Loughborough, UK) and deionized water to obtain a satisfactory dilution level in each well. Then these samples were heated in a digital dry bath at 100^{o}C for 5 min to denature proteins, spun shortly to collect the sample at the bottom of the tube and allowed to cool to RT. A 10µl of molecular weight marker was loaded into 1st lane of Precast gel Tris-Glycine extended shelf life (TGX) 12% (Bio-Rad, Hertfordshire, UK); and 5µl of prepared lysates were electrophoresed in Tris-Glycine-SDS (TGS) running buffer (see Table 2.1 for the composition) at 100V for the first 5 min then maximized to 150V for 45 min. The protein was then transferred to an Immun-Blot Polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hertfordshire, UK) by using Trans-Blot Turbo Transfer System (BIO-RAD, Hertfordshire, UK) for 7 min. After that, the membrane was blocked with 5% non-fat milk diluted in TBS-T (see Table 2.1 for the TBS-T composition) for 1 hour to prevent the nonspecific binding of antibodies. After blocking, the membrane was incubated at 4°C overnight with the primary antibody solution. The primary antibodies (anti-P2X1 and P2X7) were all polyclonal, hosted in rabbits, and used at a concentration of (1:1000-1:200), respectively and 5% non-fat milk was used as diluent. On the following day, the membrane was washed 3 times in 1x TBS-T wash buffer, with each wash lasting 10 minutes. After washing, the blot was incubated at RT overnight with the secondary antibody (goat anti-rabbit antibody (1:3000), diluted in 5% non-fat milk) (Cat. #32460) (Thermo Fisher Scientific, UK). On the third day and after washing 3 times, each for 10 min in TBS-T, membranes were incubated with Super Signal West Dura Chemiluminescent Substrate (Thermo Scientific, Loughborough, UK) for 5 min at room temperature. After that, in the darkroom, detecting signals at a range of exposures using Amersham Hyperfilm ECL film (Sigma-Aldrich, GE28-9068-35) was taken place. After exposure, the film was placed in the developer for 1 minute, then rinsed in water, then placed in fixative for 1 minute, then finally rinsed in water prior to hanging up to dry. The same blots with the same tissues were used as a re-probed blot. GAPDH (Cat. # G9545) (Sigma, UK) was selected as a loading control because it is expressed in almost all tissues in high amounts and to quantify the relative expression of P2X1 and P2X7 receptors as its molecular weight is 37 KDs which is away from the predicted protein size of my proteins of interest (P2X1=45KDs and P2X7=69KDs). The membrane was washed 3 times in 1x TBS-T wash buffer, with each wash lasting 10 minutes. After that, it was blocked for 1 hour with 5% nonfat milk to prevent nonspecific antibody binding. Then it was incubated with an anti-

GAPDH antibody as a loading control (1:5000) at RT overnight. After that, the same experimental protocol on the second and third days was followed.

Table2.1: The composition of TGS Running Buffer and TBS- Tween Washing Buffer

TGS Running Buffer	Molar concentration (mM)	TBS-Tween	Molar concentration (mM)
Trizma base	25	Trizma base	20
Glycine	192	NaCl	150
SDS	0.1%	Tween20	0.1%

2.2.3 Analysis

ImageJ was used to analyse the data. The blots were scanned at 200dpi and saved in TIFF format. The background signal in the blot was eliminated after opening the files in ImageJ. Each band in the blot was surrounded by a wide and equal size rectangular box to allow background assessment. All bands of blot were selected, and then the relative density of the contents of the rectangle over each band was plotted. Peaks and troughs represent these plots. The involved area of each peak (which represents each band) was enclosed with the line selected from the drawing tool. The (wand) tool selected each contained area and displayed the AUC for an individual band. These were then imported to Excel for additional calculations.

2.3 Immunohistochemistry (IHC)

Immunohistochemistry is a technique for detecting and locating antigens or proteins of interest in tissue samples. Previous studies have shown the enhanced expression of P2X1 and P2X7 receptors during pregnancy (Alotaibi, 2018);(Urabe et al., 2009);(Slater et al., 2002);(Khanam and Burnstock, 2007). However, all investigations have been conducted on pregnant rats, and neither of them has examined the expression of P2X1 and P2X7 in labouring myometrium. Additionally, no such expression studies have been carried out in pregnant human myometrium (labouring or non-labouring). In this study, immunohistochemistry was used to test the expression of P2X1 and P2X7 receptors in human myometrial samples taken from labouring, non-labouring and non-pregnant women. Mouse kidney was taken from the university's tissue bank and used as a positive control for P2X7R. The positive control is a tissue known to express the protein of interest. As the myometrial tissue was known to express P2X1R, the non-pregnant myometrial tissue was selected as a positive control. Later, one sample of the non-pregnant human myometrium was tested in each experimental set with the same antibody to be used as an internal positive control to ensure conditions were similar between runs. Again, the same sample was incubated with rabbit IgG (1:10000, Vector Labs) instead of primary antibody, and served as a negative control. Primary antibodies were applied at an appropriate dilution determined by titration and followed by adding the secondary antibody to all slides (horse anti-rabbit IgG for P2X1 and P2X7 antibodies); further detail is in chapter 4.

2.3.1 Tissue preparation and fixation:

The human myometrial biopsies were obtained at emergency and elective caesarean section of pregnant women and during hysterectomy procedures from non-pregnant women after informed consent at Liverpool Women's Hospital, Liverpool, UK.

In order to maintain the cells and prevent their natural autolysis, the cell fixation process plays an important role by inactivating the lysosomal enzymes and inhibiting bacterial growth without affecting the cell morphology. A 0.5mm x 0.5mm x 0.5mm of human myometrial biopsy was dissected and incubated, at room temperature, in neutral buffered formalin (NBF) containing 10 % formalin (4% formaldehyde) for 24 hours. The fixed samples were processed and embedded in paraffin wax, then mounted onto glass slides.

2.3.2 Tissue embedding and sectioning:

The fixed tissue is then prepared for tissue embedding, where infiltration with paraffin is required. The tissue was dehydrated firstly by dipping them in a series of ascending grades of ethanol baths from 60% (1 hour), 70% (1 hour), 90% (1 minute) and 100% (1 hour), 100% (1½ hours) and 100% (2 hours). Then the tissue was cleared by xylene in 3 processes, each with different times, such as the 1st process: 1 hour; the 2nd process: 2½ hours; the 3rd process: 2½ hours. After that, tissue infiltration with paraffin was taken place (the 1st process: 2½ hours; the 2nd process: 2½ hours).

The embedded tissues were then sectioned at a thickness of 5μM by a rotatory microtome (Microm HM335, MIROM UK Ltd, UK), transferred to a warm water bath at 37^{o}C and then fixed onto glass slides. The sections were placed in racks to dry overnight at room temperature until the day of the immunohistochemistry experiment. Tissue preparation, fixation, embedding and sectioning were done by Helen Cox, the lab technician at the Liverpool Women's Hospital.

2.3.3 P2X1 and P2X7 IHC staining procedure

Sections from human myometrium were selected according to the following criteria: the women should be primigravida, their age between 20-40 years, have normal body mass index (BMI), have singleton pregnancy, and they should not have diabetes. In the non-pregnant women, the age average was between 20-47 years.

On the day of the experiment, slides of sections were chosen randomly and labelled in advance with the sample ID, the antibody used, the concentration and the date of the experiment. Then slides were baked in a section dryer maintained at 60 ^{o}C for 60 mins to dry them out and ensure that the tissue sections adhered to the slides. After that, slides were dewaxed in 100% xylene, solvent-based agent, (2 times for 10mins followed by submersion in 100% ethanol (2x- 5mins) and immersed in 90% then 70% ethanol (1 min each). Finally, the sample was transferred into a bath containing distilled water for 5 minutes. This gradual rehydration is essential to prepare the tissue for water-based antibodies. After that, the antigen retrieval method was done by immersing the slides in boiling 1x citrate buffer in a Tefal Clipso Easy 6L pressure cooker for 20-30 minutes. This acid-base antigen retrieval method is crucial to expose the antigen to the desired antibodies. The slides were then transferred to distilled water. Then slides were incubated in buffered saline solution (TBS, Sigma Aldrich) 2x- 5 mins at room temperature. After that, a 0.3% H_2O_2 /TBS solution was

prepared to incubate the sections for 10 mins at room temperature to block any endogenous peroxidase activity, which is conjugated to the secondary antibody. Then the slides were incubated in TBS for 5 mins at room temperature. Each slide then was tapped off from excess water before marking a circle around the tissue section with DAKO hydrophobic barrier pen (Vector Labs), which creates sufficient space for the reagents and allows the reagents to remain localized to the tissue. Then slides were washed with the TBS for 5 mins. Then, for 20 minutes at room temperature (RT) in a humidified environment, one drop of a ready-to-use 2.5 percent Normal Horse Serum (Vector Labs) was applied to each slide to inhibit non-specific binding sites. Anti-P2X1 at 1:400 and anti P2X7 at 1:200 were diluted in a 0.5% solution of BSA, bovine serum albumin, in TBS and applied to sections which were incubated overnight at 4^{o}C. A 70µl of the solution was enough to cover the whole tissue section on each slide.

One of these myometrial sections was considered a negative control section and incubated overnight in blocking solution without adding the primary antibody, but instead DAKO rabbit IgG (1:10000, Vector Labs) were applied. The rabbit IgG negative control antibody checks for non-specific binding to human tissue.

All the incubations were performed in a humidified chamber to keep the environment moist and to prevent tissue dry out as that might lead to nonspecific binding.

On the second day, the primary antibody was washed in TBS (2x 5 mins). Then sections were incubated with two drops per slide of the ready-to-use secondary antibody, horse anti-rabbit IgG antibody (Thermo Fisher Scientific, UK, Cat. #32460) for 30 mins at RT in the humidified chamber. Then, slides were washed in TBS (2x 5 mins) and later incubated with a chromogen such as ImmPACT 3,3'-Diaminobenzidine (DAB solution) (Cat. #SK-4105) (Vector Labs) to visualize antibody binding at RT for 10 mins; then immediately return in TBS and finally into tap water to stop the further reaction. Because horseradish peroxidase (HRP) labelled secondary antibodies were utilized, (DAB) was chosen as a stain. The location of proteins can be identified using DAB as a substrate in immunohistochemical procedures. The secondary antibody conjugated with a peroxidase enzyme targets a protein of interest, and in the presence of hydrogen peroxide, DAB is readily catalyzed to its oxidized state at the position of the (HRP) resulting in a brown precipitate. Sections then were counterstained with filtered Gill 2 hematoxylin (nuclear stain). The staining reaction was halted after 1.5 mins by dipping the sections in running water until the water flowed clear.

After that, slides were briefly submerged in 1% acid alcohol solution to wash off any extra stain and immediately shifted into tap water for 5 mins at room temperature. Sections were then dehydrated through a series of ethanol; 70% then 90% (1min each), 100% (2x 3 mins) and then 100% xylene (5 then 10 mins) before being mounted. Cover-slips were then mounted with a xylene based mounting medium using DPX mountant. The steps of dehydration were performed in a fume hood. After 24hrs, slides were scanned and viewed using the Aperio scan scope system (Aperio, Technology; Vista, USA) at 20x magnification to provide a high-resolution digital image. The ImageScope software was used to help quantify P2X1R & P2X7R expressions using a scoring system from 0 to 3. The ImageScope allows visualising the whole tissue section to determine the most representative areas of the entire tissue. Images were obtained and saved as TIFF files.

The negative control was examined first to make sure there was no staining, which is an indicator of a successful experiment. The internal control was compared after each experiment to ensure conditions were similar between runs. To avoid biased results, all slides were blinded to the observer and visualized by two observers. Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

2.3.4 Analysis

Samples were scanned using the Aperio scan scope system (Aperio, Technology; Vista, USA) at 20x magnification to provide a high-resolution digital image, and the intensity of the staining was observed. The ImageScope software was used to help in the quantification of P2X1R & P2X7R expression using a quickscore system from 0 to 3 (Alnafakh et al., 2021). Both stain intensity and percentage of positive cells were evaluated within the myometrial regions. Each section of myometrium was divided into compartments. The myometrial myocytes in each compartment were included in the analysis, whereas the non-myometrial regions (if present), such as blood vessels which are easily visible by the eye, as marked in the red square in **Figure 2.3A**, were discounted during analysis. The edges of the section were avoided as well. The myometrial myocytes appear as a long spindle-shaped muscle, arranged in bundles that travel in the same direction **Figure 2.3B**. A thumbnail window guided scanning for each compartment to ensure that the whole sample was systematically assessed. The intensity was identified subjectively by comparing the compartments to determine the lowest (weak) and the highest (strong) intensity. The compartments were scored by applying the formula Q= I x P, where (Q) represents quantification, (I) represents

the intensity, and (P) represents the percentage of positive cells. The staining's intensity (brown staining) for the muscles was detected: 0 indicated no staining, 1 indicated weak staining, 2 indicated moderate staining, and 3 indicated strong staining. The overall percentage of staining was assessed across the compartment: a score of 1 is given if 25% of the compartment is stained. A score of 2 is given if 50% is stained. A score of 3 is given if 75% is stained. A score of 4 is given if 100% is stained. A compartment can have a maximum score of 12 points. This means that 100% is strongly stained. The formula: 3(strong staining) × 4(100% stained) =12. After that, the average score of each compartment is identified for each section. All slides were blinded to the observer and visualized by two people to avoid bias in the results. Discrepancies between the two observers were resolved by re-evaluating the samples together and agreeing on a final score.

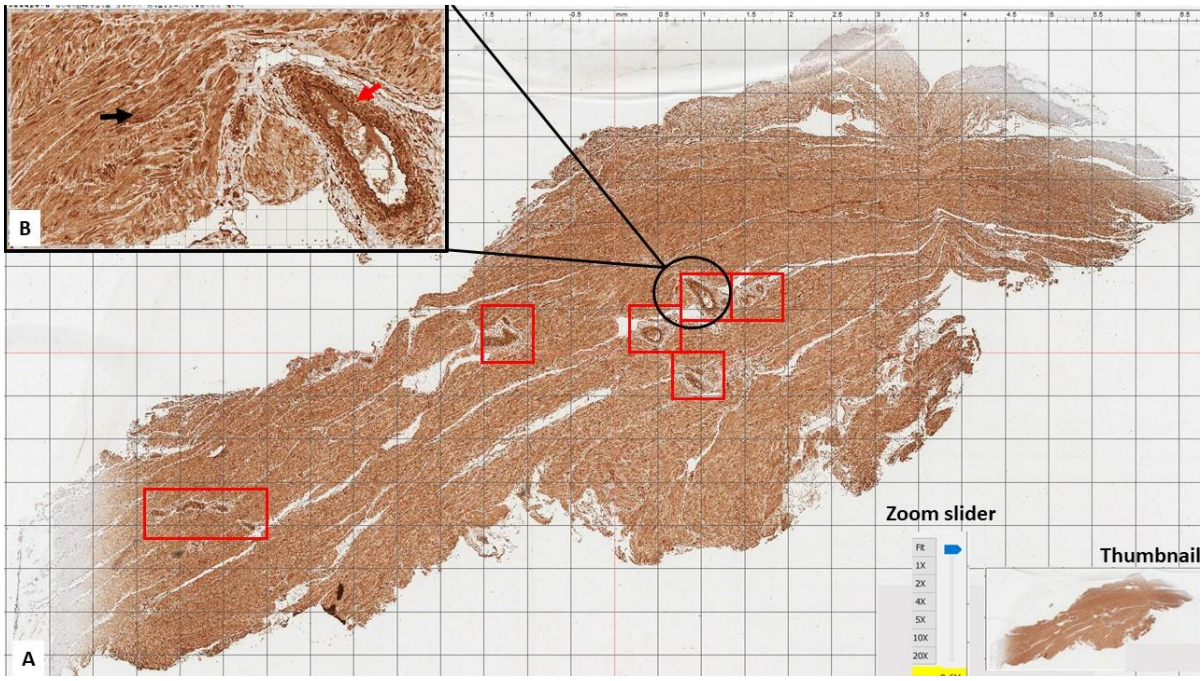


Figure 2.3 Quickscoring using Aperio ImageScope software.

A representative section of the human myometrium contains a few blood vessels labelled in red squares (A). Magnification 20x shows myometrial myocytes (black arrow) and blood vessels (red arrow) (B).

2.4 Solutions and Chemicals

Physiological Saline Solution

Fresh Physiological Saline Solution was prepared on a daily basis. The pH of the solution was adjusted to 7.40 using sodium hydroxide. The composition of the physiological saline solution is shown in Table 2.2:

Table2.2: The composition of physiological salt solution

Physiological salt solution	**Molar concentration (mM)**
Sodium Chloride (NaCl)	154
Potassium Chloride (KCl)	5.6
Magnesium Sulphate ($MgSO_4$)	1.2
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES Buffer)	10.9
Glucose (D-(+)-Glucose)	8
Calcium Chloride ($CaCl_2$)	2

The high-K^+ solution was made by replacement of NaCl with 40mM KCl; otherwise, the compositions were exactly the same as standard PSS.

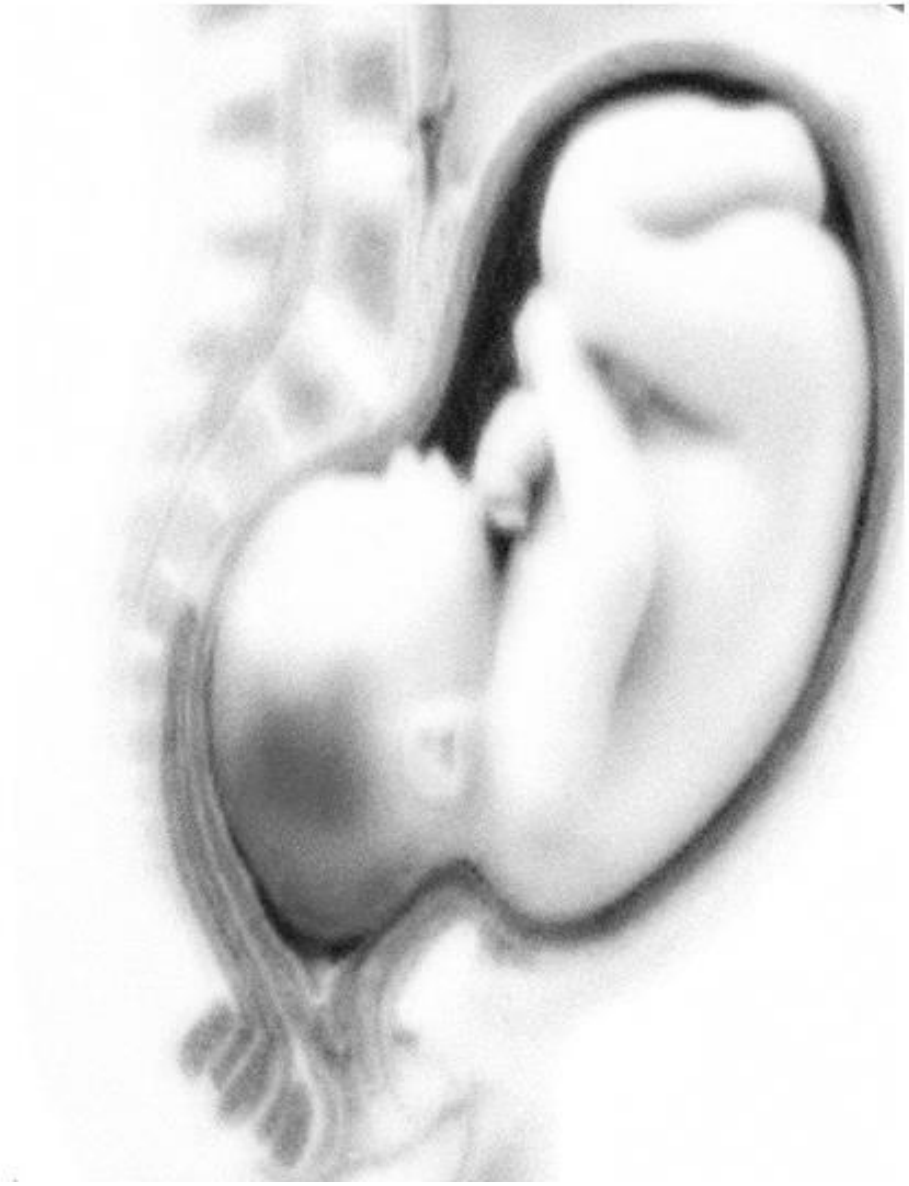
Experiment's solutions:

Concentrated solutions of Adenosine 5'-triphosphate disodium salt hydrate (ATP,10mM) or Adenosine 5′-[γ-thio] triphosphate tetralithium salt (ATPγS, 1mM) were made up freshly from powder every day and then serially diluted to the required concentration in standard PSS. Concentrated stock solutions at 10mM of [2,3-O-(4-benzoyl benzoyl)-ATP (BzATP) in H_2O and A-438079 or A-740003 in Dimethyl sulfoxide (DMSO) were made. Then they were aliquoted and frozen at -20^oC. Aliquots were removed from the freezer and diluted to required concentrations in PSS as needed on the day of the experiment, thus avoiding repeated freeze-thaw cycles of the stock solution. Matched vehicle and time controls were performed accordingly in initial experiments, as shown in (chapter 3). All chemicals were purchased from Sigma Aldrich (Dorset, UK).

2.5 Statistical Analysis

GraphPad Prism-5 was used to produce graphs and perform statistical tests. Data were expressed as a percentage of the control values (100%) in contractility studies or as immunoreactivity score values in immunohistochemistry study or as relative protein abundance values in western blot study, and the statistical differences were tested using Student's t-test or one sample t-test or Repeated Measures ANOVA or one-way ANOVA as appropriate. The Bonferroni test was used as a *post hoc* test. A probability value (P-value) of <0.05 was taken as the level of significance, and (n) is the number of myometrial tissue biopsies examined. An asterisk (*) denotes a significant difference in contractility compared to the preceding control period $p<0.05$, ** $p<0.005$ and *** $p<0.0005$. In the current study, some n numbers were very low. The number of samples suggested by the power calculations was not reached due to the shortages in tissue availability secondary to the Covid 19 pandemic, as it was difficult to obtain the tissue. As the sample size increases, the variability in the response between samples decreases, and the confidence in the conclusion increases. This is discussed further in the appropriate chapters.

CHAPTER 3



Chapter 3: An Investigation of Subtypes of Purinergic-2X Receptors and their effects on Myometrial Contractility in non-Labouring Women

3.1 Introduction

3.1.1 Effect of extracellular ATP on the myometrium

Extracellular adenosine triphosphate (ATP) has been identified as a critical extracellular signaling molecule and as a neurotransmitter regulating various biological functions in numerous physiological and pathophysiological conditions (Taruno, 2018). There is compelling evidence that ATP is released from the cell in pathological as well as physiological states (Bonora et al., 2012). Evidence show that cells can release ATP into the extracellular milieu in response to hypoxia (Gourine et al., 2005);(To et al., 2015). In myometrial cells particularly, the brief hypoxic episodes which accompany each contraction, are suggested to stimulate adenosine and ATP release into the acidic extracellular milieu during labour (Alotaibi et al., 2015). ATP has an excitatory effect on contractility of myometrium. It causes contraction of the uterus in human (Hutchings et al., 2009), guinea-pig (Piper and Hollingsworth, 1996), and rat (Alotaibi, 2018). This excitatory effect of ATP was found to be upregulated in the myometrium throughout gestation in the rat, thus it might be considered as a therapeutic target to control uterine activity during difficult labours (Zafrah and Alotaibi, 2017). A previous study on pregnant human uterus has suggested, by both laboratory and clinical observations, that ATP augments the effect of prostaglandin and thus could be a useful supplemental drug to increase uterine contractility in dysfunctional labour (Ziganshin et al., 2005). This stimulatory activity of ATP is mediated primarily by binding to its P2 receptors; P2XRs (Nagaoka et al., 2009) mainly P2X7R as reported from animal experiments (Urabe et al., 2009); (Miyoshi et al., 2010); (Miyoshi et al., 2012); (Alotaibi, 2018). For more details about purinergic receptors and their expression and physiology, see Chapter1.

In summary, there have been few studies examining the effects of extracellular ATP on human myometrium, and little is known about its mechanisms of action, or which subtypes of P2X receptors are expressed in human myometrium. In this study, the effect of extracellular ATP was investigated and some of its analogues on non-labouring human myometrial

preparations, and have studied the potential role of P2X7 in ATP-induced human myometrial contraction.

3.1.2 ATP analogues

The concentrations of extracellular ATP are maintained by the enzymatic activities of membrane-bound ectonucleotidases (Zimmermann et al., 2012). These enzymes are responsible for degradation of extracellular ATP to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine **Figure 3.1 A**, and these metabolites can also stimulate purinergic receptors (Burnstock and Kennedy, 1985). This makes it difficult to distinguish the effects of ATP from its breakdown products. Therefore, the effect of a non-hydrolysable analogue of ATP, ATPγS on human myometrial contractility, was used in the current study (**Figure 3.1 B**).

ATPγS is metabolically more stable than ATP; it does not break down to ADP or adenosine. The contractile response can therefore be attributed to ATP and not to any of its metabolites (Lambrecht, 2000). ATPγS mimics the action of ATP and is an agonist for P2X, P2Y1, P2Y2, and P2Y11 receptors (Lambrecht, 2000). A previous study has shown that ATPγS greatly increases the frequency of contractions in term-pregnant rat myometrium (Alotaibi et al., 2015). However, it has been found to be a weak agonist at the human P2X7 receptors (Donnelly-Roberts et al., 2009), but about two fold more potent than ATP at recombinant P2Y1 receptors (Schachter et al., 1996).

Studies on animal tissue have suggested that P2X7Rs are functionally regulated and contribute to ATP-induced uterine contraction (Alotaibi, 2018);(Miyoshi et al., 2012) (**Figure3.1-C**). ATP has a relatively low potency for P2X7, however the stable ATP analogue BzATP is ten times more potent (Donnelly-Roberts et al., 2009), and although not selective for the P2X7 receptor, it tends to be the agonist of choice for P2X7 receptor studies (Syed and Kennedy, 2012). Interestingly, the stimulation of P2X7R requires high concentration of nucleotides compared with all other P2X channels (Donnelly-Roberts et al., 2009) meaning a more potent agonist is a useful tool for its study. BzATP, along with ATP and ATPγS, will therefore be used to characterize the ATP response in human myometrium.

3.1.3 ATP and P2X7 receptors antagonists

There is now a large diversity of antagonists that have potent and selective activity for P2X7 receptors (Gum et al., 2012). However, evidence has shown species differences in the

potencies of these antagonists at P2X7 receptors (Donnelly-Roberts et al., 2009). A-438079 and A-740003, **Figure3.2**, are both reversible and competitive P2X7 antagonists (Donnelly-Roberts and Jarvis, 2007). A-438079 selectively antagonized BzATP-triggered Ca^{2+} entry through P2X7R with an IC_{50} of 100nM and 300nM at rat and human receptors respectively (Nelson et al., 2006). P2X7 receptors are expressed in the rat myometrium (Miyoshi et al., 2016). The stimulatory effect of ATP on rat uterine contractions was found to be reduced in the presence of A-438079, suggesting that P2X7R is involved in ATP-mediated stimulation of uterine contractility (Miyoshi et al., 2010);(Alotaibi, 2018) However, the drug also reduced uterine contraction in the absence of extracellular ATP, which suggested that P2X7R may contribute to membrane depolarization (Alotaibi, 2018).

A-740003 is another selective and competitive blocker of P2X7 receptors (Donnelly-Roberts et al., 2009). It is known to antagonize rat and human P2X7 receptors, with IC_{50} equal to 18nM and 40nM, respectively (Honore et al., 2006). There is no published data showing the effect of A-740003 in uterine tissue, however, it was found to be a potent blocker of P2X7R in neural cells and cause pain relief (Honore et al., 2006); (King, 2007).

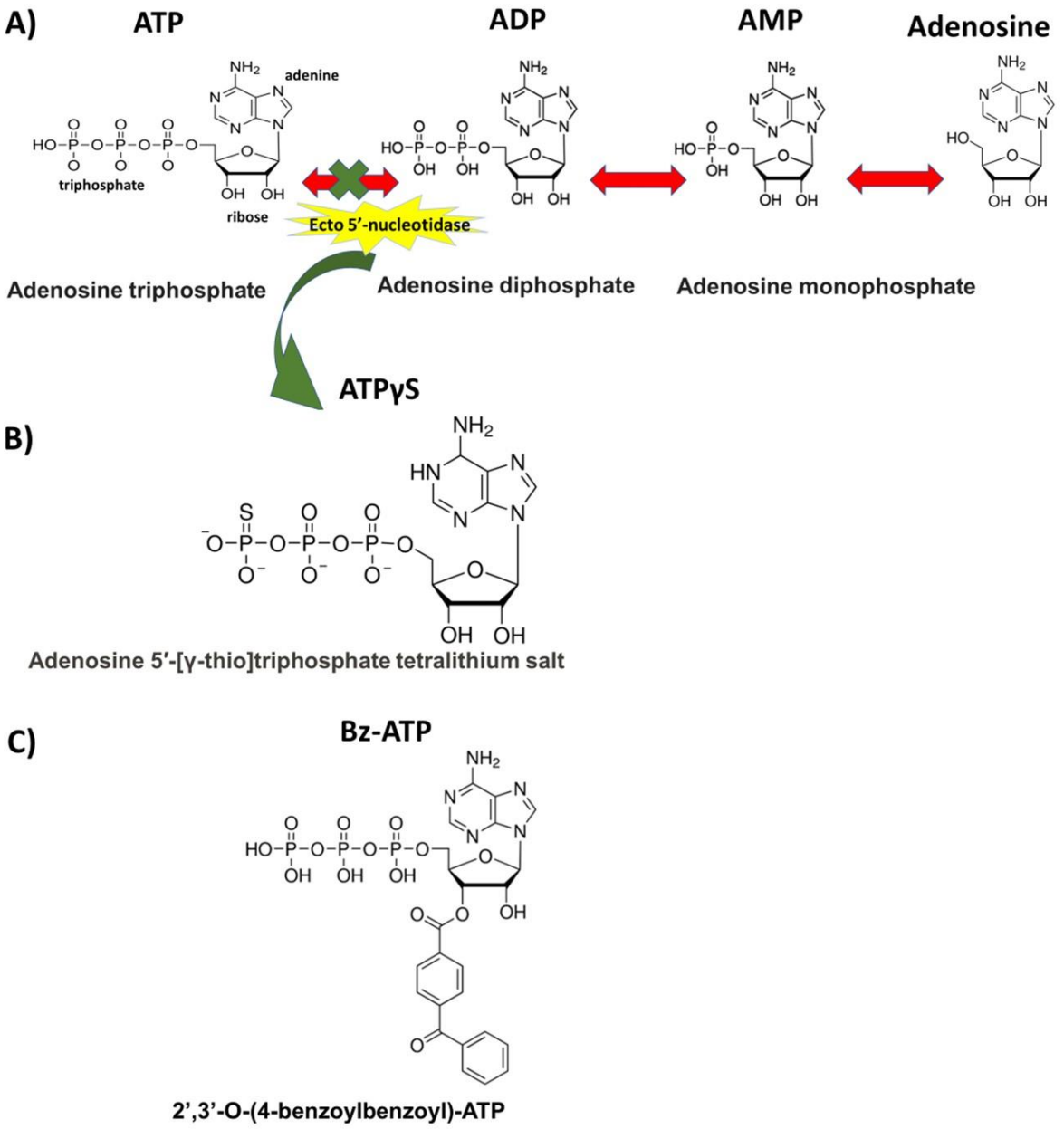
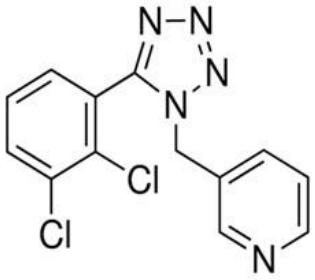


Figure 3.1: Line structures of ATP and its analogues.

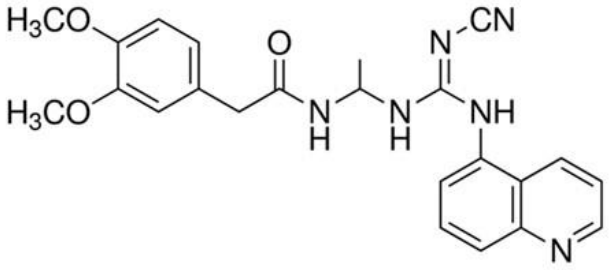
(A) Adenosine triphosphate and its metabolic products ADP, AMP, and Adenosine. (B) ATPγS and (C) BzATP.

A) **A-438079**



3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine hydrochloride hydrate

B) **A-740003**



N-(1-{[(cyanoimino)(5-quinolinylamino) methyl] amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide

Figure3.2: Line structures of selective P2X7 receptor antagonists. (A) A-438079 and (B) A-740003.

3.1.4 Aims of the study

Extracellular ATP has a powerful stimulatory effect in myometrial tissue from animals. There is however limited work investigating its effects in human uterus, or knowledge of which subtypes of P2X receptors might be involved. The aims of this chapter were:

1- To investigate the effect of extracellular ATP on spontaneous uterine contractions in myometrial biopsies obtained from non-labouring women.

2- To investigate the effect of ATP analogues, ATPγS and BzATP, on spontaneous uterine contractions and determine the rank order of potency of these analogues in non-labouring women myometrium.

3- To determine if there is a role for P2X7Rs in contraction induced by ATP or BzATP via using of selective P2X7 receptor antagonists, A-438079 hydrochloride hydrate and A-740003 on the responses of non-labouring women myometrium to ATP and BzATP were investigated.

3.2 Methods

3.2.1 Tissue

Human myometrium was obtained from women undergoing elective caesarean section at term at Liverpool Women's hospital with local ethical committee approval and written informed consent. Uterine tissues were collected, prepared and then myometrial strips were dissected and mounted as described in general materials and methods chapter (Chapter 2). The demographic data on the samples used is given in Table 3.1

3.2.2 Drugs and solutions

ATP, a pan -purinergic agonist, ATPγS, a non-hydrolysable version of ATP and BzATP, the most potent P2X7R agonist were used to stimulate myometrial contraction. The selective P2X7R blockers, A-438079 and A-740003, were used to investigate whether the responses to ATP and its analogues were mediated by P2X7 receptors. A-438079 was used at a final concentration of 10μM and 50μM to block the P2X7R. A-740003 was used at a final concentration of 10μM. A relatively higher concentration was chosen, despite an IC_{50} of 40nM at P2X7, since no inhibition was seen with A-438079. The drugs and solutions were prepared as described in general materials and methods chapter.

3.2.3 Experimental protocol

After approximately an hour of superfusion with physiological saline the spontaneous contractions reached a regular pattern displaying similar amplitude and duration and regular frequency. The uterine strips were then exposed to either 1 mM ATP, 1mM ATPγS or 1mM BzATP for 20 mins, to test their effects on spontaneous myometrial contractions, and then washed out with PSS. Additionally, cumulative concentration-response curves were obtained for each of the three agonists (ATP (10μM-1mM), ATPγS (1μM-1mM) and BzATP (1μM – 1mM)) in order to calculate an EC_{50} for each and determine the rank order of potency of these agonists. Each concentration of agonist was applied for 20min. Since the maximal response to ATP was not reached at 1mM, an alternative concentration-response curve was obtained using higher concentrations of ATP: 300μM, 1mM, 3mM, 5.6mM and 10mM. For experiments involving antagonists, tissue was preincubated (for 40 mins) with either antagonist then challenged with 1mM ATP or 100μM BzATP. The 20minute period immediately before application of the agonists was used as the control (100%) period **(Figure 3.3**). For all antagonist experiments, the concentration of DMSO did not exceed 0.1%.

3.2.4 Statistical Analysis.

Data were obtained from uterine strips that generated stable contractions for at least 1 hour to allow for a sufficient control period. Four parameters of contraction were analysed throughout the 20 mins control and treatment periods; force amplitude, duration, frequency and area under the curve and treatment effect was expressed as % of the control value. Expressing the data as % of the control, helps normalise the sometimes considerable between-sample variability that can occur in the raw data (i.e., the frequency could be 1 every 5 mins or 1 every 15 mins). In some preparations, the highest agonist concentration increased the frequency such that the contractions became tonic. In some cases, this meant an elevated baseline upon which contractions were superimposed and here, frequency was calculated as normal, as described in Chapter 2. If a tonic response was obtained and the tissue was no longer phasically contracting, then the maximal value for frequency obtained in the tissue immediately before it stopped phasically contracting, was used as an estimate of frequency for that tissue and this is noted in the figure and table legend where appropriate. This approach was undertaken to avoid having to discard the data. With data normalised to the control period (100%), significant difference from control was tested using a one-sample t-test. The time dependent effects of ATP, different agonist responses and the effects of antagonists were compared using paired or unpaired Student's t-test (or Mann Whitney test where data is non-parametric) and Repeated Measures ANOVA as appropriate. The analysis of these parameters was carried out using Origin Pro 2015 Software and statistical difference was carried out in GraphPad Prism as detailed in materials and methods chapter.

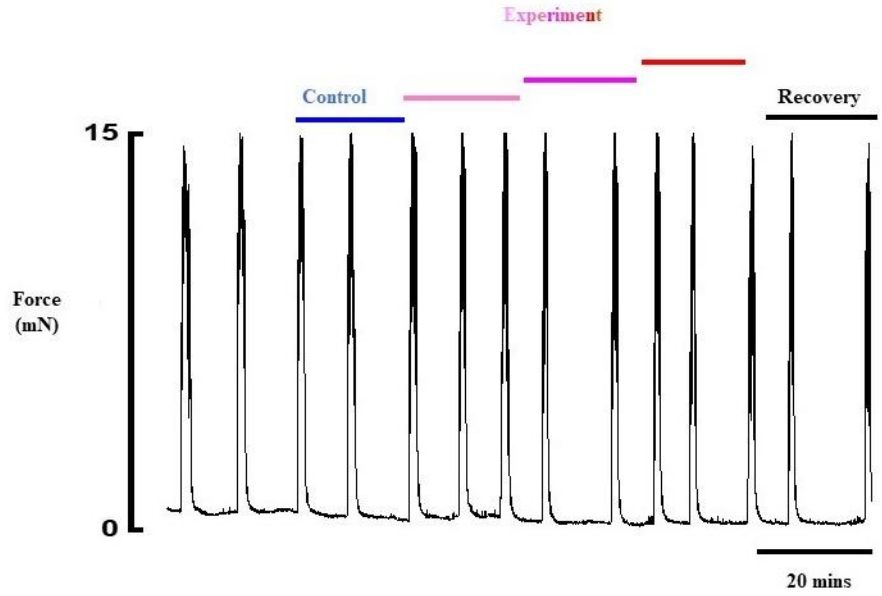


Figure 3.3: Experimental protocol. Spontaneous uterine contractions were obtained in a steady state and the last 20 minutes were considered as a control period; labelled by the blue bar. Then followed the 20-minute experimental intervention, which was labelled with colourful bars. Recovery was then followed for at least 20 minutes; labelled by black bar. Control values for the parameters of contraction were assigned 100% and any changes measured during the experimental period were expressed relative to this (mean±SEM).

3.3 Results:

The demographic data shows that the women in this study were on average slightly overweight, but otherwise largely healthy (Table 3.1). Their average age of 33 fits with the local city and country expectations, and the majority of women had already had one pregnancy. Previous traumatic vaginal birth was the major reason for the subsequent elective Caesarean section. All had a term (>37 weeks) delivery.

Table 3.1 Maternal demographic data. It illustrates the study sample characteristics of maternal age (years), gestational age (days), maternal body mass index (BMI), parity, gravidity, and indication for Caesarean section.

Maternal sample demographics (n = 48)	
Mother's age (years: mean± SD)	33±5
Gestational age at delivery (days: median IQR)	273 (271-275)
BMI (kg/m^2: median IQR)	25.2 (23-30.9)
Parity	
Nulliparous	n=14
Primiparous	n=23
Multiparous	n=11
Gravidity	
Primigravida	n=13
Multigravida	n=35
Indication for Caesarean section	
Previous traumatic vaginal delivery	n=34
Breech	n=3
Twins	n=2
Other	n=9
Type of Caesarean	
Elective non-labouring	n=48
Diabetic	None

3.3.1 The effect of ATP on spontaneous uterine contractility in the non-labouring human myometrium

Strips of myometrium from non-labouring term human myometrium, contracted spontaneously within 2hrs when mounted in the organ bath. They continued to contract regularly for hours without fatigue when superfused with the physiological saline solution and bubbled with 100% O_2 at 37 ºC (**Figure 3.4A**). A 20-minute application of ATP was

chosen as this allowed sufficient time for an average of three contractions to occur and the parameters of contraction to be accurately measured and compared to the values of the control period.

As can be seen in **Figure3.4B**, the application of 1mM ATP for 20 mins produced a large initial stimulation of frequency which then started to return to control values in the continued presence of ATP. It can also be seen that relaxation is incomplete between contractions which resulted in a small, non-significant elevation in the baseline (0.4±0.1mN, P=0.06), as frequency starts to return to control values, so too does resting force. Contractions continued on washing out of ATP with fresh PPS.

Figure 3.5 shows scatter plots of the effect of 1mM ATP on contractile parameters during spontaneous activity in the non-labouring human myometrium. Analysis of these data shows a significant increase in the frequency of contraction and AUC compared to the preceding control period (100%). During ATP application, frequency of contraction significantly increased (230±38.7% of control, P=0.01), and the AUC increased significantly to (156.3±18.1% of control, P=0.02). There was a small decline in the duration to 89.2±5.2% of control, which was not significant (P=0.06). There was also no significant difference in amplitude of contraction (97.9±9.2% of control, P=0.8).

It can be seen from **Figure 3.4B** that the effect of ATP on the frequency and AUC were transient, and values returned to baseline within approximately 10 min of 1mM ATP application. As shown in **Figure 3.6**, the effect of ATP on AUC in the 2^{nd} 10mins was significantly smaller than in the 1^{st} 10 mins (129± 10.4% and 183.6± 20.1%, P=0.02), respectively. However, no significant difference in frequency was seen between the 1^{st} and the 2^{nd} 10 minutes (323.4±71.9% and 212.4±69.8%, P= 0.1).

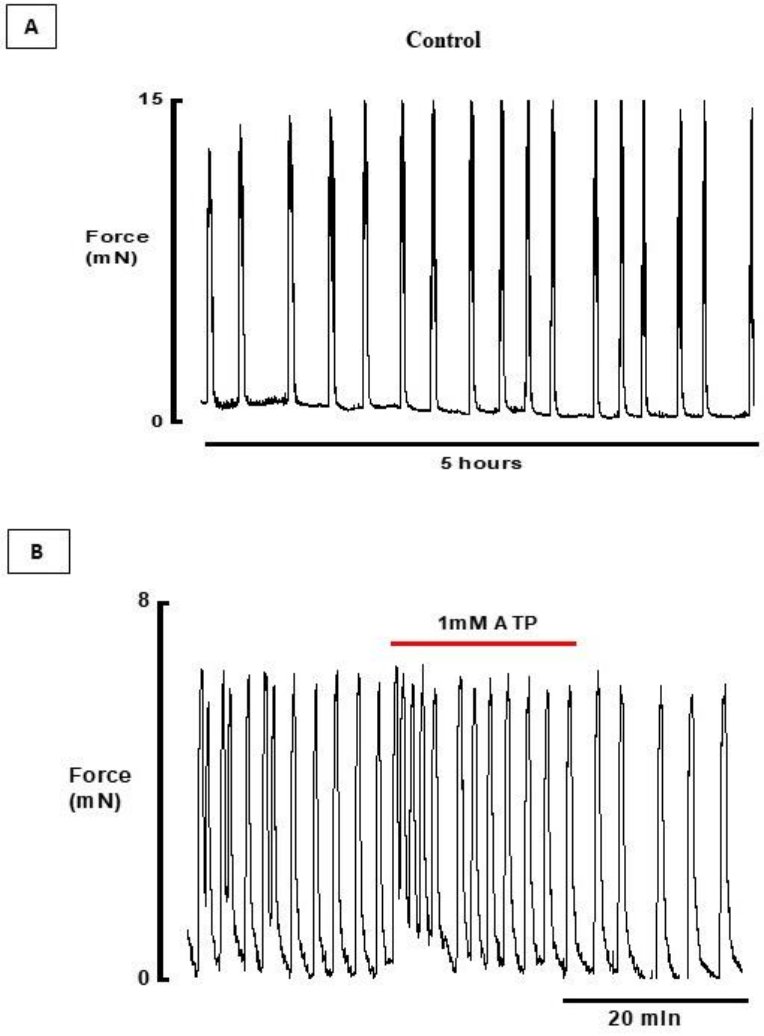


Figure 3.4: The effect of 1mM ATP application on non-labouring human myometrium.

(A) Typical trace of control spontaneous contractions obtained by dissecting myometrial strips from non-labouring women. (B) The effect of 1mM ATP on spontaneous uterine contractions applied for 20 mins. Tissue was continuously superfused with physiological saline solution at pH 7.4. The perfusion rate was 1ml/min and preparations were bubbled with 100% O_2 at 37°C in this and subsequent traces in this chapter.

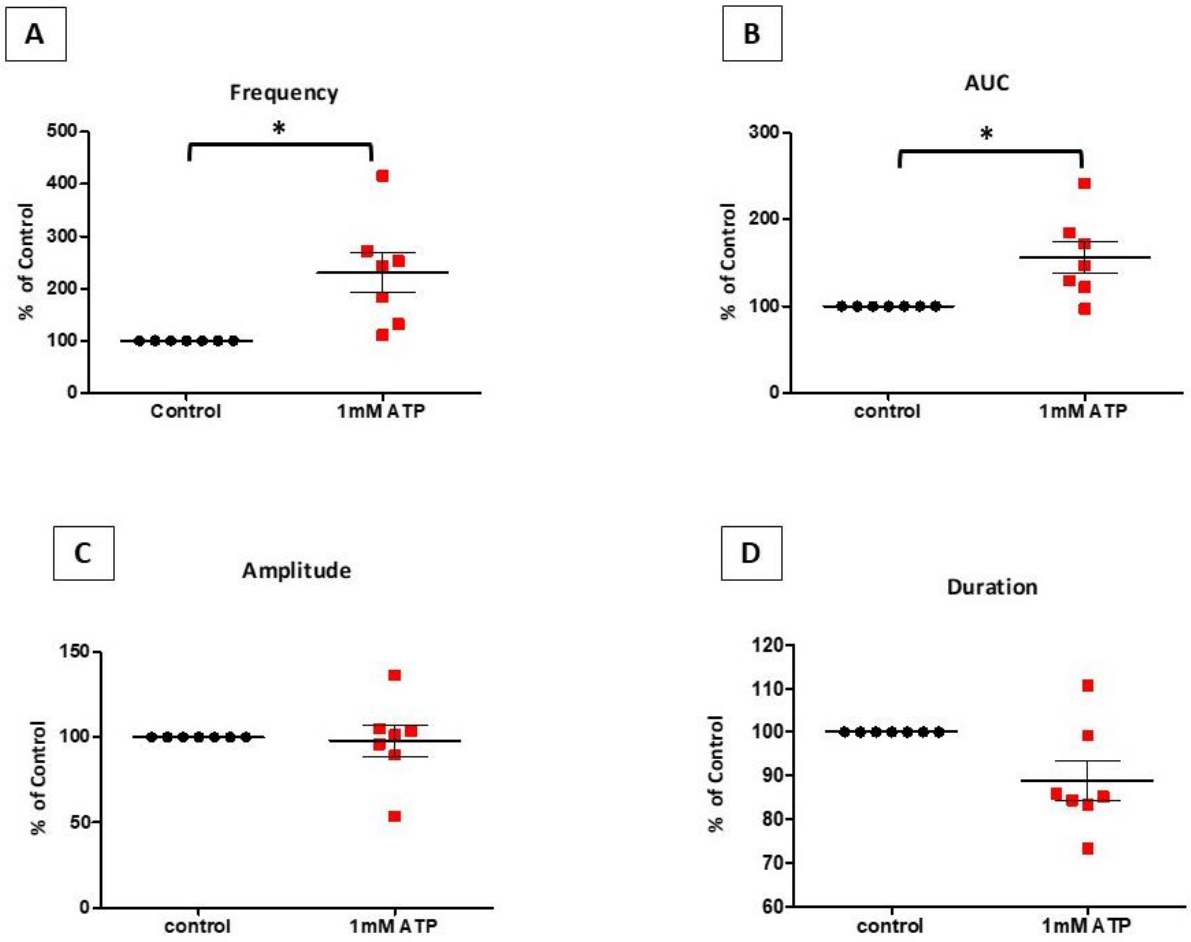


Figure 3.5: The effects of ATP on the parameters of non-labouring human myometrium contraction. Scatter plot showing the effects of 1mM ATP on **A**: frequency, **B**: the area under the curve (AUC), **C**: amplitude and **D**: duration. The horizontal line through the data points represents the mean. Significance was tested using a one sample t-test, n=7.

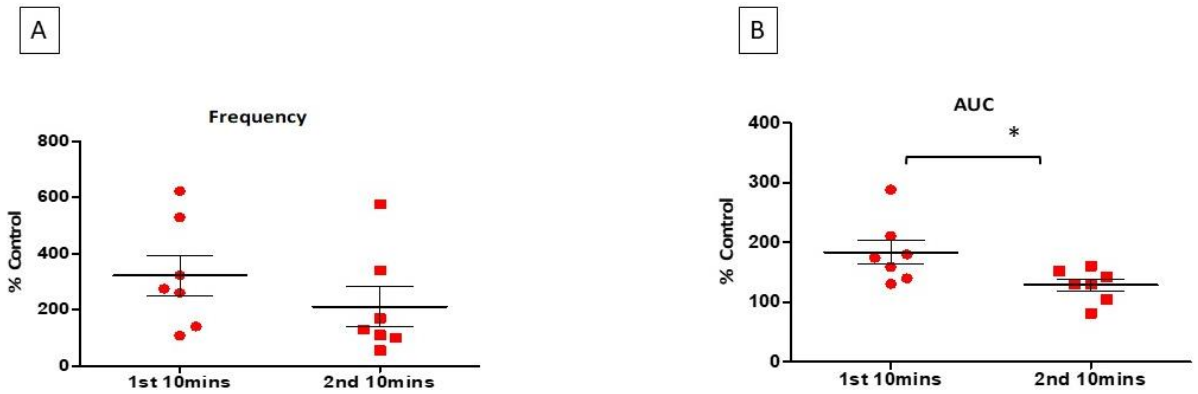


Figure 3.6: Time-dependent effect of ATP on non-labouring human myometrial contraction. Scatter plot showing the time dependent effect of 1mM ATP on contractile frequency (A) and AUC (B) over 20 mins period of application which was divided into two 10 mins sections, each analysed separately. The 2nd 10 mins of AUC was decreased significantly compared to the 1st half. Significant difference was found using a paired student t-test.

3.3.2 The concentration response to ATP in the non-labouring human myometrium.

After obtaining clear data showing the effect of 1mM ATP, concentration-response curves were obtained to generate an EC_{50} value for ATP. Each concentration of ATP was applied for 20mins.

Initially a concentration range of 10µM to 1mM was tested (n=5, **Figure 3.7A)**. As a maximal response was not defined, the concentration range was altered to include higher concentrations (300µM to 10mM, n=5, **Figure 3.7B)**. ATP produced a concentration-dependent increase of contractile frequency but the amplitude of contraction fell gradually towards 10mM as shown in **Figure 3.7B**, and contractions recovered to control values when ATP was removed.

Analysis of these data showed that starting from concentrations of 10µM and above there is an increase in frequency and decrease in amplitude towards 10mM but these changes do not achieve significance. Also, the AUC was not significantly affected as shown in **Figure 3.8A-C.** Unfortunately, from the concentration-response curve of frequency, the EC_{50} for ATP could not be calculated, as the maximum response was not achieved.

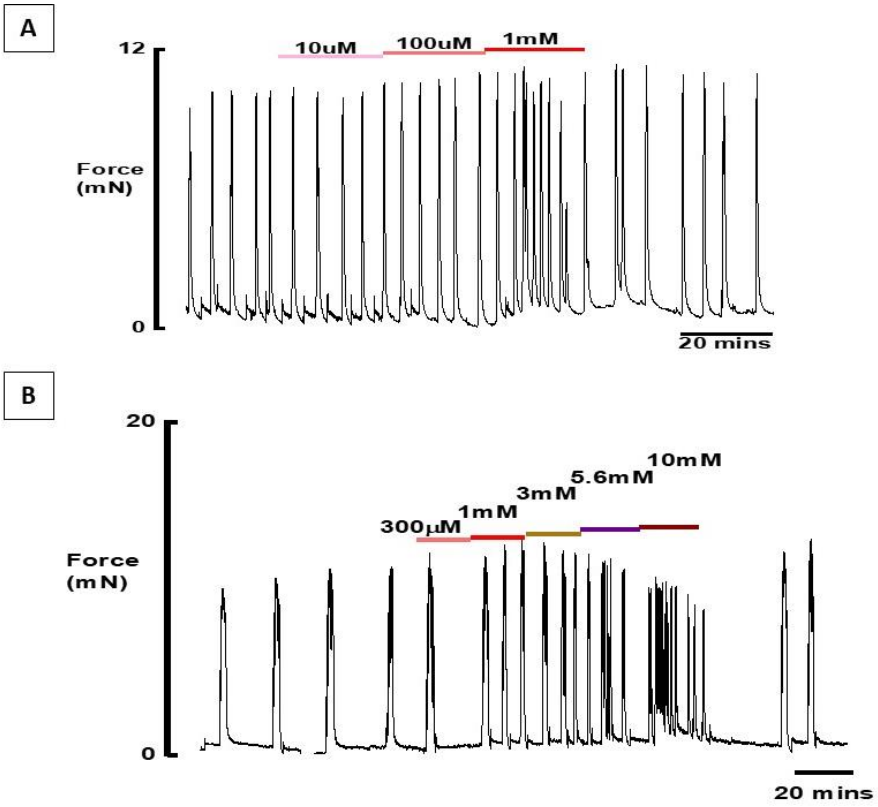


Figure 3.7: Cumulative concentration-response curves to ATP in term non-labouring women. Typical traces represent the concentration dependency of spontaneously contracting myometrial strips during 20- or 30-minute applications of (**A**) 10µM-1mM ATP, and (**B**) 300µM- 10mM ATP.

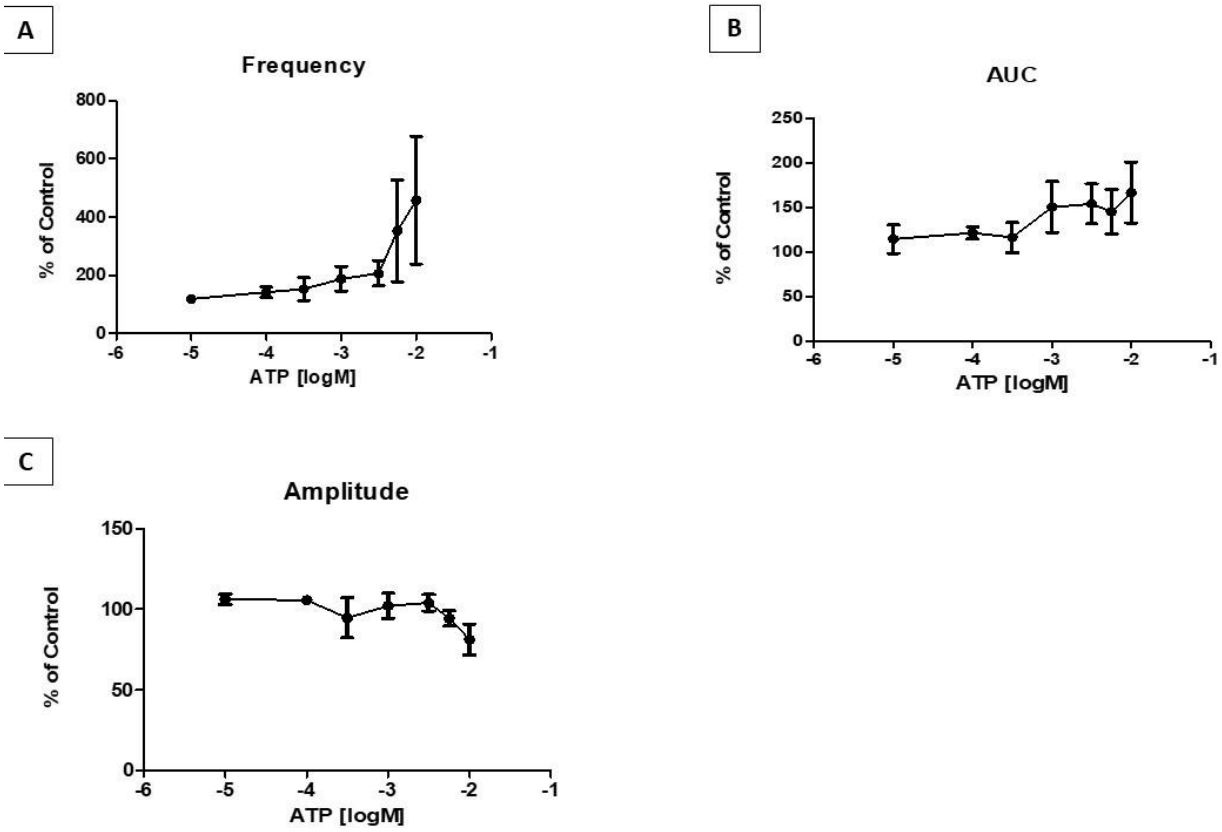


Figure 3.8: **The effects of cumulative concentrations of ATP on the parameters of contraction**. The concentration-response curves showing the effect of ATP on frequency (**A**), AUC **(B)** and amplitude (**C**) (n= 5). The data for frequency illustrated that the maximum response could not be defined, therefore, the EC_{50} value was not calculated.

3.3.3 The concentration response to ATPγS in the non-labouring human myometrium

The effects of extracellular application of ATPγS on contractility (1µM-1mM, 20 minutes for each concentration) were determined (n=5). At concentrations of 10µM and below, ATPγS had no effects on mechanical activity of the isolated uterine strips. ATPγS at 100µM and 1mM caused a clear effect on frequency, **Figure 3.9**, which reached significance at 1mM (430.3±118.2%, P=0.005, n=5, **Figure 3.10A**). 1mM ATPγS induced a significant enhancement of AUC, (294.7± 62.9%, P= 0.003, n=5, **Figure 3.10B**) when compared to the preceding control period. There was no significant change found in the amplitude of contraction.

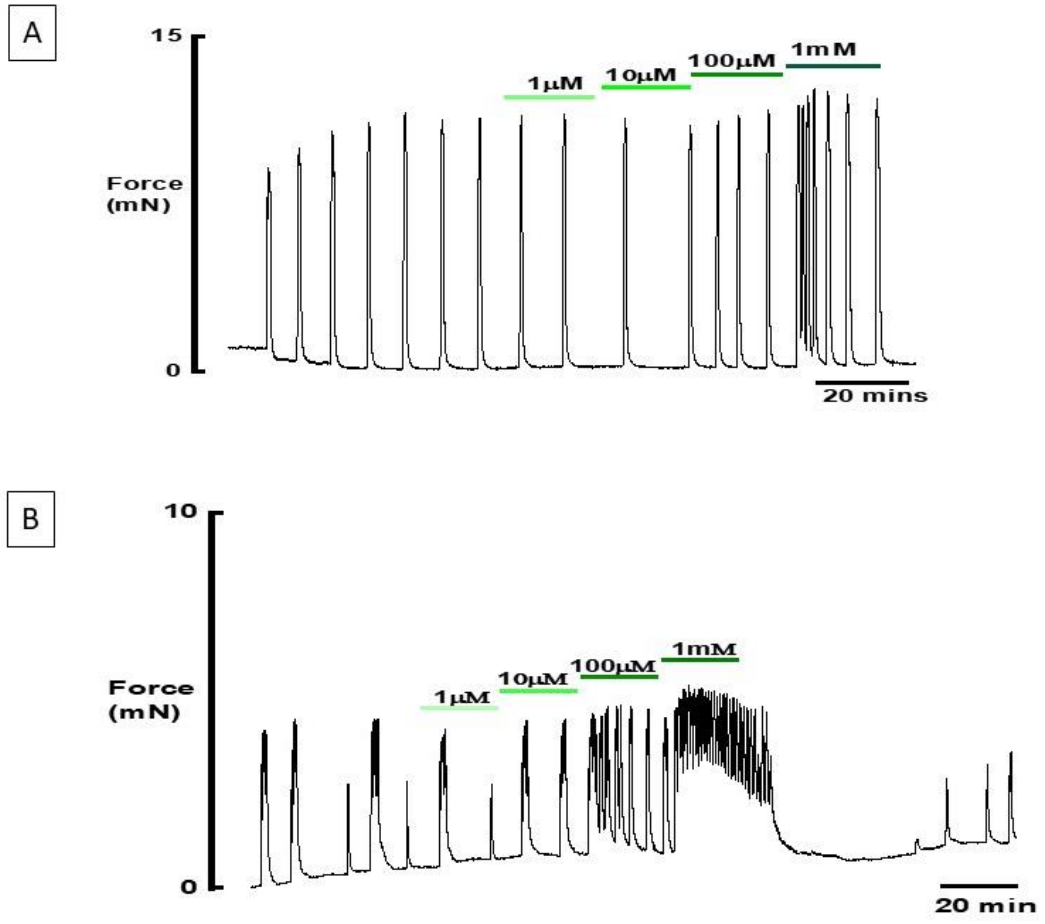


Figure 3.9: Cumulative concentration-response curve to ATPγS in term non-labouring women. Typical traces showing the effect of cumulative ATPγS concentration 1µM-1mM on spontaneously contracting uterine strips (A). In 1/5 preparations the frequency became tonic at 1mM (B).

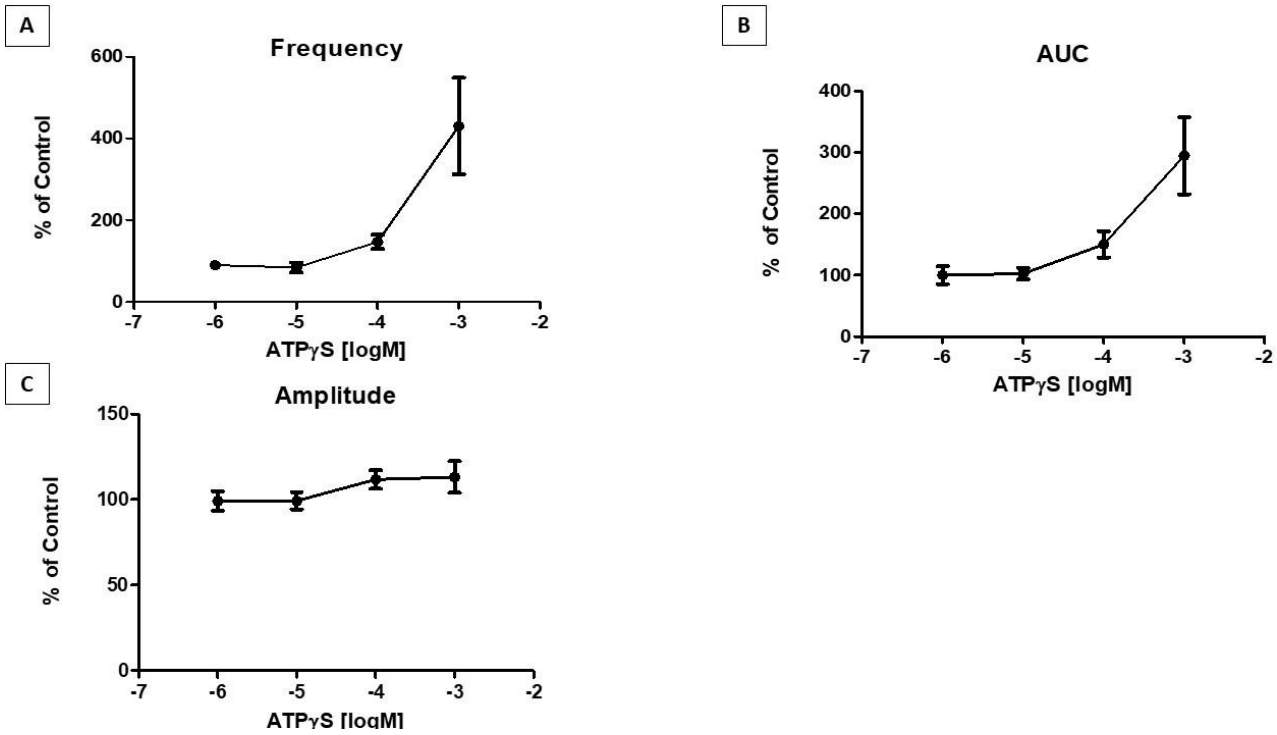


Figure 3.10: The effects of cumulative concentrations of ATPγS on the parameters of human myometrial contraction.

The concentration-response curves showing the effects of ATPγS on the frequency **(A)**, AUC **(B),** and amplitude **(C)** of contraction **(**n=4-5). A significant difference in activity was found using Repeated Measures ANOVA with Bonferroni post-hoc test.

3.3.4 The Concentration response to BzATP in the non-labouring human myometrium

Cumulative concentrations of BzATP (1µM-1mM) were applied for 20min (n=4, **Figure 3.11**). At concentrations of 10µM and below, BzATP had no effects on mechanical activity of the myometrial tissue. The frequency response to 1mM BzATP was significant (262.3± 60.7%, P=0.005, n=4). 1mM BzATP induced a significant enhancement of AUC, (156.1± 27.9%, P=0.01, n=4, **Figure 3.12**) when compared to the preceding control period. There was no significant change found in the amplitude of contraction.

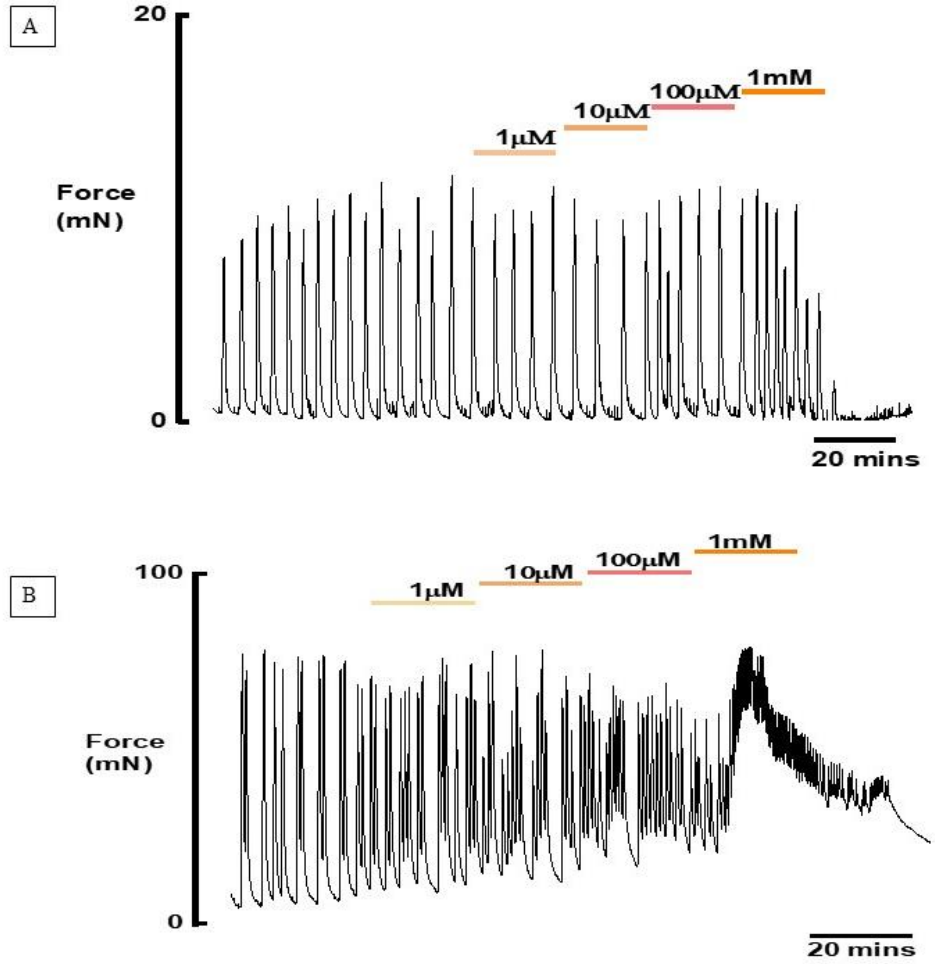


Figure 3.11: Cumulative concentration-response curve to BzATP in term non-labouring women. Typical traces showing the effect of cumulative BzATP concentrations (1µM-1mM) on spontanously contracting uterine strips from term non- labouring women (A). In 1/4 preparations the frequency became tonic at 1mM (B).

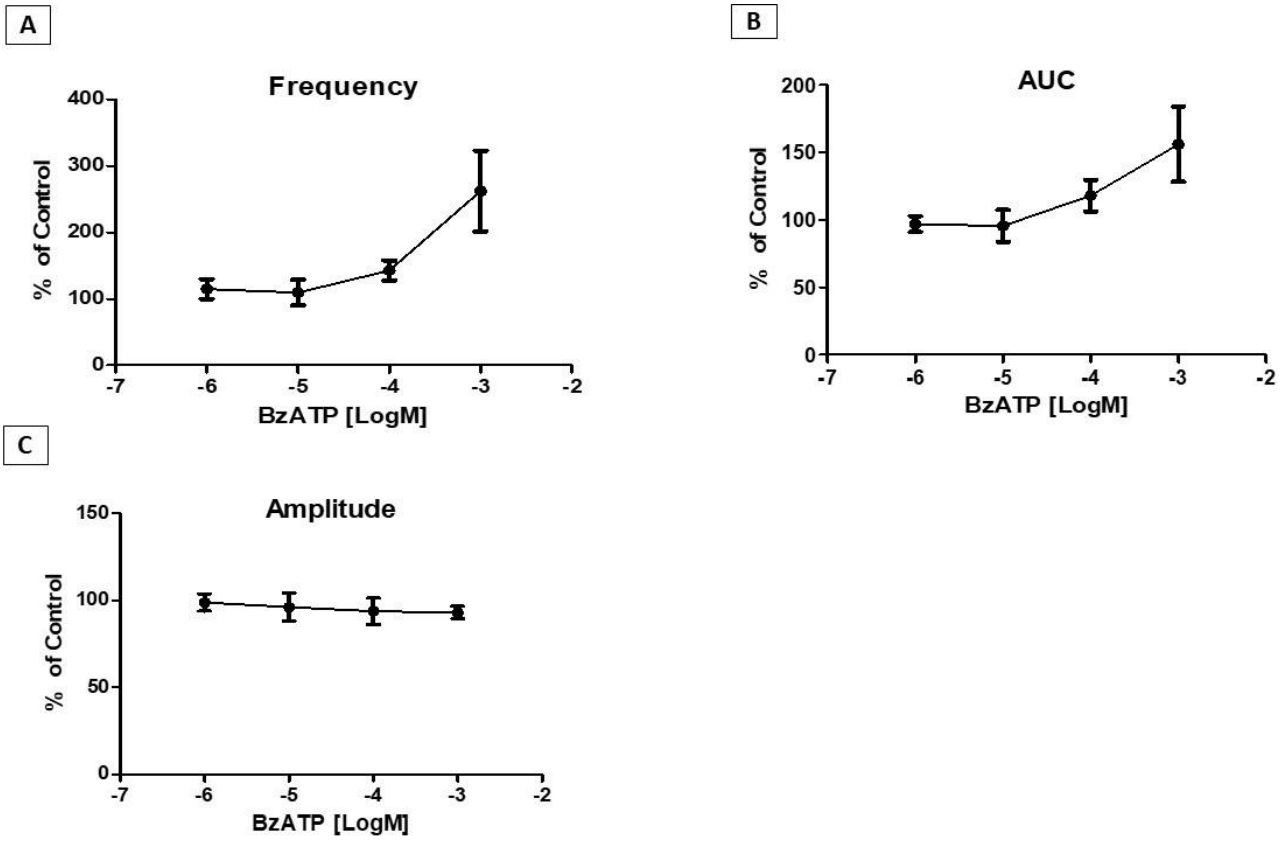


Figure 3.12: **The effects of cumulative concentrations of BzATP on the parameters of human myometrial contraction.** The concentration-response curves showing the effects of BzATP on frequency (**A**), AUC (**B**), and amplitude (**C**), (n=3-4). A significant difference in activity was found using Repeated Measures ANOVA with Bonferroni post-hoc test.

3.3.5 Comparative effects of agonists

As full concentration-response curves and EC_{50} data were not be obtained for ATP, ATPγS and BzATP, the rank order of potency could not be defined. Instead, the effect of a single concentration of ATP, ATPγS and BzATP (1mM) on myometrial response was compared. The three agonists were administered to spontaneously contracting tissue at a single concentration that gave clear effects (1mM for 20 minutes, n=6-9, **Figure 3.13A(i,ii&iii)**). The responses to a single 1mM concentration of ATP have already been described above (**Figure 3.4 & 3.5**).

Figure 3.13 illustrates that the application of 1mM BzATP and ATPγS for 20 mins increased contractility in term pregnant myometrium. With ATPγS, the contractions tended to become tonic, although contractions were still visible on top of this elevated baseline. This rise in basal tension with ATPγS was significant (3.3± 0.7mN, n=6, P=0.008) compared to the pre-treatment control period. For BzATP, as seen with ATP previously, changes in baseline tension were not significant (1.3±0.6mN, n=9, P=0.08). Application of 1mM BzATP and ATPγS increased contractile frequency significantly (BzATP: 250.2±38.8%, n=9, P=0.004 and ATPγS: 656±102.4%, n=6, p=0.0001), which then started to return to control values in their continued presence (**Figure 3.13B(ii)**). Consequently, the increase in AUC for 1mM ATPγS (349±60.2%, n=6, P=0.009) was significantly higher than the responses generated by BzATP (209.5±30.2%, n=9, P=0.005, **Figure 3.13B(iii**)) or ATP (156.3±18.1%, n=7, P=0.02, **Figure 3.13B(iii**)). There was no significant change in amplitude following the agonists' application. Contractions returned to pretreatment values after washing, taking the longest to recover with ATPγS. The current findings ranked the effectiveness of ATP analogues in the order ATPγS>BzATP>ATP, as shown in **Figure 3.13**.

It can be seen from **Figure 3.13A(ii&iii)** that the effects of 1mM BzATP and ATPγS on the frequency and the AUC of contractions were transient, and they returned to baseline values within approximately 10 min of their application, similar to what has been observed with ATP (**Figure 3.13A(i)**). As shown in **Figure 3.14B**, the effect of BzATP on AUC in the 2^{nd} 10mins were significantly less than in the 1^{st} 10 mins (159.5±19.9 and 264.6±51.2, P*<0.01), respectively. However, no significant difference was seen between the 1^{st} and 2^{nd} 10 mins of ATPγS application (395.9±67.8 and 305±64.7, P= 0.13, **Figure 3.14C**).

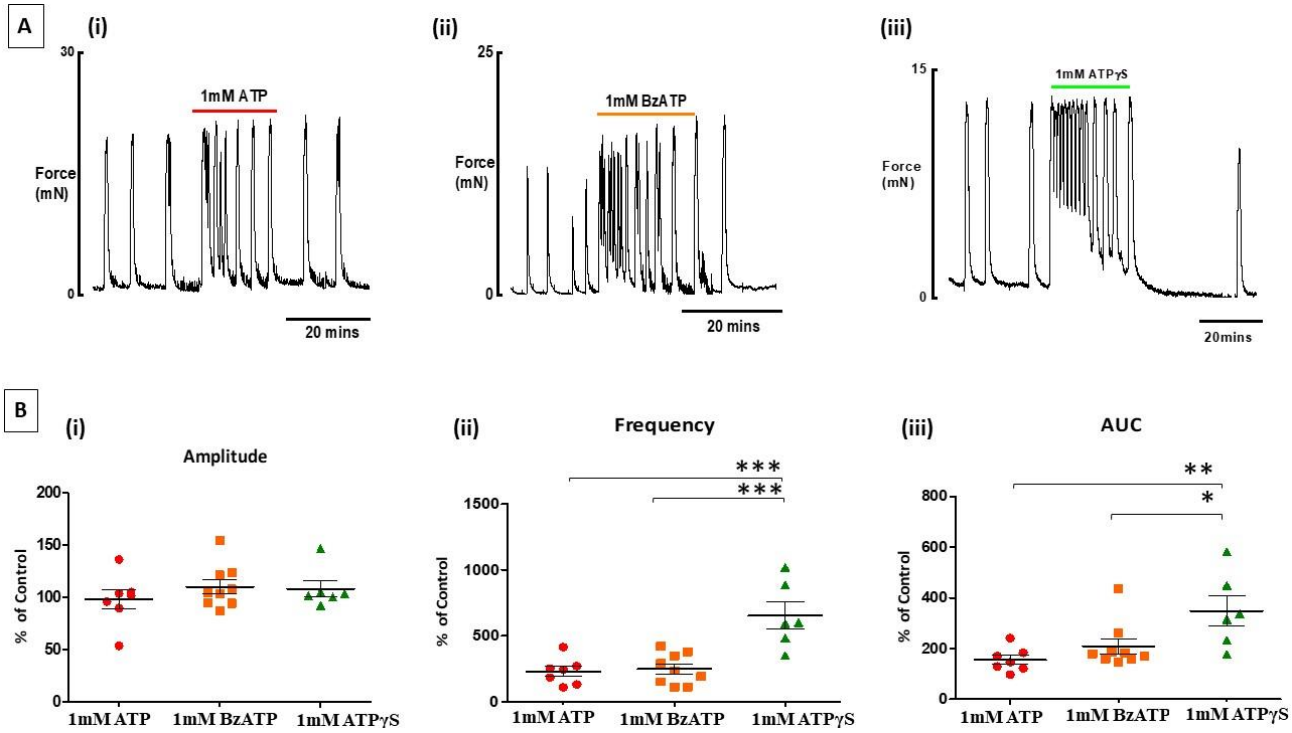


Figure 3.13: A comparison of the response to ATP and its analogues

(**A**) Typical traces showing the effect of 1mM ATP (i), BzATP (ii), and ATPγS (iii) (20 minutes) on term pregnant uterine contractility (**B**) Scattered plot showing the effect of ATP (red symbols), BzATP (orange symbols) and ATPγS (green symbols) on contraction amplitude (**i**), frequency (ii) and AUC (iii) (n=6-9). A significant difference in activity was found using One-way ANOVA with Bonferroni post-hoc test, * denotes $P<0.05$, ** $P<0.005$ and *** $P<0.0005$.

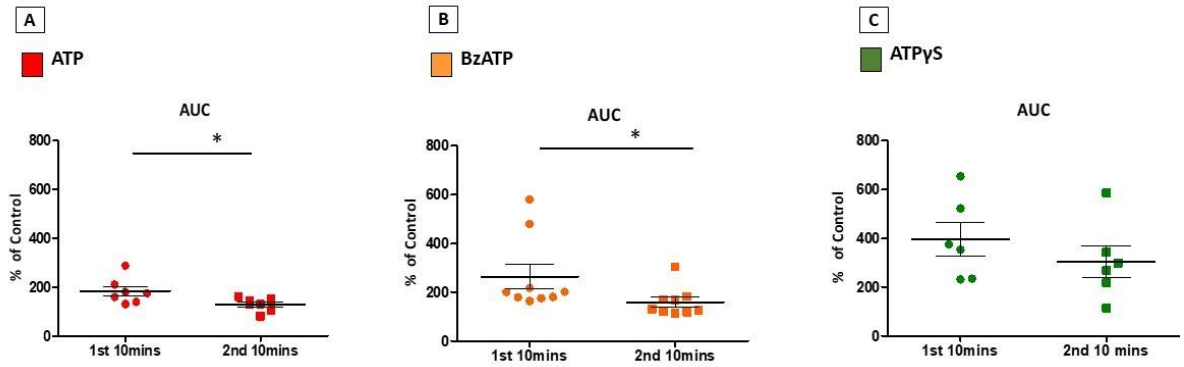


Figure 3.14 Time-dependent effect of ATP and its analogues on non- labouring human myometrial contraction. Scatter plot showing the time-dependent effects of 1mM ATP (**A**), BzATP (**B**) and ATPγS (**C**) on AUC over 20 mins period of application, which was divided into two 10 mins sections, each analysed separately. The 2nd 10 mins of AUC was decreased significantly compared to the 1st 10 mins in ATP and BzATP. There was no significant difference between the 1st and 2nd 10 mins in ATPγS. Significant difference was found using paired student t-test in ATP & ATPγS while Mann Whitney test used in BzATP. * Denotes a significance $P<0.05$.

3.3.6 The Effect of ATP on uterine contractility in the presence of selective P2X7 Purinoceptor Antagonists A-438079 and A-740003.

The data clearly showed that ATP has a stimulatory effect on human myometrial contraction, but which subtypes of P2X receptors in human myometrium mediates this is still unknown. The involvement of P2X7 purinoceptors in the stimulatory effect of extracellular ATP action, was assessed by using two selective P2X7R blockers, A-438079 and A-740003.

As these antagonists are prepared in DMSO, vehicle control experiments were carried out to determine the effect of the vehicle on contraction. There was no effect of DMSO on spontaneous contractions until reached to concentration of 1%, **Figure 3.15 A**. Therefore, in all antagonist experiments that follow, a DMSO concentration of 0.1% was not exceeded**.**

1) The Effect of ATP on uterine contractility in the presence of A-438079

Previous studies conducted on rat myometrium have used A-438079 at 10µM to block P2X7R (Alotaibi, 2018); (Miyoshi et al., 2012) and this is the concentration that initially was used in the current study. 10µM A-438079 was perfused into the organ bath for 40mins before applying 1mM ATP. A-438079 caused a non-significant decrease in frequency of the spontaneous contractions (80.6±7.5%, n=5, p=0.6, **Figure 3.15B&E**). It did not significantly

alter the response to 1mM ATP (see Table 3.2, **Figure 3.16A&B**). As this concentration of A-438079 did not show an effect on the myometrial contractile activity induced by 1mM ATP, a higher concentration of 50µM was tested. The 50µM A-438079 was perfused into the organ bath for 40mins before applying 1mM ATP. A-438079 caused a small, but non-significant decrease in frequency of the spontaneous contractions (92.9±4.8%, n=8, p=0.1 **Figure 3.15C&F**). It did not significantly alter the response to 1mM ATP (see Table 3.2, **Figure 3.16A &C**)

2) The Effect of ATP on uterine contractility in the presence of A-740003

A-740003 is known to be a highly specific and potent inhibitor of human P2X7 receptors with IC_{50} value at 40nM (Honore et al., 2006). A relatively high concentration of A-740003 was chosen to test, 10µM, since the previous antagonist, A-438079 at 50µM was unable to significantly reduce the stimulatory effect of ATP, at the concentrations expected. The 10µM A-740003 was perfused into the organ bath for 40mins before applying 1mM ATP. A-740003 caused a small, but non-significant decrease in contractile amplitude, frequency and AUC of the spontaneous contraction (85.4±10.3%, p=0.2, 89.1±17.5%, p=0.5 and 66.1±13.7%, p=0.09, n=4, respectively, **Figure 3.15 D&G**). It did not significantly alter the response to 1mM ATP (see Table 3.2, **Figure 3.16 A&D**).

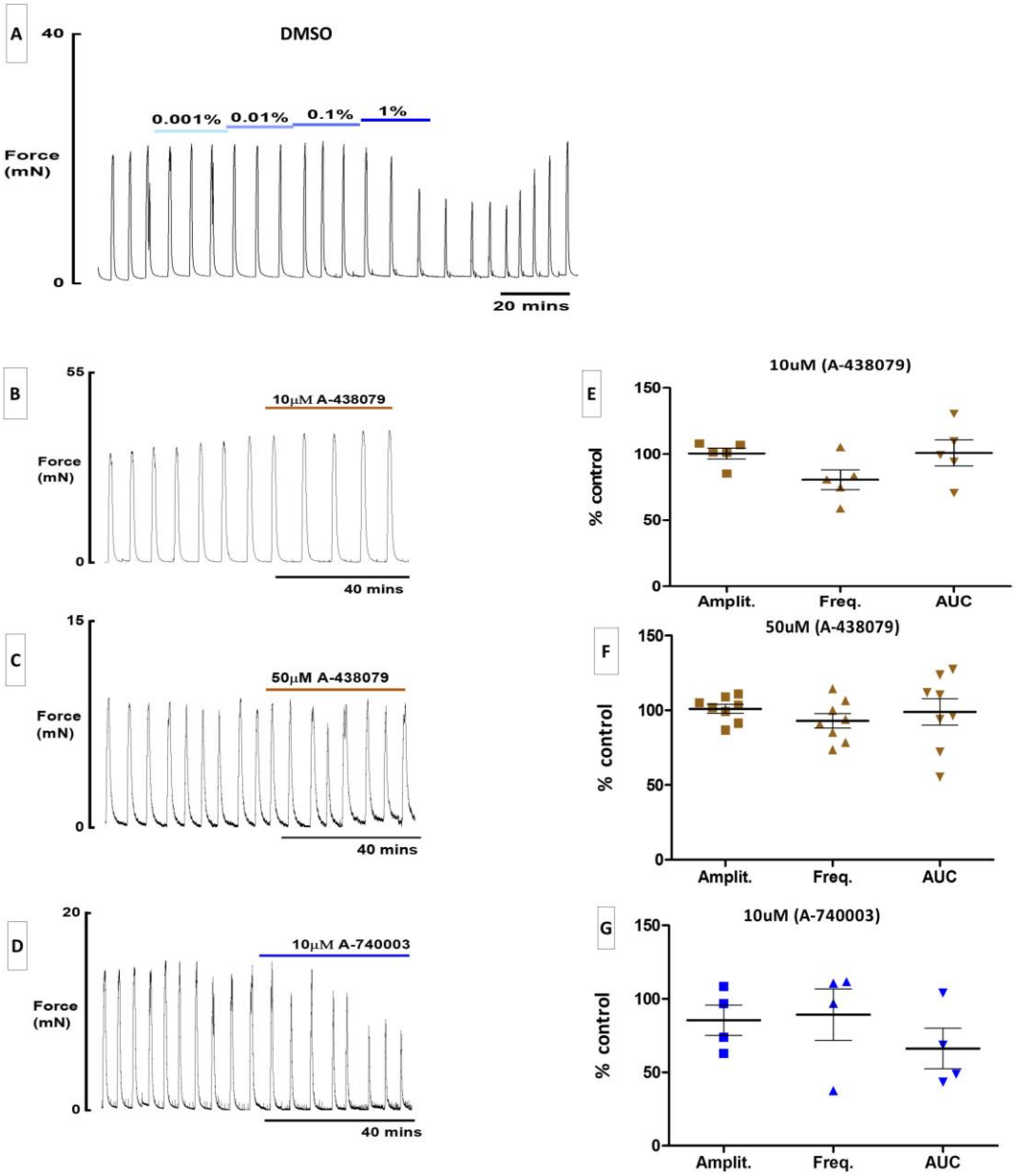


Figure 3.15: Effect of the vehicle control and selective P2X7 antagonists on spontaneous contraction of term non-labouring women. Typical trace showing the effect of the vehicle (DMSO) on spontaneous contractions (A). The short-coloured bars indicate the 20 minutes period where increasing concentrations of DMSO were added (0.001%, 0.01%, 0.1%, 1%). Typical traces represent original recording for the effect of 10μM, 50μM A-438079 and 10μM A-740003 on spontaneous uterine contraction in non-labouring myometrium (B, C &D). Scattered Plots in (E, F&G) represent the non-significant change in amplitude, frequency and AUC in the presence of 10μM, 50μM A-438079 and 10μM A-740003, respectively (n=4-8). Significance was tested using one sample t-test.

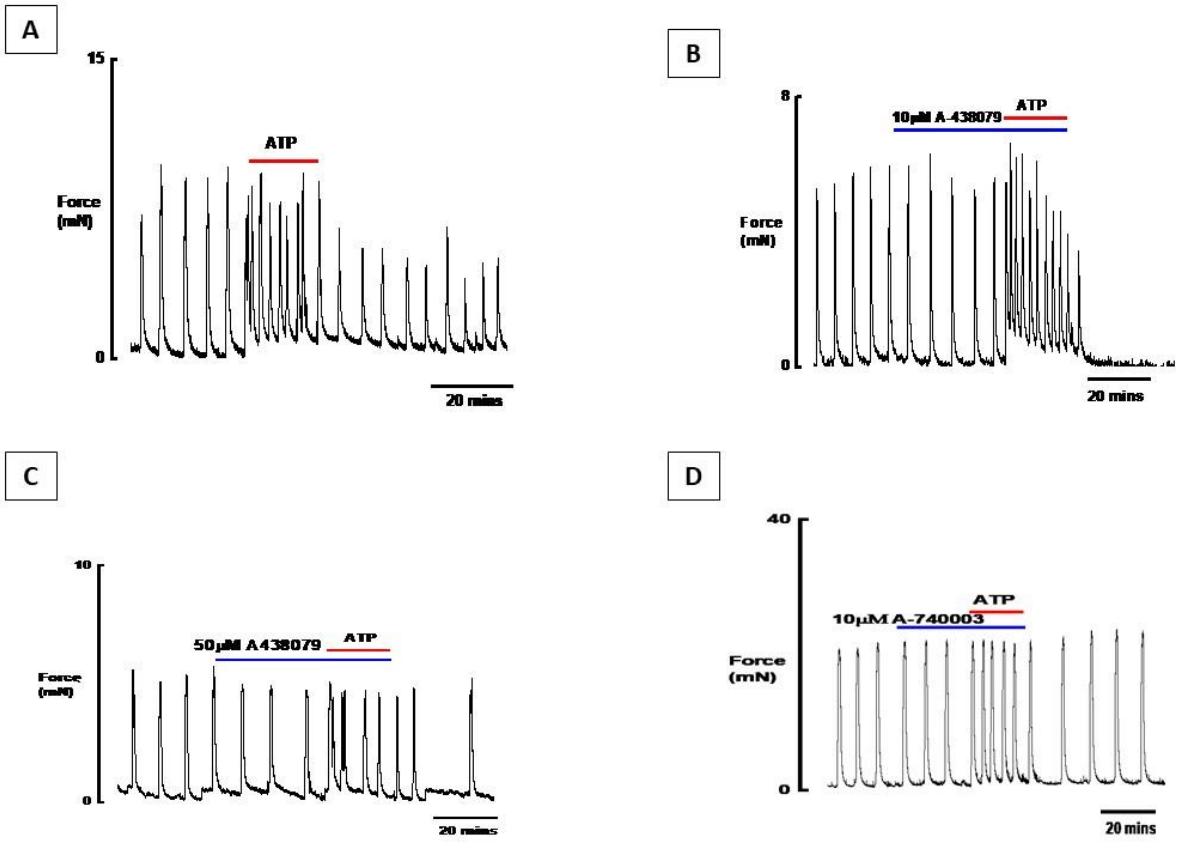


Figure 3.16: The response to ATP in the absence and presence of P2X7-selective antagonists. Typical traces of the effect of 20-minute application of 1mM ATP on uterine contraction in term-pregnant women, alone (**A**), in presence of 10μM A-438079 (n=5) (**B**), 50μM A-438079 (n=8) (**C**) and 10μM A-740003 (n=4) (**D**).

Table 3.2: Parameters of contraction in response to 1mM ATP, in the absence and presence of selective P2X7 purinoceptor antagonists in non-labouring human myometrium tissue.

	Contractile parameters (% of control)			
Treatment	Amplitude (Mean±SEM)	Frequency (Mean±SEM)	AUC (Mean ±SEM)	Baseline (mN ±SEM)
1mM ATP n=5	107.4± 1.6%	147.3± 32.6%	132.2± 16.7%	0.4± 0.07mN
10µM A-438079+ 1mM ATP n=5	102± 1.6%	190± 38.3%	168.8± 18.9%	0.5± 0.08mN
P-value	0.1335	0.4217	0.1858	0.2827
1mM ATP n=8	99.8±3.9%	286.1± 67.7%	174.4± 27.4%	0.6± 0.1mN
50µM A-438079+ 1mM ATP n= 8	94.7± 3.3%	203.8± 27.2%	150.3± 17.4%	0.5± 0.09mN
P-value	0.3422	0.2786	0.4710	0.9057
1mM ATP n=4	115.8±16.9%	428.8±119.8%	365±157.3%	0.60± 0.1mN
10µM A-740003+ 1mM ATP n= 4	109.9± 13.4%	355.2± 85.9%	280± 39.9%	0.66± 0.1mN
P-value	0.7947	0.6354	0.6188	0.7111

Mean data ± SEM of the contractile parameters (% of control) and raw mN ±S.E.M of the baseline showing no significant change in response to 1mM ATP, in the absence and presence of selective P2X7 purinoceptor antagonists in non-labouring human myometrium tissue. Significance level was determined by Unpaired t-test.

3.3.7 The Effect of BzATP on uterine contractility in the presence of selective P2X7 purinoceptor antagonist (A-740003).

As ATP acts through receptors other than P2X7, the effects of the selective P2X7 receptor blocker on the stimulation produced by the most potent P2X7 agonist BzATP was examined. 10μM A-740003 was perfused into the organ bath for 30 mins before applying 100μM BzATP. It did not significantly alter the response to 100μM BzATP (see Table 3.3, **Figure 3.17**).

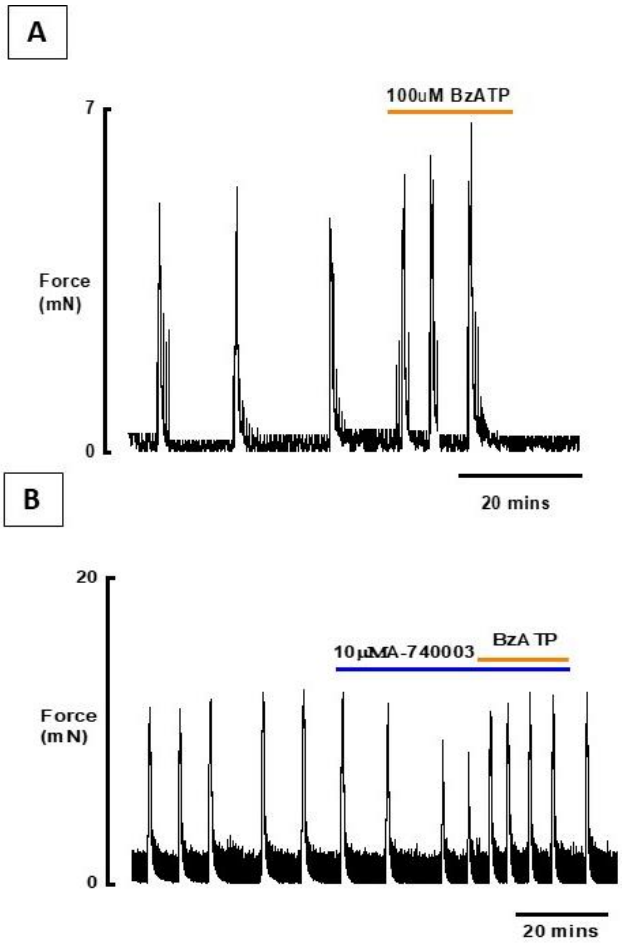


Figure 3.17: The response to BzATP in the absence and presence of A-74003.

Typical traces of the effect of 20minute application of 100μM BzATP on uterine contraction in term-pregnant women alone, (A) and in presence of the selective P2X7 purinoceptor antagonist A-740003 (10μM, n=7) (B).

Table 3.3: Parameters of contraction in response to 100µM BzATP, in the absence and presence of A-740003 in non-labouring human myometrium.

	Contractile parameters (% of control)			
Treatment	Amplitude (Mean ±SEM)	Frequency (Mean ±SEM)	AUC (Mean ±SEM)	Baseline (mN ±SEM)
100µM BzATP n=3	103.4±4.5%	241.8 ± 72.1%	199.4± 26.6%	0.4± 0.2mN
10µM A-740003+100µM BzATP n=7	100.6 ± 11.7%	210.4± 19.3%	181.3 ± 21%	0.2± 0.1mN
P-value	0.8833	0.5683	0.7942	0.3521

Mean data ± SEM of the contractile parameters (% of control) and raw mN ±S.E.M of the baseline showing no significant change in response to 100µM BzATP, in the absence and presence of A-740003 in non-labouring human myometrium tissue. Significance level was determined by Unpaired t-test.

3.4 Discussion

This study shows that the application of extracellular ATP and its analogues causes a significant increase in the frequency of contraction. This is consistent with them causing depolarization, presumably due to the entry of cations through the cation channel associated with the receptors. The three purinergic agonists caused concentration-dependent contractions with the rank order of ATPγS> BzATP>ATP. The study also shows that the data do not support a major role for P2X7R in human non-labouring myometrial responses to purinergic stimulation.

The uterus is sustained in a quiescent state throughout pregnancy to ensure the fetus remains *in utero* and can grow and develop. However, this quiescence is shifted to regular and progressive strong contractions at the onset of labour to deliver the baby and placenta. Any issues in this process might lead to dysfunctional labour, which ultimately ends by induction of labour using oxytocin or even an emergency CS (Wray, 2007). Unfortunately, 50% of women receiving oxytocin do not respond effectively to oxytocin and do not progress to a normal vaginal delivery (Bugg et al., 2008). Therefore, identifying alternative uterotonics such as ATP is of great importance.

The data available from the current study agrees with previous myometrial studies that extracellular ATP is a myometrial stimulant (Alotaibi, 2018); (Ziganshin et al., 2005); (Alotaibi et al., 2015);(Hutchings et al., 2009). Extracellular ATP also shows a concentration-dependent relationship to contractile frequency without affecting the amplitude. The increase in frequency suggests it is depolarizing the myocytes, consistent with the opening of its non-specific cation channel (NSCC) and allowing the influx of cations such as Na^+ and Ca^{2+} via binding to P2X receptors, which are ATP gated ion channels (Surprenant and North, 2009); (Burnstock, 2007). This depolarization in turn, would activate voltage-gated calcium channels to facilitate further Ca^{2+} influx, strengthening the depolarization, increasing the rate of action potential firing and hence, contraction frequency (Burdyga et al., 2007);(Wray et al., 2015).

Comparison of the AUC under ATP application between the initial 10 minutes of perfusion compared to the last 10 minutes was found to be significantly increased, which suggests a biphasic response. Reasons for this could be that the receptors are desensitizing to the agonist over time, or that ATP is being hydrolyzed and its metabolites do not stimulate the receptors. In collaborative, unpublished work conducted by a colleague, Masters student Erinn Melville

(whose project I co-supervised) identified a similar biphasic response of ATP in pregnant mouse myometrial tissue (n=9, **Figure 3.18A**). The biphasic response could essentially be that P2X receptors have a high binding affinity initially for ATP over its metabolites (Lohman et al., 2012). Using ATPγS, a non-hydrolysable version of ATP, allows testing the effect of ATP itself on myometrial contractions, before its degradation by ectonucleotidases. Interestingly, there were no significant differences between the 1st and the 2nd 10 mins of application of ATPγS. This supports my hypothesis that the biphasic response might be caused by an initial stimulation of ATP, which then diminishes over time as ATP is hydrolyzed (Young, 2000). It also suggests that the biphasic response is not likely to be due to receptor desensitization.

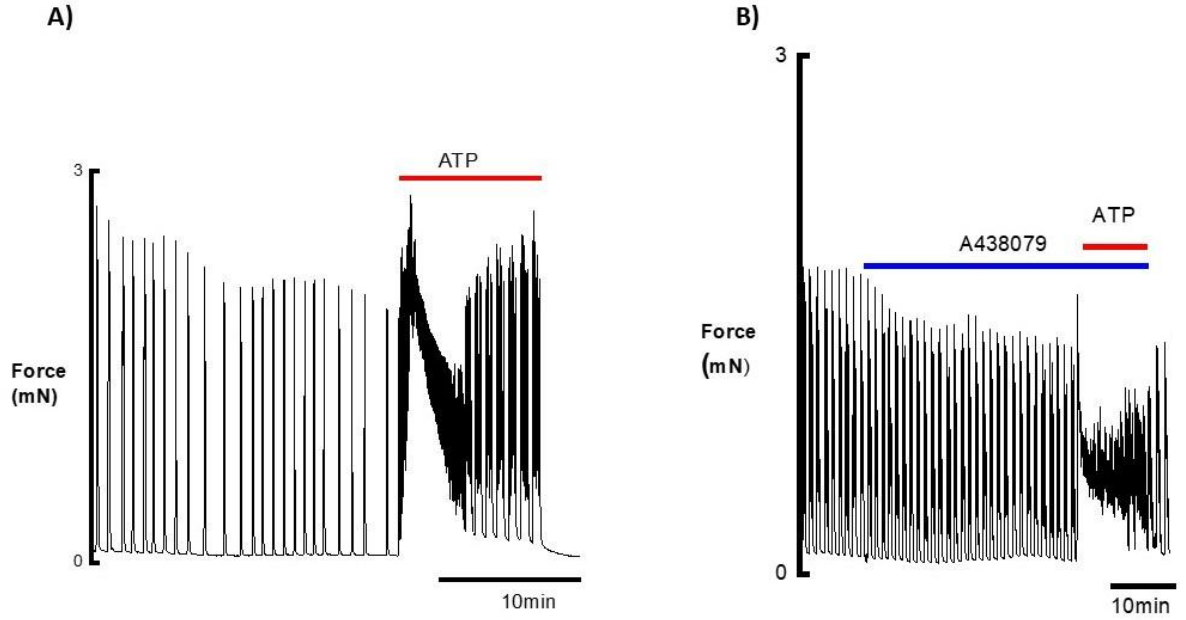


Figure 3.18 Effects of ATP on mouse spontaneous myometrial contractions in the presence and absence of a P2X7R antagonist. The trace recording A) shows the effect of 1mM ATP, alone, on term pregnant (18-day gestation) mouse myometrium, n=9. B) shows the effect of 40min A438079 [10μM] perfusion during perfusion of 1mM ATP for 10min in term pregnant tissue. Unpublished work: Erinn Melville, Hind Zafrah, and Susan Wray.

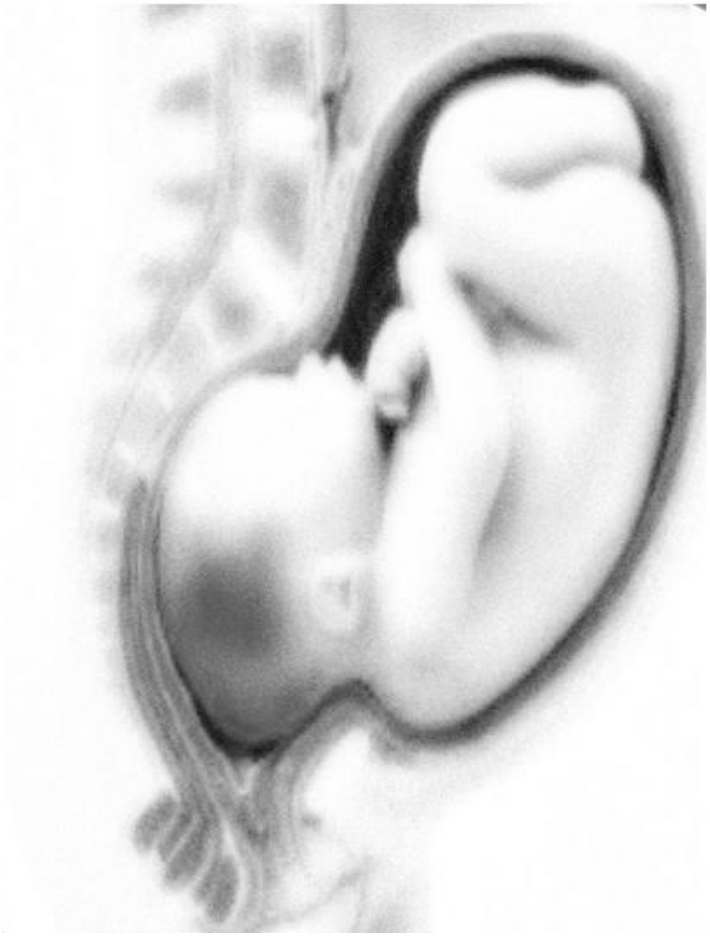
Taken together, it is concluded that the transient excitatory effect of ATP could have a role in initiating contractions and particularly initiation of labour (Hutchings et al., 2009). It has been shown in rats that the sensitivity to ATP increases towards term pregnancy (Zafrah and Alotaibi, 2017), and increased expression of purinergic receptors during the early stage of pregnancy such as P2X1 (Slater et al., 2000) or during term pregnancy such as P2X7 (Alotaibi, 2018) occurs in the myometrium. Therefore, the contribution of ATP in the onset of labour must not be ignored.

Since there is evidence of the role of P2X7R in pregnant rat uterine contraction (Alotaibi, 2018); (Miyoshi et al., 2012);(Miyoshi et al., 2010) , it is considered that these receptors could also be involved in the human pregnant uterus. Applying two specific and potent P2X7 receptor inhibitors, A438079 and A-740003 however, did not block the stimulatory effect of ATP on human uterine contractility. This would suggest either 1) there are species differences in P2X7 receptor sensitivity to antagonists, or 2) there are other purinergic receptors that mediate the action of ATP in human myometrium. The first suggestion was supported by collaborative unpublished work (Melville, Zafrah, and Wray) showing ATP-induced mouse uterine contraction was significantly reduced by using the same antagonist, A-438079 (10μM, **Figure 3.18A-B)**. Studies have shown that species might have minor variations in the receptor itself, which in turn affects antagonist binding and leads to species differences in P2X7 receptor sensitivity to antagonists (Honore et al., 2006);(Dayel et al., 2019). This interesting area needs further investigation. The failure of a potent P2X7R antagonist to inhibit the robust uterine contractions produced by the most potent agonist for P2X7R, BzATP in the current study, could again be due to species differences in the activity of this antagonist, or it might reveal the involvement of other receptors mediating this effect of BzATP in the myometrium. The notion that BzATP is a selective agonist for P2X7R has been widely reported, however, Bianchi and his team have concluded that using BzATP as a P2X7 receptor agonist in native tissues is complicated by its lack of selectivity and specificity, as they found it was 100- to 1000-fold more potent at the human P2X1 and rat and human P2X3 receptors than human P2X7R (Jacobson and Müller, 2016);(Bianchi et al., 1999). Furthermore, BzATP can bind to and activate other P2XR, such as P2X2 (Bhargava et al., 2012). This might explain the current study result of the greater effect on contractility with BzATP on human myometrial tissue compared to ATP which potentially means the presence of other P2X receptors mediating this additional activity (Bianchi et al., 1999).

The current results ranked the effectiveness of ATP analogues in the order ATPγS >> Bz-ATP >ATP, where ATPγS action suggests little role for ADP, AMP or adenosine in the effects of ATP. ATP metabolites predominantly act via P1 purinergic receptors. This suggests that the P1 receptors are less involved in the stimulatory effect of ATP (Burnstock, 2018). ATPγS produced a significantly marked increase in uterine activity, which was not altered in the presence of P2XR antagonists and suggest that P2X7Rs have little involvement in its response. Instead, the effect may be attributed partly to the triggering of P2YRs in line with reports which have suggested that the uterus probably contains a mixture of P2-purinoceptors (Piper and Hollingsworth, 1996). The above rank order of stimulation of myometrial response is not matched with any single P2-purinoceptor subtype. As ATPγS was the agonist producing the largest effect in the non-labouring human myometrium, it is normally an agonist at P2X, P2Y1, P2Y2 and P2Y11 receptors (Lambrecht, 2000), the data suggests that P2Y-purinoceptors are present. However, BzATP produces the next largest response. It is normally the most potent agonist at P2X7, but it can also react with P2X1, P2X3 and P2Y1 receptors (Jacobson and Müller, 2016);(Vigne et al., 1999);(Allsopp et al., 2011). It is, therefore, likely that a mixture of purinergic receptors is present in the uterus. There have been reports of P2 purinoceptors co-existing in the non-pregnant guinea-pig myometrium (Piper and Hollingsworth, 1996). Airat U. Ziganshin has also suggested the presence of P2X and P2Y receptors in the human uterus, where activating P2X receptors causes uterine contraction while activating P2Y receptors causes uterine relaxation (Ziganshin et al., 2006). However, they did not recognize which P2Y receptor subtype could mediate the relaxant effect. Some P2YR are known to have an excitatory effect, and their stimulation could lead to Ca^{2+} release from the SR via PLC activation (Erb et al., 2015) and also activate PLD, PLA2, mitogen-activated protein kinase (MAPK) (Dubyak and el-Moatassim, 1993) which were all identified to be progressively upregulated in myometrium along with other important proteins during a late stage of pregnancy and labour (Breuiller-Fouche and Germain, 2006). On the other hand, P2X receptor subtypes such as P2X1, P2X4 and P2X7 have been reported to have upregulated expression in mammalian myometrial tissue in the preterm rat model (Urabe et al., 2009) during the pregnancy. They also found it to mediate the powerful contractile effect of the ATP in rat myometrial cells (Alotaibi, 2018);(Miyoshi et al., 2012). Therefore, diverse subtypes of P2X and P2Y receptors could be expressed in the pregnant uterus, and their functional expression might depend on the different gestational stages of the pregnancy.

In conclusion, the current studies have shown that ATP is a powerful myometrial stimulant that has the potential to induce uterine contractions in dystocic labours. The transient increase in frequency suggests it depolarizes the myocytes, consistent with the opening of its non-specific ion channel. Interestingly, human myometrium probably contains a mixture of P2-purinoceptors but not P1 purinoceptors as ATPγS action suggests little role for ADP, AMP or adenosine that act mainly via P1 purinergic receptors. The data do not support a role for P2X7R in the response of human myometrium to ATP. This finding contrasts with findings in the rat. Further research is required to investigate the role of other purinergic receptors in mediating the effects of ATP in human myometrium.

CHAPTER 4



Chapter 4: Investigating the expression of purinergic receptors, P2X1 and P2X7, in non-pregnant, term pregnant, non-labouring and labouring human myometrium

4.1 Introduction

The onset of labour is initiated with synchronised and coordinated uterine contractions. These contractions become stronger and more frequent when labour progresses to the stage where the baby and placenta are delivered. The excitatory effects of ATP on uterine contractility open a window for researchers to consider it as a new stimulant for successful uterine activity during difficult labours (Alotaibi, 2018); (Ziganshin et al., 2005). A previous study on isolated uteri of non-pregnant guinea pigs proposed that myometrial tissue contains a mixture of P2 receptors (Piper and Hollingsworth, 1996). Data from experiments using single myometrial cells from pregnant rats suggests that uterine contraction is generated through the activation of P2X7 receptors (Miyoshi et al., 2010). The same group later confirmed that the P2X7R carried ATP-induced currents in rat myometrial cells (Miyoshi et al., 2012). Also, P2X7R expression has been shown to increase in the myometrium of pregnant rats in a preterm delivery model (Urabe et al., 2009). Furthermore, Alotaibi demonstrated that P2X7R was expressed in nonpregnant rat uterine tissues, and their expression was progressively upregulated towards term, which support their contribution to ATP-induced uterine contraction (Alotaibi, 2018). On the other hand, experiments carried out on preparations of isolated pregnant human uterus taken during planned CS have suggested that P2X1 might be involved in the contractile responses to ATP and other agonists (Ziganshin et al., 2003). However, they did not check for P2X7R. Immunohistochemistry was used to describe the expression pattern of P2X receptors in the non-pregnant rat reproductive organs and found that P2X1 receptor expression was determined within the perifollicular and vascular smooth muscle of the ovary as well as the uterus (Bardini et al., 2000). P2X1R immunoreactivity has been documented in early pregnant uterine epithelia (Slater et al., 2000); (Vial and Evans, 2001). Khanam et al. in their immunolabeling study in rat myometrium have reported that Cx43 expression, gap junction protein, is markedly increased during the time of parturition which may reflects their significant role in the synchronized and coordinated uterine contractions at the onset of labour. However, they found the expression of P2X1 receptors peaked on the first day postpartum and suggested that this might mediate the ATP effect to

inhibit expression of Cx43 at one day postpartum, hence diminishing gap junctions and preventing coordinated contractions (Khanam and Burnstock, 2007).

Current studies therefore suggest that there is an upregulation of P2X receptors in the rodent uterus at a late stage of pregnancy and the enhanced response to ATP during labour indicates a critical role for them in successful labour. Findings from animal myometrium could be assumed to occur in human myometrium. However, there are few data examining the receptors involved in mediating the contractile responses of the human myometrium to extracellular ATP, and no research to date in humans has been performed to determine the P2X receptor subtypes that may contribute to ATP-induced contraction during the onset of labour.

4.1.1 Aims of the study

Given their potential importance in response to ATP, the aims were, for the first time, to use western blot and immunohistochemistry techniques to characterise the presence and tissue distribution of P2X1 and P2X7 receptors in human myometrium at different gestational and physiological states. It was hypothesized that there would be differences in expression between the four groups of myometrial samples: non-labouring term pregnant, labouring with fetal distress, labouring dysfunctionally and non-pregnant women. Samples for women with CS for foetal distress were considered as the control group as they were expected to be closest to normal labour.

4.2 Methods

Human myometrial tissues were taken from:

1) non-pregnant women undergoing hysterectomy.

(2) term pregnant women with normal labour but undergoing emergency CS deliveries due to fetal distress.

(3) term pregnant women in labour undergoing emergency CS deliveries due to dysfunctional labour.

(4) term pregnant non-labouring women undergoing elective CS deliveries.

A total of 30 biopsies of human myometrial tissue were used for the immunohistochemical study (IHC). The demographic data on the samples used are given in Table 4.1. The reason for hysterectomy (n=3) were menorrhagia and blocked fallopian tubes. For C-section delivery of non-labouring (n=10) women, the causes were, breach fetal presentation and previous C-section. The causes for C-sections in dysfunctional labour were failure to progress and stalled cervical dilation (n=9), and in normal labour, were Cardiotocography (CTG) outside normal limits (n=8).

In the western blot study (WB), a total of 22 biopsies of human myometrial tissue were used. The demographic data on the samples used are given in Table 4.1. The reasons for hysterectomy (n=3) were menorrhagia and blocked fallopian tubes, while the causes for C-section delivery of non-labouring were breach fetal presentation and previous C-section (n=10), and the causes for C-section in dysfunctional labour were failure to progress in labour (n=6) and in normal labour was CTG outside normal limits (n=3). All data relating to the samples were collected from the Liverpool Women's Hospital Research Tissue bank. And we were blinded to it until all the work had been completed and analysed.

Details of how the tissue was collected, prepared, fixed, embedded, sectioned, the IHC staining procedure and the scoring method were extensively described in the Methods Chapter. In the case of protein extraction and western blotting, uterine biopsies were immediately snap-frozen in liquid nitrogen on the collection and stored at -80^{o}C until sufficient samples were collected for the project. The western blot procedure is extensively described in the Methods Chapter.

4.3 Results

The demographic data shows that the women in the IHC study had a BMI within the normal range, whereas in the WB study were overweight on average (Table 4.1). As expected, women who had hysterectomies were older than the pregnant women. Nearly half of the recruited pregnant women were nulliparous, while the other half was distributed between primiparous or multiparous, with the vast majority having an elective caesarean section due to previous caesarean sections. Around 15 of the pregnant women had prolonged labour. In 10/15 of dysfunctional labour, samples were obtained from women who were nulliparous, which is expected; however, 5 women of them were primiparous.

Table 4.1 Summary table of characteristics of the study's sample.

Labouring

	IHC (n=9) Dysfun.labr	IHC (n=8) Norml.labr	WB (n=6) Dysfun.labr	WB (n=3) Norml.labr.
Mother's age (years: mean±SD)	29± 5	30± 6	31± 5	31± 4
Gestational age at delivery (days: mean±SD)	281.6±10.6	287.5±9.9	283.3±2.4	289.3± 6.4
BMI (kg/m^2: mean±SD)	22± 1	23± 1	25± 1	26± 3
Parity: para 0	n=5	n=4	n=5	n=3
para 1	n=4	n=4	n=1	n=0
para 2	n=0	n=0	n=0	n=0

Non-labouring women

	IHC (n=10)	WB (n=10)
Mother's age (years: mean±SD)	31± 4	32± 5
Gestational Age at delivery (days: mean±SD)	273.7±1.8	274.3±2.6
BMI (kg/m^2: mean±SD)	23± 1	25±1
Parity: para 0	n=2	n=2
para 1	n=4	n=6
para 2	n=4	n=2

Non-pregnant women		
	IHC (n=3)	WB (n=3)
Age (years: mean±SD)	38± 1	38±4
BMI (kg/m^2)	22	23± 5

Study sample characteristics of mother's age /year (mean± SD), gestational age at delivery /days (mean± SD), maternal weight/Kg (mean± SD) and parity.

4.3.1 P2X1 antibody titration

Initial titration experiments of the primary antibody were done to determine the minimum antibody concentration required to generate optimal IHC results. The following antibody concentrations were used for non-pregnant women myometrium P2X1 polyclonal antibody titration, 1:100, 1:200, 1:400 and 1:800 (**Figure 4.1A-D, respectively**). Figure 4.1E is the negative control which was stained with rabbit IgG immunoglobulin at (1:10000). A concentration of 1:400 was found to be optimal. The immunoreactivity of P2X1 in blood vessels (blue arrows) was determined, as seen in Figure 4.1F.

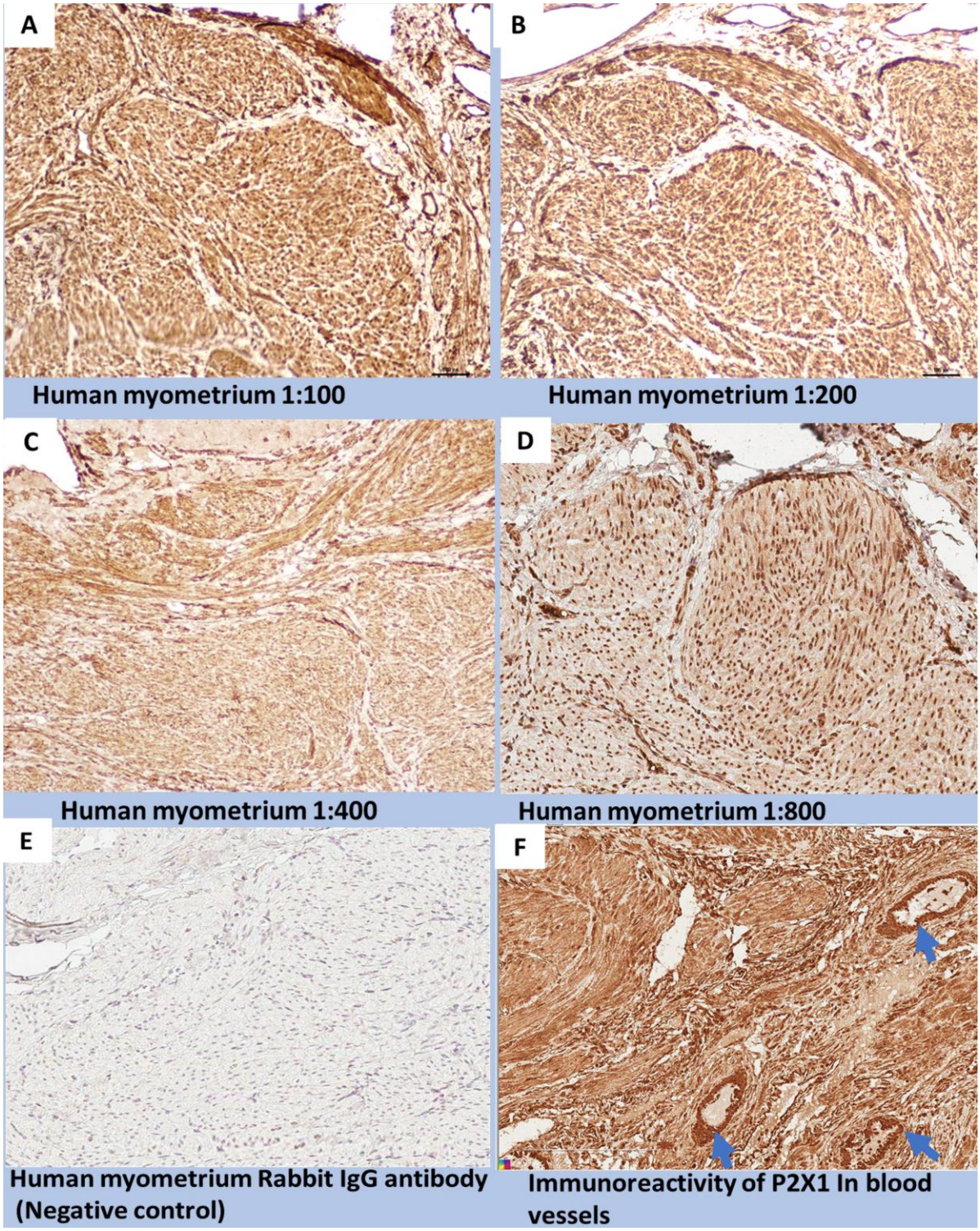


Figure 4.1: P2X1 antibody titration. (A-D) titration of the rabbit anti-P2X1 primary antibody concentration for IHC of human myometrial sections. (E) negative control of human myometrial section (Instead of primary antibody, rabbit IgG was used, 1:10000). (F) The immunoreactivity of P2X1 in blood vessels is indicated by blue arrows (5μM thickness, 20X).

4.3.2 Comparing the myometrial expression of P2X1R in non-pregnant, pregnant labouring and non- labouring women:

The expression of P2X1R was examined by DAB staining, which is equivalent to P2X1R expression. Myometrial sections were obtained from 30 women and examined by Aperio image scope software. There were 10 samples not in labour, 17 in labour either due to dysfunctional labour (n= 9) or fetal distress (n=8), and 3 samples of the non-pregnant uterus.

The myocytes are small and compressed in small bundles in the non-pregnant section compared to the term pregnant sections, which are hypertrophied and arranged in bigger bundles surrounded by thick connective tissue, **Figure 4.2A-D**. The distribution of the P2X1R was not determined whether it is confined to the myometrial plasma membrane - as expected for the receptor, even at higher magnification, 20X. Using a high-resolution immunofluorescence assay might help localize the receptors.

When the primary antibody (P2X1 antibody) was replaced with a rabbit IgG antibody, no staining was detected (**Figure 4.1E**).

Typical positive staining of myometrial smooth muscles is shown in **Figure 4.2A-D** as well as the blood vessels (blue arrow) in **Figure 4.1F**. Staining was detected in the myometrial tissue of all samples. The P2X1Rs were evenly distributed in all myometrial cells in all groups. The P2X1R myometrial staining intensity was not significantly different between non-pregnant, non-labour, normal or dysfunctional labour ($p>0.05$), **Figure 4.2E.**

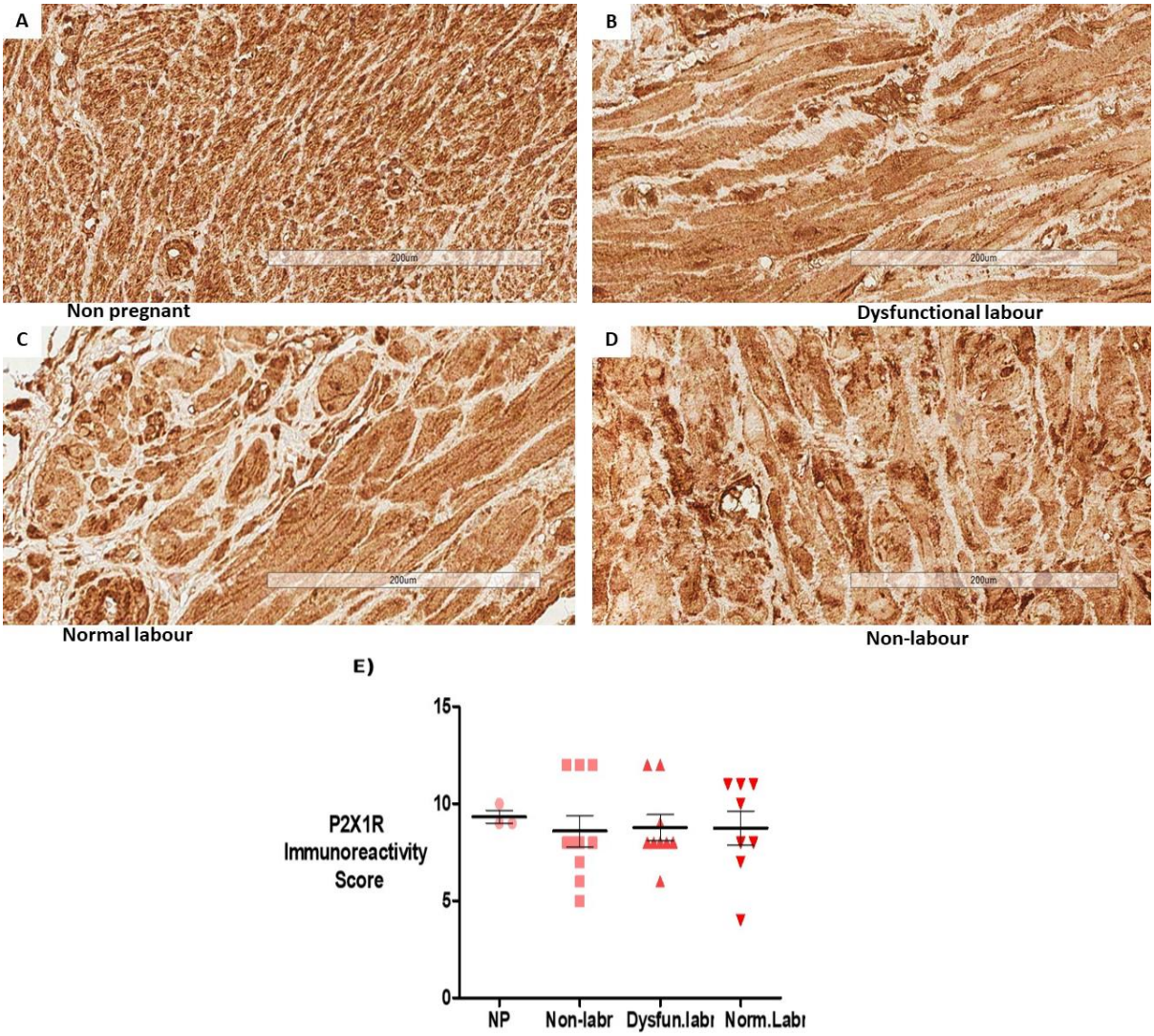


Figure 4.2 Immunoreactivity of P2X1 in the human myometrium. Immunoreactivity of P2X1 (1:400) is shown using DAB staining in myometrial tissue from A) non-pregnant NP, B) dysfunctional labour, C) Normal labour, and D) Non-laboring myometrial tissue. The mean immunoreactivity score in myometrium from non-pregnant NP, non-labour (Non-labr), dysfunctional labour (Dysfun.labr) and normal labour (Norm-labr) (E), 5µM thickness-20X.

4.3.3 P2X7antibody titration

Titration of the non-pregnant human myometrial P2X7R polyclonal antibody was performed to optimize antibody concentrations: 1:100, 1:200, and 1:400 (**Figure 4.3**A-C, respectively). Figure 4.3D shows the negative control stained with rabbit IgG immunoglobulin at (1:10000), and section 4.3E shows a mouse kidney stained with 1:200 antibody concentration (positive control). The antibody concentration of 1:200 was found to be optimal for staining. The immunoreactivity of P2X7 in blood vessels (blue arrows) was determined, as seen in Figure 4.3F.

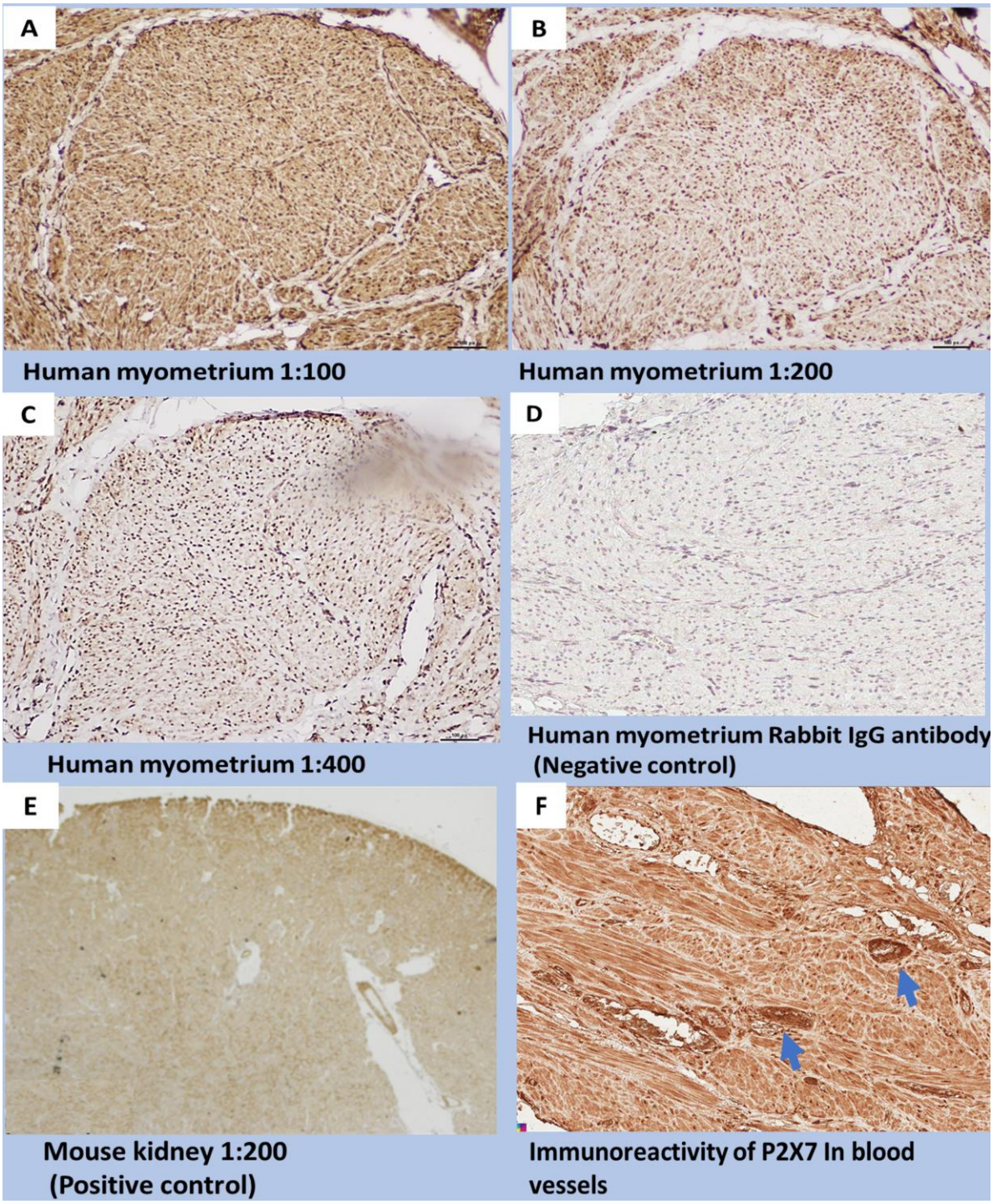


Figure 4.3: P2X7antibody titration. (A-C) represent titration of the rabbit anti-P2X7 primary antibody concentrations for IHC of human myometrial sections. (D) represents the negative control of human myometrium (Instead of a primary antibody, the rabbit IgG antibody was used,1:10000). (E) shows staining in the mouse kidney, which was used as a positive control (1:200, brown staining). (F) Immunoreactivity of P2X7 in blood vessels indicated by blue arrows. (5µM thickness, 20X).

4.3.4 Comparing the myometrial expression of P2X7R in non-pregnant, pregnant labouring and non- labouring women:

The DAB-stained myometrial sections for P2X7 immunoreactivity were detected by Aperio image scope software among myometrial tissues taken during a planned Cesarean section operation, an emergency operation of Cesarean section due to dysfunctional labour or fetal distress (labouring tissues), and non-pregnant women**.** The mouse kidney was used as a positive control, showing clear DAB staining of nephrocytes (**Figure 4.3E**). The primary antibody (P2X7 antibody) was replaced with a rabbit IgG antibody as a negative control and showed no staining. (**Figure 4.3D**).

The IHC showed typical positive staining of myometrial smooth muscles **Figure 4.4** as well as the blood vessels (blue arrow) (**Figure 4.3F**). Staining was detected in the myometrial tissue of all samples. In the non-pregnancy section, myometrial cells are tiny and condensed into small bundles, whereas in term pregnant sections, they have hypertrophied and are grouped in larger bundles surrounded by thick connective tissue, **Figure 4.4A-D**. Even at higher magnification, 20X, the distribution of the P2X7R was not identified whether it is restricted to the myometrial plasma membrane, as expected for the receptor. Using a high-resolution immunofluorescence assay might help localize the receptors. The P2X7R myometrial staining intensity was not significantly different between non-pregnant, non-labour, normal or dysfunctional labour ($p>0.05$) **(Figure 4.4E**).

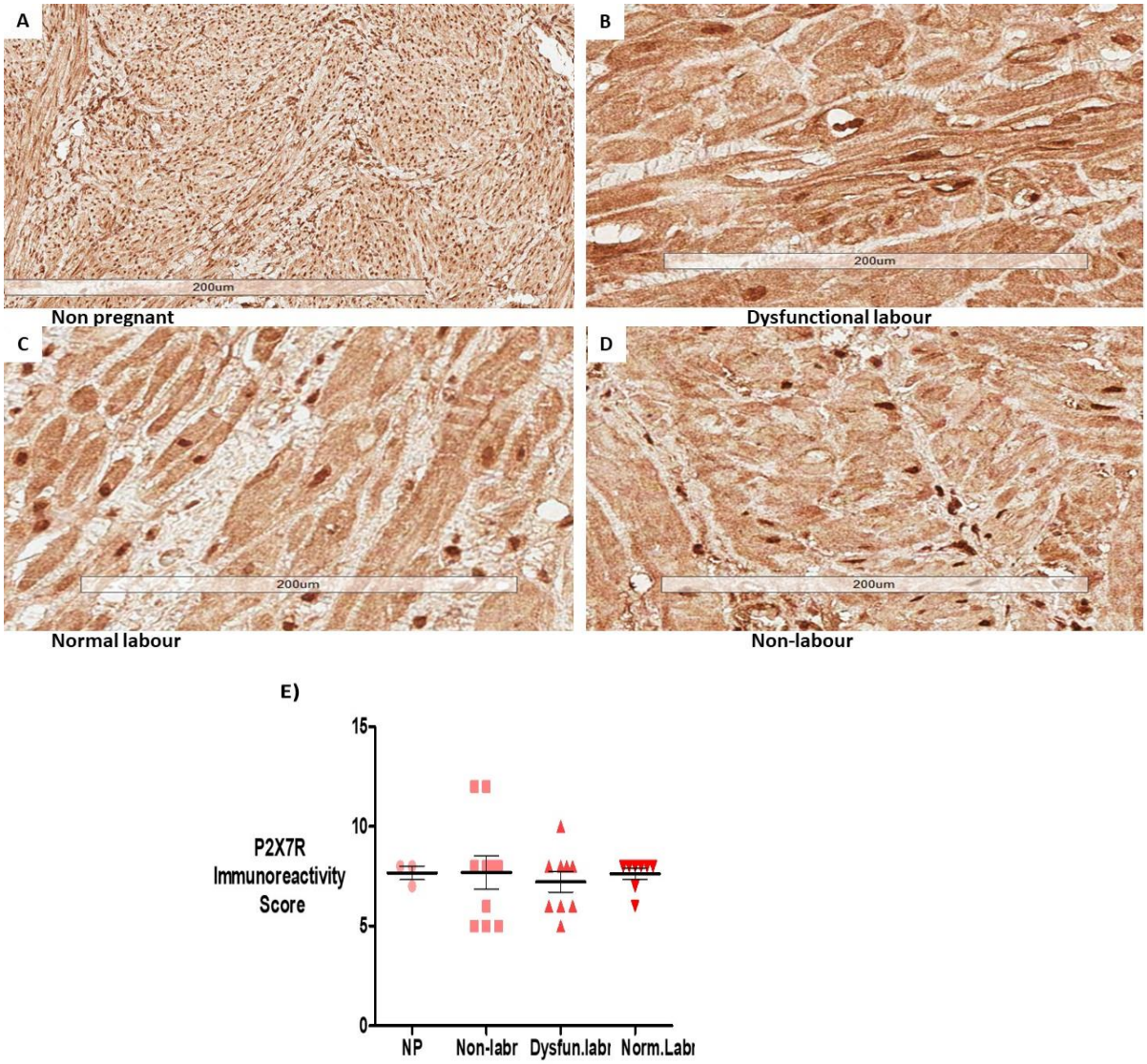


Figure 4.4: Immunoreactivity of P2X7 in the human myometrium. Immunoreactivity of P2X7 (1:200) is shown using DAB staining in myometrial tissue from A) non-pregnant, B) dysfunctional labour, C) Normal labour, and D) Non-labour myometrial tissue. The mean immunoreactivity score in myometrium from non-pregnant NP, non-labour (Non-labr), dysfunctional labour (Dysfun.labr) and normal labour (Norm-labr) (E), 5µM thickness-20X.

4.3.5 Quantification of P2X1R

For accurate quantification of P2X1 expression in human myometrium, western blotting using antibodies against P2X1R was carried out (**Figure 4.5**) using myometrium of 1) non-pregnant (n=3), 2) pregnant non-labouring (n=10), 3) labouring but dysfunctional (n=5) and 4) normal labour (n=3).

Figure 4.5 is a representative blot for P2X1R in human myometrium (n=3-10). P2X1R was identified in all groups. Different isoforms of the P2X1Rs were observed in the human myometrium of NP compared to pregnant non-labouring and labouring. A total of 3 bands were detected in NP (n=2/3); two bands have low-intensity staining at the molecular weight of (100 &75kDa) and the third one, the strongest, at the molecular weight of (50kDa) (predicted weight around 45KDa), (**Figure 4.5A**). Regarding the pregnant non-labouring and labouring groups, there is a single band at the molecular weight of (50kDa) (**Figure 4.5A**). Reprobed blots were used with the same experimental protocol and tissues, with anti-GAPDH antibody for loading control and to quantify the relative expression of P2X1.

Quantification of P2X1R expression was applied (**Figure 4.5B**). Analysis of signal intensity at 50KDa relative to GAPDH shows there is a small increase in expression in all term pregnant tissues, mainly in foetal distress labouring myometrial tissue when compared to non-pregnant tissue. Despite this trend, there was no significant difference between the different tissues ($p>0.05$).

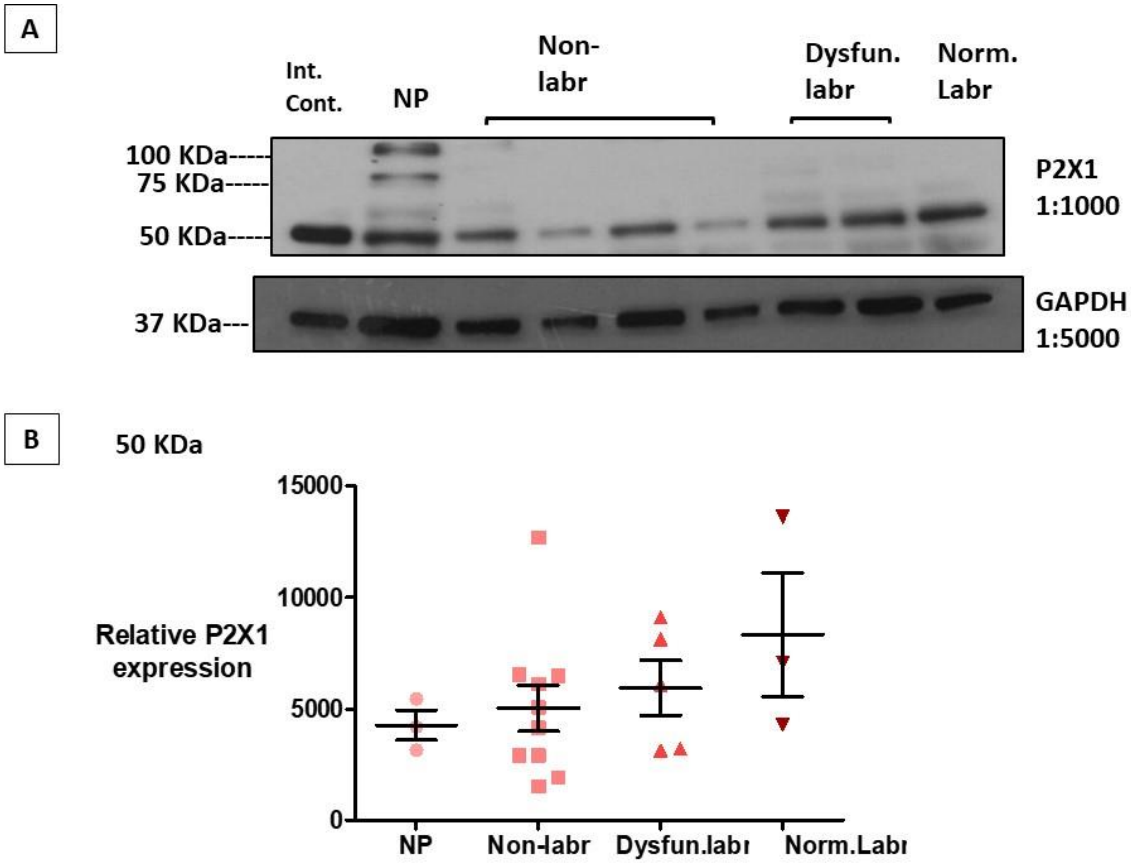


Figure 4.5 Presence of P2X1R in human myometrial tissue.

A: Representative image of blots for P2X1R in human non-pregnant (NP, n=3), non-labouring (Non-labr n=10), dysfunctionally labour (Dysfun.labr, n=5) and normal labour myometrium, (Norm-Labr, n=3). Anti P2X1 antibody at 1:1000 concentration was used to detect a variety of P2X1R expressions. **B:** Relative P2X1R abundance for 50KDa band. GAPDH was used to normalize protein concentration as a loading control. The internal control tissue (Int.cont) was used to ensure conditions were similar between runs.

4.3.6 Quantification of P2X7R

Figure 4.6A is a representative blot for P2X7R in human myometrium (n=3-10). P2X7R was seen in all groups. Two different isoforms of the P2X7Rs in human myometrium were detected, one at 50kDa (predicted weight around 69KDa) and another at 100kDa, the ratio of which appears to change between non-pregnant and term pregnant tissues. All samples expressed the 50kDa protein. Non-pregnant myometrial tissue only expressed the 50kDa protein (n=3), whereas both sized proteins were detected in all non-labouring tissues (n=10), whereas only in 2/5 dysfunctional labouring and 2/3 fetal distress tissues samples were both protein sizes detected. In both labouring tissue types, the predominant protein was 50kDa (**Figure 4.6B&C**). Reprobed blots were used with the same experimental protocol and tissues, with anti-GAPDH antibody for loading and quantifying the relative expression of P2X7.

Quantification of P2X7R expression was applied (**Figure 4.6B**). Analysis of signal intensity at 50KDa relative to GAPDH shows there is a significant increase of the P2X7R expression in normal labour tissues (32845±8655%, n=3, P= 0.0025) compared to non-labour, dysfunctional labour and non-pregnant tissue; (12256± 2118%, n=10), (15599±3146%, n=5), and (4911±751%, n=3), respectively. Analysis of signal intensity at 100KDa (where evident) shows that expression in dysfunctional labour tended to be less than non-labouring and normal labour. However, due to their limited n numbers, statistical analysis could not be performed on the signal intensity of this isoform.

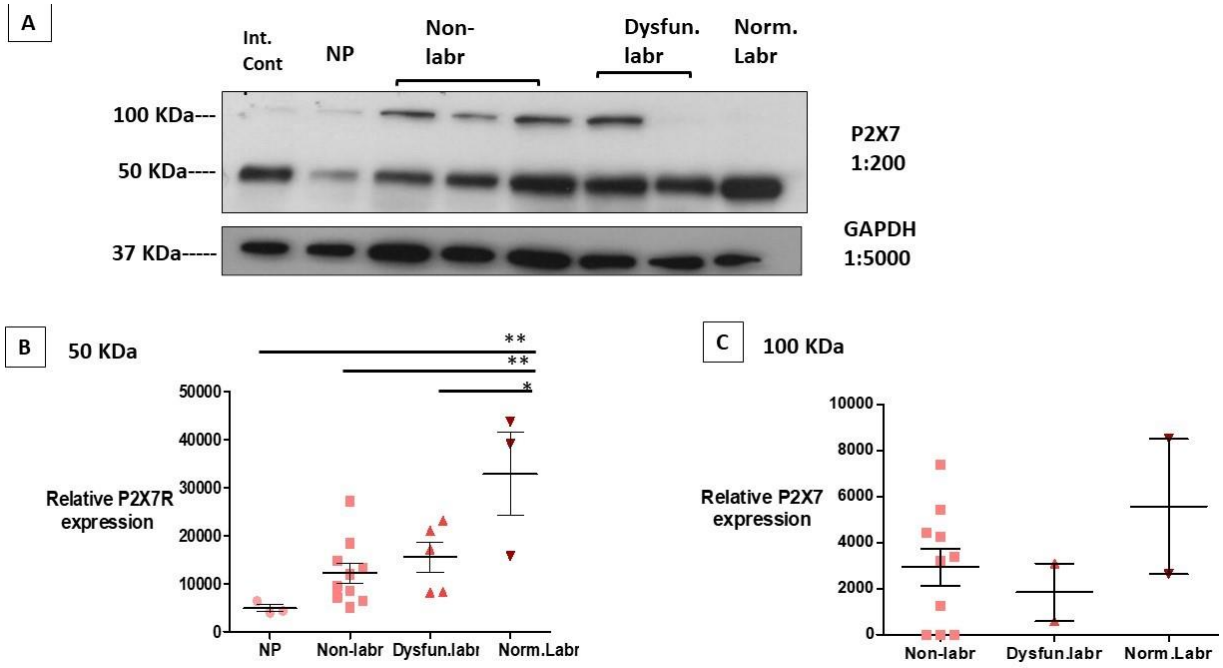


Figure 4.6: Presence of P2X7R in human myometrial tissue.

A: Representative image of blots for P2X7R in human non-pregnant (NP, n=3), non-laboring (Non-labr n=10), dysfunctionally labor (Dysfun.labr, n=5) and normal labor myometrium, (Norm.Labr, n=3). Anti P2X7 antibody at 1:200 concentration was used to detect P2X7R expression. **B:** Relative P2X7R abundance at 50KDa. **C:** Relative P2X7R abundance at 100KDa. GAPDH was used to control for protein loading and normalize P2X7 expression. The internal control tissue (Int.cont) was used to ensure consistency between blots.

4.4 Discussion

In the previous chapter, it was shown that extracellular ATP increased the frequency of uterine contraction. It is hypothesized that the expression of P2X1Rs or P2X7Rs would increase towards term pregnancy, and ATP binding to one of these receptors could potentiate the myometrium contraction, which would help contractile augmentation during the onset of labour. On the other hand, the depletion of one or both receptors could suggest a potential mechanism underpinning the poor myometrial contractility of those who have dysfunctional labour. So far, there are few data to suggest P2X1R or P2X7R are involved in mediating the contractile responses of the human myometrium to extracellular ATP (Alotaibi, 2018); (Khanam and Burnstock, 2007). The aim of this study was to examine the P2X1R or P2X7R expression in human myometrium from non-pregnant, non-labouring, labouring normally or labouring dysfunctionally women. The expression location of P2X1R & P2X7R in all mentioned groups was investigated using immunohistochemistry and quantified by a western blot study. The data shows that both receptors are present in myometrial smooth muscle and the wall of blood vessels. P2X1R expression in myometrium has no differences between all groups. Interestingly, the myometrial expression of P2X7R in non-pregnant, non-labouring or dysfunctional labour is significantly less than in the normal labour group. This is compatible with the hypothesis of this study. This result suggests that the expression of uterine P2X7Rs is functionally significant and might be implicated in the mechanisms of labour. Alotaibi, in his study, also found the density of P2X7Rs in rat uterine tissues markedly increased during labour (Alotaibi, 2018). However, the P2X7R expression has no significant differences between non-labour and dysfunctional labour in human myometrium.

Throughout pregnancy, the uterus is in a quiescent state to ensure a safe environment for foetal development and avoid preterm delivery. In contrast, during labour, the myometrium starts contracting forcefully in a coordinated pattern in order to expel the foetus and later the placenta into the birth canal (Riemer and Heymann, 1998). Many factors could contribute to the control of this switch from uterine quiescence throughout pregnancy to an actively contracting uterus in labour, including the marked upregulation of gap junctions, ion channels, oxytocin receptors, and the increased production of prostaglandins (Wray and Prendergast, 2019). The current finding of a high density of P2X7 receptors in normal labour, compared to non-labouring myometrium or dysfunctional labour, suggests their implication with other factors in coordinated contractile activity of the muscle cells at labour.

Immunohistochemistry is a subjective approach known as a semi-quantitative method used mainly to determine the location of protein expression and cannot quantify protein expression accurately so western blot approach was used which is more accurate and can give an overview of total protein, but it cannot determine any functional expression differences e.g. surface (and likely functional) verses internalized i.e. trafficking or recycling receptors (and therefore likely non-functional). Hence using other techniques to measure surface expression would be beneficial.

The mechanisms that prepare the myometrium for labour are still unknown, but it is currently thought that the normal physiological process of labour is linked to uterine inflammation (Shynlova et al., 2013). P2X7R play a key part in the cellular inflammatory process through its importance in interleukin (IL)-1β secretion (Miyoshi et al., 2016). Interestingly, a study on pregnant rat myometrium found that the lipopolysaccharide-induced inflammatory preterm model is associated with the overexpression of P2X7 mRNAs and P2X7 receptor proteins and suggested that these receptors play important roles in uterine contractions at the time of delivery (Urabe et al., 2009). Thus, when ATP is released by traumatized myometrial cells associated with parturition, could result in P2X7 receptor activation at the cell membrane and augment the uterine contraction. The current results indicating overexpression of the P2X7 receptors associated with the normal labour group are consistent with our hypothesis. This is supported by a study conducted on a mouse model of intrauterine inflammation, which is concluded that the blocking P2X7 receptor may provide a clinically useful tool to prevent preterm birth (Tsimis et al., 2017).

Ovarian hormones such as oestrogen and progesterone control myometrial contractility essentially by regulating the expression of genes that encode OTR and Cx43 (Mesiano and Welsh, 2007). During labour, the high circulating oestrogen enhances the expression of OTR and Cx43 dramatically to promote uterine sensitivity to oxytocin and help cell-cell communication, which is essential for successful and coordinated contractions (Ilicic et al., 2020). There are no studies regarding oestrogen's role in regulating the purinergic receptor expression in the myometrium. However, Raymond et al have examined the expression of P2X3R in rat uterine cervix during different gestational stages and during labour by using western blot study. They found that P2X3R protein expression was significantly enhanced in the cervix at labour and suggested that the increase in oestrogen might contribute to the elevation of P2X3R expression (Papka et al., 2005). If this is the case for P2X7R, hence the elevated oestrogen in pregnancy towards labour may contribute towards enhancing their

expression in the normal labour group. Furthermore, Urabe and his team have suggested that progesterone withdrawal is likely responsible for the up-regulated P2X7 mRNA expression seen in the hormone-induced preterm delivery model in rats. They concluded this markedly increased P2X7R might enhance uterine contractions during normal delivery (Urabe et al., 2009). However, it is worth to mention that humans do not undergo progesterone withdrawal which involves changes in the ratio of expression of different progesterone receptor isoforms, instead they undergo a functional withdrawal (Mitchell and Taggart, 2009).

On the other hand, in the term pregnant non-labouring group, the uterus is not in a labour state. Therefore, P2X7R expression is unlikely to be upregulated to the maximum level of the onset of labour; thus, the uterine sensitivity to ATP may alter. This is supported by evidence that examined the influence of ATP on the contractile response of the uterine arteries collected from guinea pigs at various gestational stages. The researchers came to the conclusion that oestrogen during pregnancy substantially increased the sensitivity of the uterine vasculature to adenosine triphosphate, which is mediated by P2X receptors (Haynes et al., 2003).

The physiological cause of dysfunctional labour has been suggested to be due to a low pH and increased lactate in myometrial capillary blood (Quenby et al., 2004). The P2X7R has been found to play a function in the cellular inflammatory response, which has been claimed to be a possible mechanism of labour (Gomez-Lopez et al., 2014);(Miyoshi et al., 2016);(Urabe et al., 2009). The extracellular acidification associated with dysfunctional labour could affect the expression of P2X7 receptors and inhibit their role in the inflammatory response, which eventually alters the myometrial response to ATP and leads to poor progress in uterine contractility during the onset of labour. Interestingly, extracellular acidification has been shown to decrease the protein expression of P2X7R and inhibit their activation, and this functional inhibition by acidic pH is mediated by five amino acid residues in P2X7's extracellular domain, which suppresses IL-1-dependent innate immune activation (Liu et al., 2009); (Virginio et al., 1997); (Takenouchi et al., 2014); (Brand et al., 2016); (Flittiger et al., 2010);(Sekar et al., 2018).

The relatively small differences in the abundance of P2X7R expression in non-labour and dysfunctional labour myometrial tissues suggest that they could mirror the similar contractile response to ATP in the contractility study. In other words, the ATP contractile response in both groups might be driven by the level of their P2X7R expression. However, no normally

labouring tissues were available for the contractility study to compare the actual contractile response to ATP mediated by the high density of P2X7R found in this group.

The result also shows the inconsistency of findings with non-pregnant myometrium tissues as there is a single protein band at 50KDa. Unlike the pregnant non-labouring and labouring myometrial tissues, which showed potentially two isoforms in some samples, one at 50KDa and the other at 100KDa, the reason for the expression of double bands in pregnant tissue is unknown but might be related to immunological responses. P2X7R is known to have an important role in inflammatory processes and immunological responses, mainly through its C-terminal domain (Kopp et al., 2019); (Miyoshi et al., 2016), and inflammatory reactions are associated with premature initiation of labour. The N-glycosylation of the P2X7 receptor has been shown to attenuate its activity (Lenertz et al., 2010). This glycosylation could be cross-reacting with another protein that for some reason is not present in all samples. Also, it could be responsible for the 100kDa isoform, and its existence in the pregnancy might explain its role in maintaining the pregnancy to allow sufficient time for the foetus to grow before the onset of labour. However, this pattern of expression does not include all labouring samples as the exact mechanisms underlying the onset of labour are not fully understood, and further studies need to be performed to discover this.

The alteration in oestrogen and progesterone ratio towards term pregnancy and intrauterine inflammation during parturition may have an important role in increasing the density of P2X7 receptors expression on the myometrial cell membrane. This might indicate their role in promoting uterine sensitivity to ATP and mediating the augmentation of uterine contraction during the onset of labour (Urabe et al., 2009). Studies have shown that the surface expression of these receptors is regulated by different mechanisms, including receptor internalisation, degradation, down-regulation and truncation mutant in of the cytosolic c-terminal (Feng et al., 2005); (Khadra et al., 2013);(Smart et al., 2003), which might affect the function of the receptor and change its response to different stimuli (Feng et al., 2006); (Smart et al., 2003). However, further investigation of surface expression by higher resolution immunofluorescence assay during dysfunctional and normal labour is required to determine its effect on myometrial contraction.

The immunohistochemistry also shows the expression of P2X1 and P2X7 receptors in the myometrial blood vessel wall. This is consistent with previous studies where their expression has been found in the vascular smooth muscle within the uterus of non-pregnant rats (Bardini et al., 2000) and in the smooth muscles of the human umbilical cord and chorionic blood

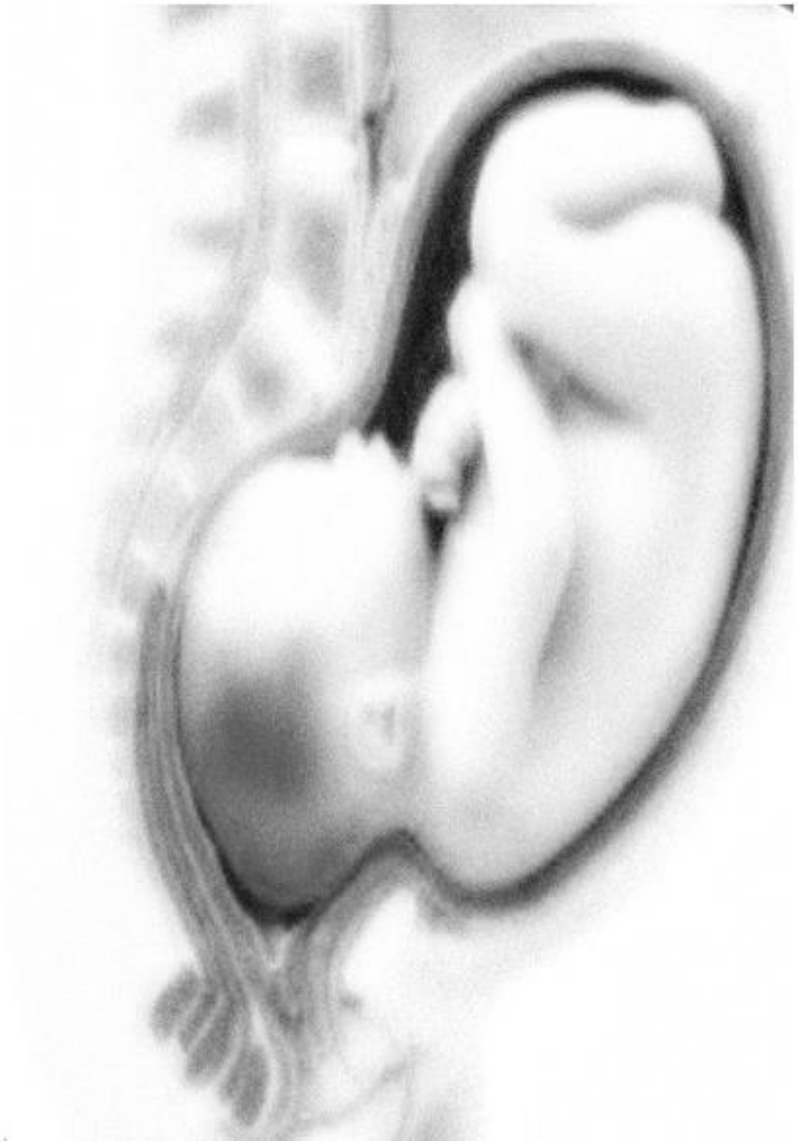
vessels (Valdecantos et al., 2003). And their abundance in guinea pig uterine artery is identified to increase in the late stage of pregnancy (Haynes *et al.,* 2003). This indicates their potential role in changing the blood flow to the myometrium during labour. It has been reported that P2X1 and P2X7Rs mediate vasoconstriction in human saphenous vein smooth muscle (Cario-Toumaniantz et al., 1998) and human omental arteries (Nichols et al., 2014). The hypoxia associated with each forceful myometrial contraction during labour might trigger the release of ATP from endothelial cells (To et al., 2015), which binds to these receptors to induce vasoconstriction, which eventually leads to a reduction in the blood perfusion and cause ischemia. It is worth mentioning that these periods of hypoxia and ischemia during labour are normal, and a recovery period is followed during the reperfusion between contractions (Alotaibi et al., 2015). However, it could be speculated that a high expression of one or both of these receptors in uterine vessels could mediate the contractile responses to ATP which then prevents these contracted vessels from recovering so no perfusion would occur and a poor progress in myometrial contraction might take place which in turn could contribute to the dysfunctional labour.

4.5 Limitations of the study

The number of samples used in this study was relatively small, and larger sample size should be examined in future studies. This was due to the Covid epidemic, which significantly influenced sample collection. Obtaining consent and taking biopsies were extremely challenging with all the additional clinical precautions in place (Raghuraman et al., 2021).

Immunohistochemical studies are limited in that they can only give a semi-quantitative assessment of protein expression, and Western blotting is a quantitative procedure that is more precise but does not allow for localisation of receptors, e.g., surface (and functional) vs internally located receptors. IHC at the magnification used could not determine the cellular distribution of P2X1 and P2X7 receptors. Using a high-resolution immunofluorescence assay might help localize the receptors.

CHAPTER 5



Chapter 5: An Investigation of the effect of ATP on Myometrial Contractility of Laboring Women

5.1 Introduction

Powerful uterine contractions are essential for delivering the baby. Unfortunately, not all labours will end with normal vaginal delivery, some might have weak and uncoordinated uterine contractions, and labour will be prolonged and accordingly will need augmentation with oxytocin of their labour to reduce the risk of maternal and neonatal morbidity and mortality (Organization, 2011). Interestingly, up to 50% of women who are labouring poorly do not respond to oxytocin administration and ultimately require emergency CS (Wiberg-Itzel et al., 2016); (Wray, 2007).

Dysfunctional labour is defined as poor progress in labour due to weak and uncoordinated uterine contraction, which in turn prevents cervical dilatation (Steer et al., 1985); (Wray et al., 2020). Scientists have worked hard investigating the underlying mechanisms of dysfunctional labour. Interestingly, a clinical trial involved 16 women with dysfunctional labours who received ATP and prostaglandin by intravenous infusion were found to have a full cervical dilatation in a shorter time compared to 18 women who received prostaglandin alone (Ziganshin et al., 2005). It is suggested that purinergic signalling plays a role in uterine contraction and that the low expression of their receptors might lead to an obstructed labour (Alotaibi, 2018).

During normal parturition, the myometrium starts contracting frequently and forcefully in a coordinated pattern in order to expel the foetus and later the placenta through the birth canal to the external world (Riemer and Heymann, 1998). The precise mechanisms of initiating this contractility remain mysterious. The marked upregulation of gap junctions and oxytocin receptors, the increased stretch of the uterine wall as the foetus grows, alteration of the oestrogen/progesterone ratio, and the increased production of prostaglandins probably all play a part in the strong contractions necessary for labour (Wray and Prendergast, 2019). The strong uterine contraction during labour compresses the myometrial vessels. This squeeze reduces blood flow and results in local transient ischemia and hypoxia, which recovers when the muscle relaxes (Wray et al., 2020). If the myometrial contractions are sustained for a long time or are very frequent, the decreased myometrial blood perfusion will associate with a decrease in the placental blood flow, which causes a less efficient gas and nutrient exchange.

As a result, fetal heart rate will increase with each peak of force, which might lead to fetal distress and eventually require a C-section delivery (Krishna and Bhalerao, 2011). The frequency of uterine contractions and fetal cerebral oxygenation was discovered to have an inverse correlation, according to Peebles et al. research (Peebles et al., 1994). In addition, overstimulation with uterotonics such as oxytocin, which can be used to induce slow progress labour, can also cause fetal distress due to uterine hyperstimulation. Furthermore, tonic-like contractions that occur on occasion spontaneously might impair uterine blood flow and leads to fetal distress (Wray et al., 2020).

No published work determines the functional effect of extracellular ATPγS, a nonhydrolyzable version of ATP, on labouring human myometrial contractility. In chapter 3, ATPγS was found to have the highest stimulatory effect on non-labouring human myometrial contractility among other agonists (BzATP and ATP). Therefore, it is hypothesized that spontaneous myometrial contractility in normal labour would have the highest stimulatory response to the effect of ATPγS compared to non-labouring and dysfunctional labouring myometrial tissues.

In this chapter, the labouring myometrial tissues associated with fetal distress will be taken as a “normal” labour sample as the contractions are coordinated and frequent and strong, and the cervix is dilating. This is not the case in women labouring dysfunctionally.

5.1.1 Aims of the study

1) To investigate the effect of ATPγS on spontaneous contractile activity in dysfunctionally labouring human myometrium.

2) To compare the effect of ATPγS on myometrial contractility between dysfunctionally labouring and non-labouring women.

5.2 Methods

5.2.1 Tissue

Human myometrial biopsies were obtained from women undergoing emergency caesarean section at term at Liverpool Women's hospital. The local ethical committee's approval and the patients' informed consent were obtained. Uterine tissues were collected and prepared, and then myometrial strips were dissected and mounted as described in detail in the general materials and methods chapter (Chapter 2). The patient's details were blinded at the time of studying the biopsies to avoid any bias. The demographic data on the samples used are given in Table 5.1.

5.2.2 Drugs and solutions

1mM ATPγS was used to stimulate myometrial contraction. A 40mM high potassium (KCl) solution was used to assess tissue viability. They were prepared as described in the general materials and methods chapter.

5.2.3 Experimental protocol

After approximately an hour, the spontaneous contractions reached a regular pattern in physiological saline. The uterine strips were then exposed to 1 mM ATPγS for 20 mins to test their effects on spontaneous myometrial contractions and washed out with PSS. ATPγS was used as it was the agonist that produced the largest effect in the non-labouring human myometrium. The 20 mins immediately preceding the application of ATPγS was used as the control (100%) period (**Figure 5.1**). If there was a delay in contraction recovery after washing out the ATPγS, a 40mM high potassium (KCl) solution was used to assess tissue viability.

5.2.4 Statistical Analysis

Data were analysed as described in Chapter 3.

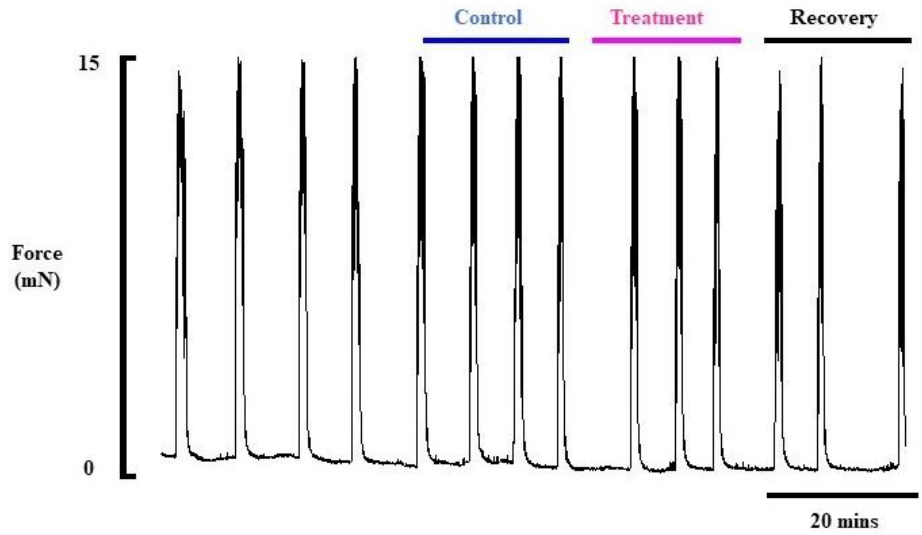


Figure 5.1 Experimental protocol. Spontaneous uterine contractions were obtained in a steady-state, and the last 20 minutes were considered a control period; labelled by the blue bar. This was followed by a treatment period of 20 minutes pink bar. Recovery was then followed for at least 20 minutes, labelled by a black bar.

5.3 Results:

During this study, five biopsies from women in labour were obtained. Analysis was performed blinded to the indication for the C-section. After performing the analysis, data were related to the cause of the C-section. All five samples were found to be taken from women labouring dysfunctionally.

The demographic data demonstrated that the women in this study were obese (the record does not show the BMI of 2 samples) but not diabetic (Table 5.1). Their mean age was 30, and the majority were nulliparous, which is expected – indeed, dysfunctional labour was the primary reason for the subsequent emergency caesarean section. All women had a term (>37 weeks) delivery; one of them had twins.

Table 5.1 Maternal demographic data. It illustrates the study sample characteristics of maternal age (years), gestational age (days), maternal body mass index (BMI), parity, gravidity, and indication for Caesarean section.

Maternal Sample Demographics (n = 5)	
Mother's age (years: mean ± SD)	30±3
Gestational age at delivery (days: median (IQR)	275 (272.5-27.5)
BMI (kg/m^2: mean ± SD)	30±7 (n=3/5)
Parity	
Nulliparous	n=3
Primiparous	n=1
Multiparous	n=1
Gravidity	
Primigravida	n=2
Multigravida	n=2
Twins	n=1
Indication for Caesarean section	
Dysfunctional labour	n=5
Normal labour	n=0

Type of Caesarean Emergency-labouring	 n=5
Diabetic	None

5.3.1 The effect of ATPγS on spontaneous uterine contractility in the dysfunctionally labouring human myometrium

After an hour, the majority of (4/5) labouring term human myometrial strips produced regular spontaneous contractions, and 1mM ATPγS was applied for 20 mins (**Figure 5.2A**). The ATPγS caused a clear increase in contractile frequency, which tended to become tonic in some tissues (although contractions were still visible on top of this elevated baseline **(Figure 5.2B))**, and achieved significance 652.4± 165.5%, P= 0.04, n=4, **Figure 5.3A**. One tissue out of 5 failed to establish spontaneous contractions after 2 hours. However, ATPγS was able to stimulate contractions in this sample (**Figure 5.2C)**. Due to the absence of spontaneous contractions and control activity, this one sample was excluded from the analysis. The 1mM ATPγS induced a significant enhancement of AUC (439.5±95.9%, P=0.03, n=4, **Figure 5.3B**) when compared to the preceding control period. There were no significant changes found in the amplitude of contraction. Interestingly, in 4 out of 5 tissues, contractions did not continue after washing out of ATPγS with fresh PPS for approximately 1 hour; therefore, tissue viability was assessed by adding a 40mM high potassium (KCl) solution. The high KCl solution was able to recover contractions, as shown in **Figure 5.2 B,** which means the tissue was viable.

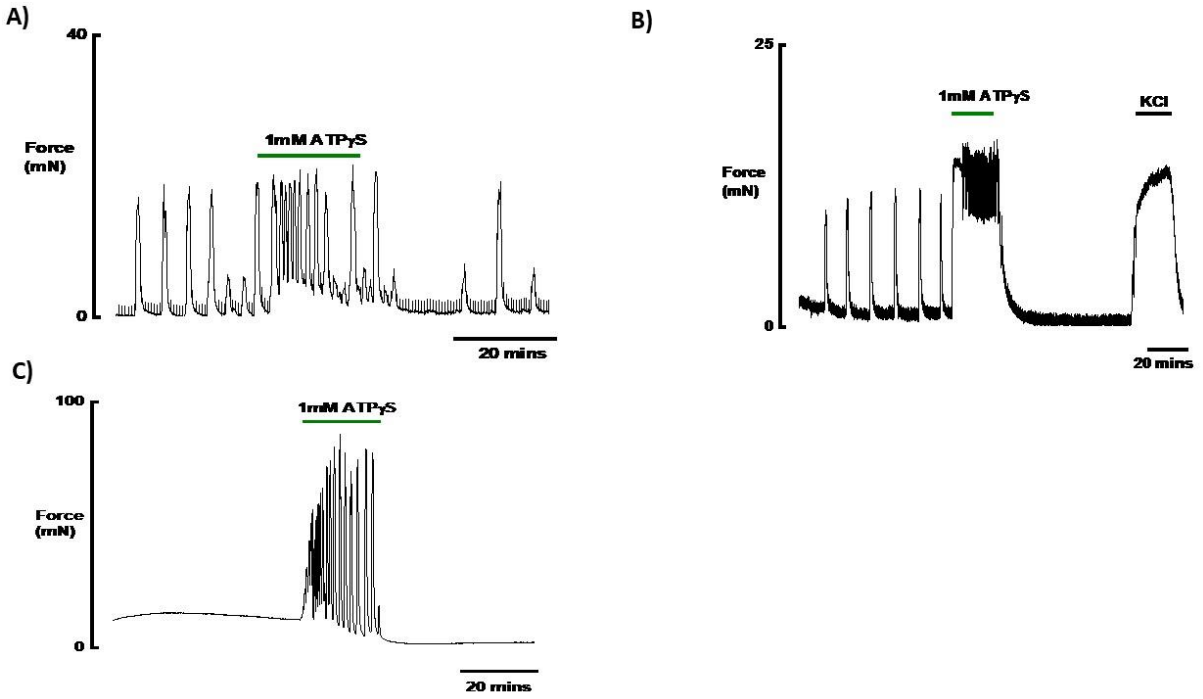


Figure 5.2: Effect of 1mM ATPγS application on dysfunctional labouring human myometrium. Traces of the effect of ATPγS on spontaneous uterine contractions in dysfunctionally labouring human myometrium. (A): Trace showing the stimulatory effect of 1mM ATPγS on contraction frequency n=2. (B): Trace showing a tonic contraction caused by 1mM ATPγS n=2. A 40mM KCl was added to assess the tissue viability. (C): Trace showing 1mM ATPγS ability to stimulate contractions in dysfunctional labouring human myometrium that was not spontaneously contracting, n=1.

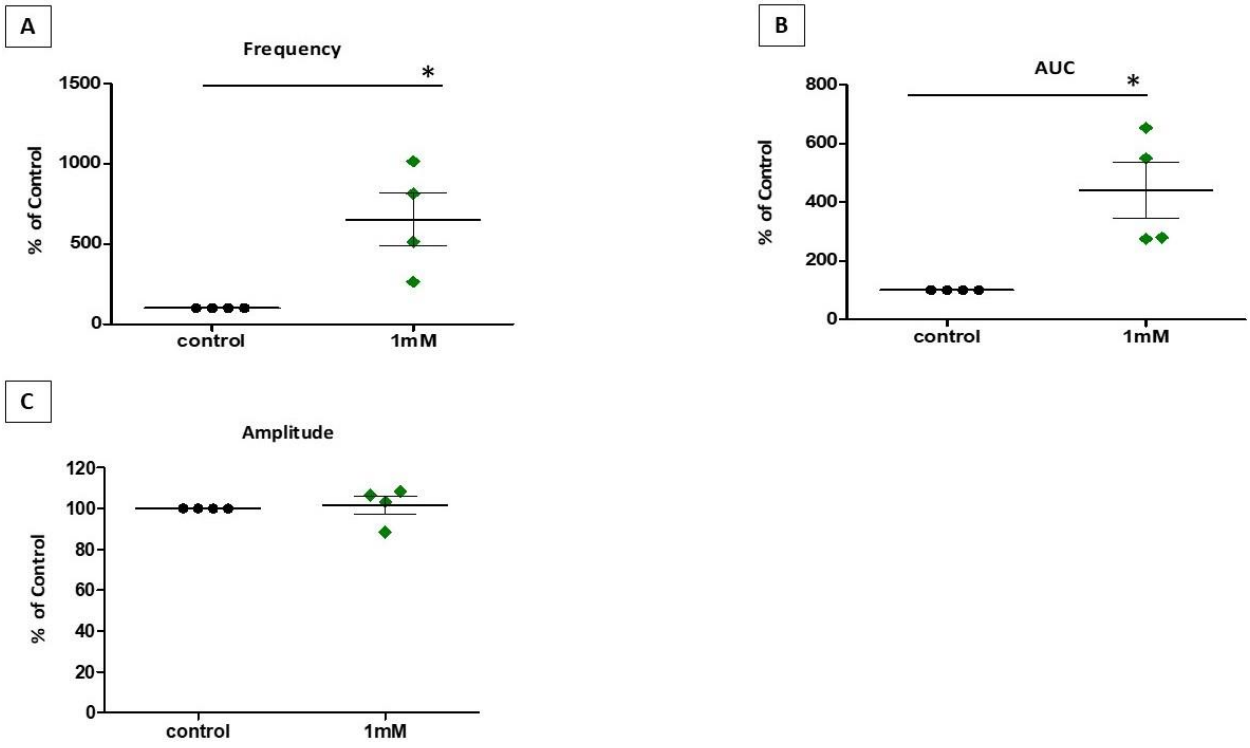


Figure 5.3: The effects of ATPγS on dysfunctionally labouring human myometrial contraction parameters. Scatter plot showing the effects of ATPγS on A: frequency, B: AUC, and C: amplitude. A Significant difference was found using the one sample t-test, n=4.

5.3.2 The comparative effect of ATPγS on spontaneous uterine contractility in the dysfunctionally labouring and non-labouring human myometrium.

The effect of 1mM ATPγS for 20 min on pregnant dysfunctionally labouring human myometrium and non-labouring myometrium was compared (n=4-6, **Figure 5.4**). The mean data shows there were no significant differences in frequency of contraction (p=0.9), AUC (p=0.5), and amplitude (p=0.3) in the dysfunctional labouring versus non-labouring groups **(Figure 5.5A-D**).

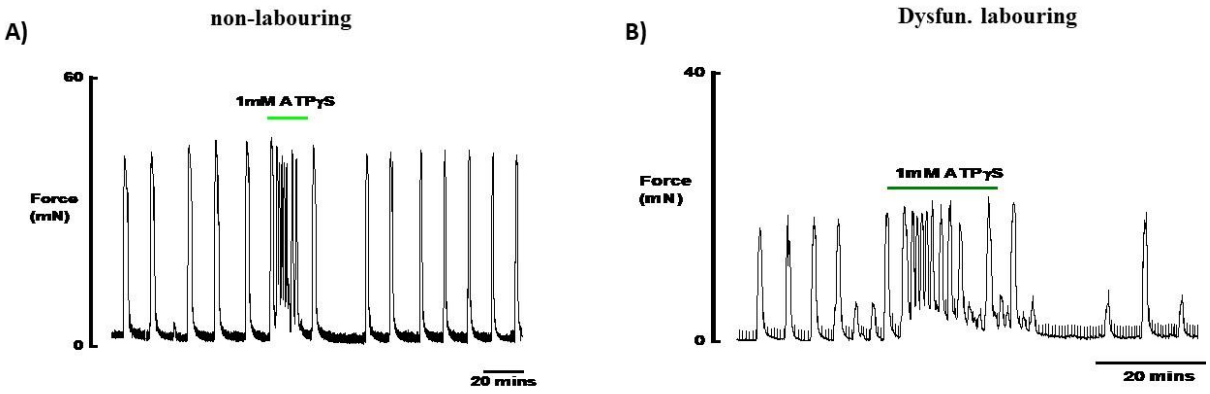


Figure 5.4. A comparison of non-labouring and dysfunctional labouring human myometrial response to ATPγS.

Typical traces show 20 mins pregnant non-labouring (A) and dysfunctional labouring (B) women uterine contractility induced by 1mM ATPγS.

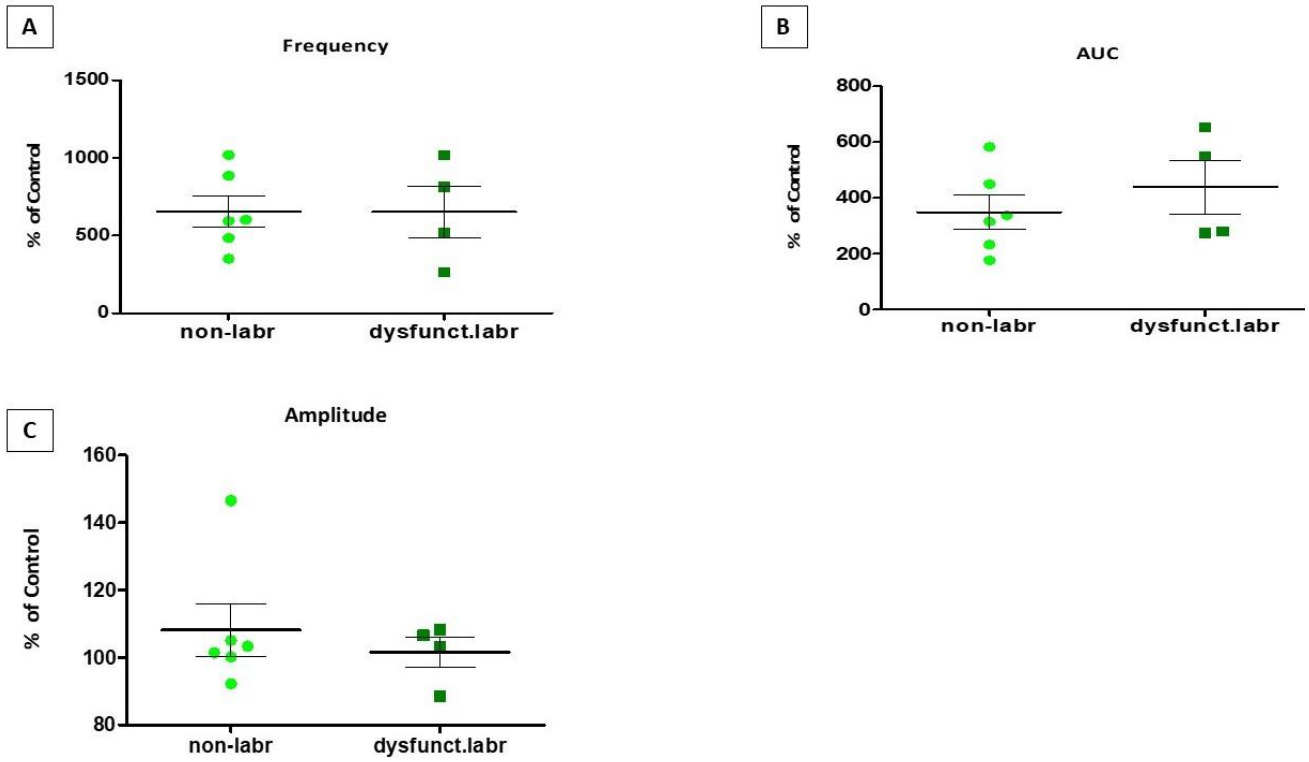


Figure 5.5. Comparison of ATPγS response between non-labouring and dysfunctional labouring human myometrium.

Scatter plot showing the effects of 1mM ATPγS on contraction (A) frequency, (B) AUC, and (C) amplitude in non-labouring (n=6, light green) and dysfunctional labouring human myometrial tissue (n=4, dark green). There was no significant difference found between the two groups by using students' unpaired t-test.

5.4 Discussion

Chapter 3 has shown that ATPγS, the non-hydrolysable form of ATP, significantly increased the frequency of uterine contractility in non-labouring human myometrium and was the most potent agonist among other analogues. This data suggests that the powerful excitatory effect of ATP could be considered a novel uterotonic agent in dysfunctional labour. The current work shows that the application of 1mM ATPγS also induces significant increases in the spontaneous contraction frequency of dysfunctionally labouring women. However, there were no significant differences between the response of non-labouring and dysfunctionally labouring myometrial contraction to ATPγS.

It was hypothesized that the expression of P2X1Rs or P2X7Rs would increase towards term pregnancy, and ATP binding to one of these receptors could potentiate myometrial contraction, which would help in contractile augmentation during the onset of labour. Although no normally labouring tissues were obtained in the contractility study, the relatively small differences in the abundance of P2X7R expression in non-labour and dysfunctional labour myometrial tissues in the previous work suggest that the current contractile response to ATPγS in both groups might be driven by the level of their P2X7R expression. However, the role of other purinergic receptors such as P2Y or other P2X subtype receptors should be taken into account since a mixture of these receptors has been identified in the uterus of various species, both pregnant and non-pregnant (Ziganshin et al., 2003);(Piper and Hollingsworth, 1996);(Aitken et al., 2001).

The P2X and P2Y receptors are suggested to be present in the human uterus during pregnancy (Ziganshin et al., 2003). Ziganshin and his team hypothesized that P2X receptor expression increases slowly during pregnancy peaking at labour, while P2Y receptor expression reaches maximal expression in late pregnancy before declining at labour (Ziganshin et al., 2006). ATPγS is known as an agonist at P2X, P2Y1, P2Y2 and P2Y11 receptors (Lambrecht, 2000). The clear stimulation of myometrial contraction in dysfunctional labouring women by ATPγS suggests that ATPγS is binding to functional receptors, which may be P2X or P2YR. As the peak expression of P2XR was demonstrated in labour in rats, then it may be speculated that the responses to ATPγS would be even more pronounced in normally labouring myometrium (Ziganshin et al., 2006);(Alotaibi, 2018). This is supported by the work in Chapter 4, showing the expression of P2X7R was markedly increased in the normal labour group compared to dysfunctional labouring and non-labouring

myometrial tissue. However, exploring the myometrial expression of P2YR is important to consider in future work.

Taken together, the pattern of P2X7R expression in the non-pregnant, term pregnant, and the normal labour group and the relatively low density in non-labouring tissues could explain why P2X7R antagonists failed to inhibit the stimulatory effect of ATP and its analogues. This could be proven if these P2X7R blockers were examined on normal labouring tissues and the effect of ATP or its analogues was abolished. Further work on normal labouring tissue and using specific inhibitors of P2YR in the non-labouring tissues is needed to provide evidence for this hypothesis.

An interesting finding was that ATPγS could elicit contractions in a sample of myometrium that was previously quiescent. This gives the exciting possibility that ATPγS will be of use to help women labouring dysfunctionally, to regain contractions and avoid an unplanned C-Section. Future work could compare ATPγS and oxytocin, the current stimulant given to women suffering from dysfunctional labour. Questions would be, for example, can ATPγS stimulate myometrium in cases where oxytocin does not? Do their actions synergize? If so, this may also mean lower concentrations of both agents could be given, and effects such as desensitization and side effects, reduced.

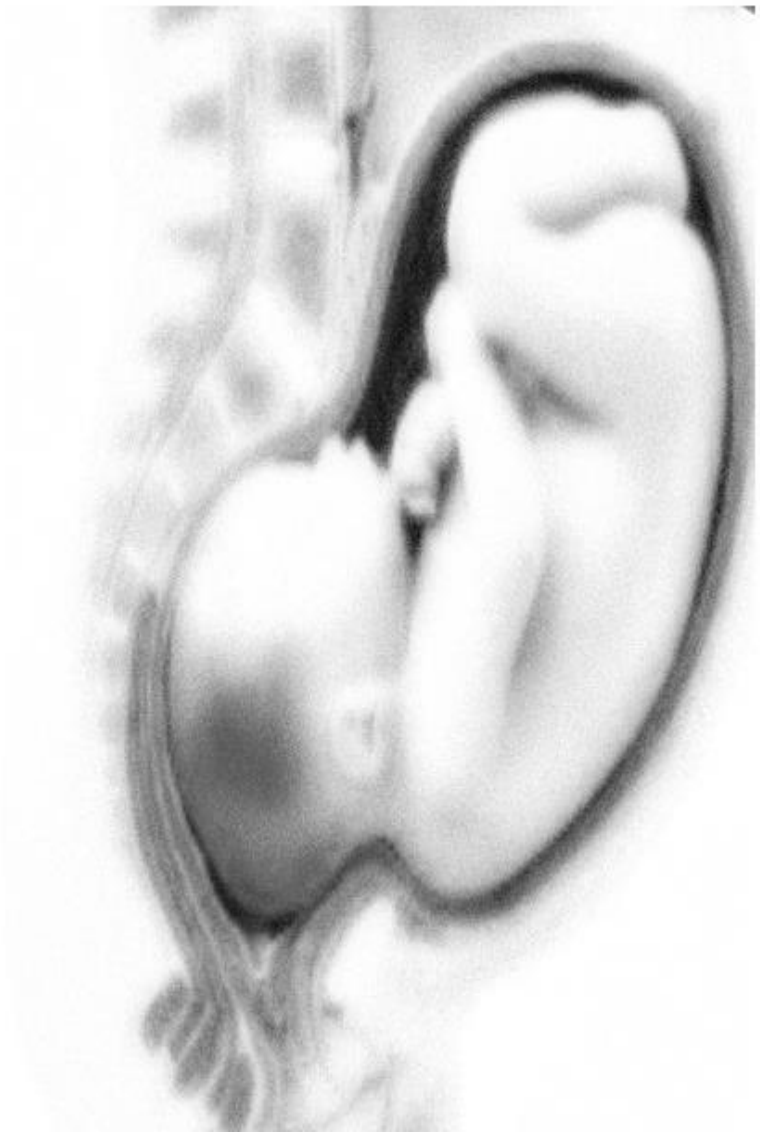
5.5 Limitations of the study

This work has a low sample size for two main reasons. This is a reflection of how difficult it is to obtain labouring samples. It is not always appropriate to ask for consent if a woman is going to have an unplanned C-section, and she will have lots on her mind and may well be distressed. In the latter case, women are not approached for a sample by the surgical team. It is also the case that some women who did give their consent did not have a biopsy taken because the surgical team forgot or decided against taking a biopsy. Secondly, there was a large impact of the Covid pandemic on obtaining samples. With all the additional clinical measures in place, both obtaining consent and taking biopsies was largely impossible (Raghuraman et al., 2021). In addition, women were understandably much more anxious in their pregnancy and not as likely to give informed consent.

Human tissue, in its nature, has a lot of variation compared to animal models, and this increases the variation between samples. Some of this can be seen in **Figure 5.2**. Increasing n number would boost confidence in the conclusions between labouring and non-labouring

myometrium. Obtaining five biopsies was challenging. It was helpful however that they were all from women labouring dysfunctionally.

CHAPTER 6



Chapter 6: Final Discussion

This thesis aimed to investigate the effect of ATP on labouring and non-labouring human myometrial contractility and examine its potential to augment contractility in cases of dysfunctional labour. Also, to investigate the implication of P2X7R in mediating the ATP action.

The initial work was to investigate the effect of ATP on term pregnant non-labouring human myometrial contractility. Having shown its significant effects on contractile frequency, exploring the contribution of purinergic receptors, mainly P2X7R, in ATP-induced uterine contraction was investigated by using different analogues and selective P2X7R antagonists. Finally, the effect of ATP as a novel uterotonic agent on the labouring state, dysfunctional labour, was identified.

Another method to understand how ATP enhances contractility was to look for evidence of changes in purinergic receptor protein in myometrial tissue. This was accomplished by examining the expression of P2X1R and P2X7R in the myometrium of women who were term pregnant (non-labouring, labouring normally or dysfunctionally) and non-pregnant. Despite the limitation in the sample size used in some experiments, many particular goals have been addressed. The work presented in the current study has shed light on the effect of ATP on human myometrial contractility besides exploring the implication of myometrial purinergic receptor protein expression, including P2X1R and P2X7R.

The contractility study results showed that the myometrial strips from term non-labouring and dysfunctionally labouring women spontaneously contracted even before agonists were applied. It is known that the myometrium is a dynamic tissue (Wray, 1993). It can contract spontaneously in the nonpregnant state during the menstrual cycle, known as endometrial waves, and can also occur throughout pregnancy, resulting in Braxton Hicks contractions (McEvoy and Sabir, 2021). These spontaneous contractions occur as a reflection of the electrical activity state of membrane potential (Wray et al., 2015), as discussed in section 1.2.4 in the general introduction. During labour, these contractions are sustained at regular intervals, increasing strength and frequency and associated with cervical dilation. This is due to the propagation of multiple coordinated membrane depolarizations necessary for forceful and sustained contractions (McEvoy and Sabir, 2021). It is believed that there is more than one key event that turns on the switch to labour, including the marked upregulation of gap junctions, ion channels, oxytocin receptors, and the increased production of prostaglandin

(Wray and Prendergast, 2019). Identifying the high density of P2X7 receptors in normal labour vs non-labouring myometrium or dysfunctional labour implies that they may play a role in the coordinated contractile activity of muscle cells during labour. This is supported by a recent study that found that the expression of P2X7Rs significantly increased in rat uterine tissues during labour, suggesting their essential role in the mechanisms of labour (Alotaibi, 2018). However, no normally labouring tissues were obtained in this contractility study due to the Covid pandemic and the difficult collecting emergency samples during this critical condition. Therefore, it could be speculated that in labouring tissue, the spontaneous contractions become stronger, and the time between each contraction decreases.

6.1 The Effect of ATP on the human myometrial contractility

ATP had a stimulatory effect, increasing frequency, on term pregnant non-labouring human myometrial contractility. This is consistent with previous studies on its effect on pregnant and non-pregnant myometrial contraction from various species (Moritoki et al., 1979); (Alotaibi, 2018); (Hutchings et al., 2009). Interestingly, ATP increased the contractile frequency in a concentration-dependent manner, suggesting it is depolarizing the myocytes, consistent with the opening of its non-specific ion channel (Surprenant and North, 2009);(North, 2002). To explore the contribution of purinergic receptors in ATP-induced uterine contraction, a potent P2X7R agonist and selective antagonists were applied. The current results showed it is unlikely that P2X7R contributes to the response of term pregnant non-labouring human myometrium to ATP. The rank order of potency of the agonists was ATPγS> BzATP>ATP, which is not consistent with the P2X7 receptors. ATPγS is normally an agonist at P2X, P2Y1, P2Y2 and P2Y11 receptors (Lambrecht, 2000). However, BzATP comes next, and it is normally the most potent agonist at P2X7, but it can also react with P2X1, P2X3 and P2Y1 receptors (Vigne et al., 1999);(Allsopp et al., 2011);(Jacobson and Müller, 2016). Therefore, it appears that numerous subtypes of P2X receptors coexist in the term pregnant non-labouring human myometrium, which could be involved in the contractility response to the ATP. This finding is supported by the western blot analysis, where a low density of P2X7 receptors was identified in this group, which could explain the failure of the selective P2X7R antagonists to inhibit the stimulatory effect of ATP and BzATP in this study. This contrasts with findings in term pregnant rat myometrial cells, where patch clamping analysis was used and found that P2X7 is the main receptor that carries the ATP-induced currents (Miyoshi et al., 2010);(Miyoshi et al., 2012). In this study, the use of BzATP produced increased contractile frequency, and again the selective P2X7 antagonist failed to block its effects.

Also, ATPγS, the non-hydrolysable version of ATP, caused an increase in the contractile frequency in dysfunctionally labouring and non-labouring term pregnant human myometrium. However, there was no significant difference in this response between the two groups. In addition, the western blot analysis revealed that the expression level of P2X7R in dysfunctionally labouring myometrium was not different compared to term pregnant non-labouring myometrium. Since the abundance of this receptor is low in these tissues, it is likely that other receptors, such as P2YR, which has a bell-shaped expression profile throughout pregnancy and is reduced at the onset of labour, where the P2X7R upregulated in normally labouring myometrium, takes over the role of mediating the ATP effect (Ziganshin et al., 2006). Moreover, in the dysfunctional labour group, the low expression of P2X7R could be due to the low pH, accompanied by the poor myometrial contraction, which is found to suppress the P2X7R protein expression in microglial cells (Sekar et al., 2018). ATPγS is a potent agonist at P2Y1, P2Y2 and P2Y11 receptors (Lambrecht, 2000) and the myometrial contraction in term pregnant non-labouring and dysfunctional labouring women suggests that the response to ATPγS could be mediated through these receptors. It is interesting to speculate that testing the effect of ATP and the potent P2X7R agonist on normal labouring tissue, then it might have seen a greater effect, as the western blot analysis showed P2X7R is upregulated markedly in this group compared to other pregnant and non-pregnant ones.

The findings in this study showed that ATP is a myometrial stimulant, including in dysfunctionally labouring tissue samples. It could potentially help to stimulate contractions in dystocic labours. This is consistent with a prior study on a group of dystocic labouring women who had intravenous infusion with ATP combined with prostaglandin and were found to have a shorter first stage of labour with no side effects (Ziganshin et al., 2005). ATPγS action suggests little role for ADP, AMP or adenosine in the effects of ATP. As they act via P1 purinergic receptors, it also indicates that P1 receptors are not involved in ATP action. Still, we cannot exclude the role of P2YR and other P2XR such as P2X4R or P2X7R. They are progressively upregulated in the myometrium, along with other important proteins during the late stage of pregnancy and labour (Urabe et al., 2009); (Alotaibi, 2018). However, further work is needed to investigate the role of other purinergic receptors in mediating the effects of ATP in human myometrium.

6.2 ATP and P2XR

To further confirm the implication of purinergic receptors in ATP action during pregnancy or labour, the myometrial expression of P2X1R and P2X7R proteins during non-pregnancy and term pregnancy (non-labouring and dysfunctionally labouring) was examined and compared to normally labouring women. To the best of my knowledge, this is the first known study investigating the expression of P2X1R and P2X7R in different gestational states of human myometrial tissue. Expression data clearly showed the presence of P2X7R and P2X1R in human myometrial tissue. Since the immunohistochemistry approach is mainly used to determine the location of protein expression, it may not be sensitive enough to find differences in receptor abundance among the pregnant and non-pregnant myometrial tissues. The findings from the western blot analysis will be used to explain in the current discussion.

The results of P2X7R expression from the current work are in agreement with other published studies (Alotaibi, 2018);(Urabe et al., 2009). The P2X7R expression increased from non-pregnant to term pregnant and significantly increased just in the normal labour group. This suggests that the expression of uterine P2X7Rs is functionally significant and might be contributing to the mechanisms of labour. In contrast, the low P2X7R expression could be involved in the dysfunctional labour group's pathological mechanisms. The mechanisms that prepare the myometrium for labour are unknown, although it is currently considered that uterine inflammation is involved in the normal physiological process of labour (Shynlova et al., 2013). P2X7R is important for interleukin (IL)-1 secretion, which plays a role in the cellular inflammatory process (Miyoshi et al., 2016). When ATP is released by injured myometrial cells associated with normal parturition, that will probably result in P2X7 receptor activation at the cell membrane of myometrial cells. An inflammatory process might take place, leading to the initiation of contractility that is essential during the onset of labour. The current finding, which shows that overexpression of P2X7 receptors is related to normal labour, supports our hypothesis. This is backed up by a study on a mouse model of intrauterine inflammation, which found that inhibiting the P2X7 receptor could be a clinically helpful technique for preventing premature birth (Tsimis et al., 2017).

The physiological cause of dysfunctional labour was suggested to be due to the low pH level and increased lactate in myometrial capillary blood (Quenby et al., 2004). The P2X7R has been identified to play a role in the cellular inflammatory response, proposed as a possible labour mechanism (Gomez-Lopez et al., 2014);(Miyoshi et al., 2016);(Urabe et al., 2009). Extracellular acidification associated with dysfunctional labour may change the expression of

P2X7 receptors and limit their participation in the inflammatory response, altering the myometrial response to ATP and resulting in a slow progression of uterine contractility during labour. Interestingly, extracellular acidification has been found to decrease the protein expression of P2X7R in microglia cells and inhibit their activation via suppressing IL-1-dependent innate immune activation (Liu et al., 2009); (Virginio et al., 1997); (Takenouchi et al., 2014); (Brand et al., 2016); (Flittiger et al., 2010);(Sekar et al., 2018).

It is interesting to identify double bands of P2X7R in all non-labouring tissue. This indicates that glycosylation of this receptor might attenuate its activity and, eventually, prevent its participation in any immunological responses that might enhance the onset of labour. Moreover, the glycosylation of the C-terminal domain is found to reduce the surface expression of P2X7R (Wickert et al., 2013), which could lead to a decrease in the uterine sensitivity to ATP and reduce the myometrial contractility. Therefore, the presence of the double bands of P2X7R in the non-labouring group would help protect the pregnancy allowing sufficient time for the baby to grow before delivery. It is well known that P2X7R participate in the immune responses, and any modification, like glycosylation, would affect the receptor function and weaken its activity (Kopp et al., 2019); (Lenertz et al., 2010). The double bands of protein were detected in some but not all labouring tissues, either dysfunctional labour or normal labour. Low pH may contribute to receptor inhibition and modulation of P2X7R expression, reducing uterine sensitivity to ATP and myometrial contractility. We would have detected a significant decrease in the expression of the double protein bands in the normally labouring group if more samples had been available for study. A limitation of this study is that the sample size for normally labouring tissues is insufficient to determine this. Therefore, further sample collection and research are required to test this hypothesis.

The low density of P2X7R and the relatively small differences in expression in non-labour and dysfunctional labour myometrial tissues compared to the markedly high expression in the normal labour tissue suggests that they are unlikely to be involved in ATP response in these tissues. This could be clarified as previously indicated that ovarian hormones are raised during labour in normal labour, where the OTR and Cx43 expression is greatly elevated to enhance uterine sensitivity to oxytocin and facilitate cell-cell communication that is needed for effective and synchronized contractions. This is not the case with the term pregnant non-labouring group, where the uterus is not in a labour state; thus, the contractile associated proteins (CAPs) are not elevated to the maximum level as they are anticipated to be during

the onset of labour, which may affect the P2X7 receptor expression (Haynes et al., 2003). In mice microglia cells, it has been reported that oestrogen increases P2X7 mRNA expression (Crain and Watters, 2010); if this is the case during labour, that could explain the enhanced expression of P2X7R in the normal labour group. Regarding the dysfunctional labour group, the associated extracellular acidification environment might negatively impact the P2X7R and reduce their expression (Sekar et al., 2018). This is supported by the findings of the contractility study, which revealed no significant differences between the two groups in the stimulatory response to ATPγS, which could be mediated by other P2XRs or P2YRs.

Literature suggests P2X1 may play a role in the response of human myometrial tissue to ATP (Ziganshin et al., 2006); (Ziganshin et al., 2005); (Ziganshin et al., 2003). At the beginning of this thesis, it was hypothesised that P2X7 or P2X1 receptor expression would increase as gestation progressed, peaking in labouring tissues and thus play an important role in labour. These western blot studies have demonstrated that P2X1R expression is unchanged from non-pregnant to term pregnant non-labouring and labouring tissues. Therefore, it is clear that P2X1R is not the receptor of interest in labour. P2X7R expression is increased in term pregnant tissue compared to non-pregnant and expression peaks massively in normally labouring samples. This data suggests that it is P2X7R that are important for labour.

6.3 ATP and Dysfunctional labour

The functional study on dysfunctional labour myometrial tissue found that ATP could be a useful additional stimulant to augment uterine contractility during parturition. This finding is consistent with a Russian clinical trial conducted on 16 women who experienced dysfunctional labour and received a combination of ATP and $PGF_{2\alpha}$ and 18 women in dysfunctional labour who received $PGF_{2\alpha}$ alone. They found that women who were administered ATP had a shorter first stage of labour with no side effects (Ziganshin et al., 2005). Oxytocin is recommended in clinical practice to help to increase uterine contractility during dysfunctional labour and reduce the number of caesarean sections; however, about 50% of women who are labouring poorly do not respond to oxytocin administration and ultimately require an emergency CS (Arrowsmith, Kendrick et al. 2010). Interestingly, the previous work on term pregnant rat myometrial tissue showed that the combined effect of ATP and oxytocin produced a significant increase in the myometrial contractile activity (Zafrah and Alotaibi, 2017). Further work to test the interaction of ATP with oxytocin in women with dysfunctional labour is needed, both in vitro and in vivo, as those women might benefit from this combination treatment.

Despite the limitation in the sample size of women who experienced dysfunctional or normal labour in this study, the Western blot was a useful technique to quantify protein expression accurately and provide an overview of total protein. The analysis found a significant upregulation of P2X7R in normal labouring compared to dysfunctionally labouring women. However, the technique cannot identify any variations in functional expression. Further investigation of surface expression by higher resolution immunofluorescence assay during dysfunctional and normal labour is required to determine its effect on myometrial contraction. The increased P2X7R expression in the normal labouring group from the western blot study indicates their critical role in the inflammatory process that occurs during labour, leading to an increase in the myometrial contractility response to ATP and helping in the augmentation of contraction during the onset of parturition. On the other hand, the low density of P2X7R due to the significant elevation of the extracellular acidification in the dysfunctional labour group could play a role in the underlying pathological mechanism of dysfunctional labour, leading to slow contraction during labour progress. It is anticipated that reducing acidity in the myometrium would help to upregulate the P2X7R expression and mediate the inflammatory reaction necessary to initiate the uterine contractions, hence improving labour outcomes in women who were labouring dysfunctionally. This could be achevied by using bicarbonate ingestion to neutralize the acidity associated with labour dystocia (Wiberg-Itzel et al., 2018). However, no normally labouring tissues were available for the contractility study to compare the actual contractile response to ATP mediated by the high density of P2X7R found in this group with that in dysfunctionally labouring women.

6.4 Future work

The findings from the current study point to the potential development of new treatments in clinical obstetrics to increase uterine contractility during dysfunctional labour, indicating the need for more research in this area. Examining the effect of ATP on myometrial contractions in biopsies from normal labouring women would be beneficial to compare with other gestational states and confirm the important role of P2X7R.

It would be interesting to compare ATPγS to oxytocin, the current stimulant used to help women with dysfunctional labours. Can ATPγS stimulate myometrium in circumstances where oxytocin fails to do so, for example? Is there a synergistic effect between their actions? If this is the case, lower doses of both drugs could be used, resulting in fewer adverse effects and desensitisation.

More work could be done to determine the expression of other myometrial P2XR subunits such as P2X4 or P2YRs in humans at a different gestational state or use the available selective antagonists at these receptors in functional assays to demonstrate if they have a role. The P2X4R, which is found to be co-expressed with P2X7R in the rat myometrium and different P2Y subunit receptors, is suggested to mediate the uterine contractile activity in response to ATP and its agonists in the human myometrium.

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