The Myometrial Effects of Progesterone

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Abstract

Introduction: Preterm birth is the leading cause of perinatal morbidity and mortality and rates are rising. The UK now has the highest rate of premature birth in Europe with 5.3% of overall births in Scotland occurring spontaneously before 37 weeks gestation (1, 2) .Preterm babies have higher rates of perinatal mortality and morbidity and those that survive are at risk of multiple conditions including respiratory distress syndrome, central nervous system abnormalities, necrotising enterocolitis and sepsis. The mechanisms of preterm birth are poorly understood. Preterm birth can be spontaneous or induced and spontaneous preterm labour has multiple aetiologies. Current evidence suggests that prolonged treatment with progesterone and 17 α-hydroxyprogesterone caproate (17OHPC) may reduce the incidence of premature delivery in high risk patients with a history of spontaneous preterm birth (3) or with a short cervix. However, progesterone is not uniformly effective in preventing preterm labour and at present its principal mode of action on myometrium is unknown. I aimed to determine some of the specific mechanisms of action of progesterone.

Aims: I hypothesised that progesterone has a direct inhibitory effect on spontaneous myometrial contractility, induces increased sensitivity to tocolytic agents and decreases sensitivity to contractile agonists such as oxytocin. I also hypothesised that progesterone has inhibitory effects on endogenous uterine stimulants, stimulatory effects on endogenous uterine relaxants, induces upregulation of endogenous receptors that inhibit uterine contractions and inhibits contraction associated proteins both *in vitro* and *in vivo*.

Methods: I recruited women already enrolled in the STOPPIT (a double blind randomised placebo controlled study of progesterone for the prevention of preterm birth in twins) who were given vaginal progesterone, or placebo and who were scheduled for caesarean section. I also recruited women with healthy twin or singleton pregnancies undergoing elective caesarean section. Myometrial biopsies were obtained from the upper border of the lower uterine segment incision during caesarean section. Samples were divided and used for contractility measurements, or subsequent mRNA, protein and immunohistochemical analysis.

Myometrial strips were cut and suspended under resting tension within organ baths. Concentration-response curves were carried out in response to oxytocin, levcromakalim, nifedipine and ritodrine to ascertain any reduction in effect by progesterone on oxytocics or enhancement of tocolytic effects. I also carried out concentration-response curves to progesterone alone and in the presence of potassium channel blocking agents. I then assessed *ex vivo*, the inherent contractility of the placebo versus progesterone groups from myometrium sampled from the STOPPIT cohort of patients.

I carried out cell culture experiments on myometrium from healthy singleton women who were not in labour at the time of sampling. Myometrial explants were placed in cell culture medium, cultured for 1, 4 and 24 hours, and the supernatants were then analysed using Bio-Plex array technology to ascertain cytokine release. I selected time points and concentrations conditions to incubate myometrial tissue using progesterone and 17OHPC and was able to assess cytokine release.The myometrial explants were used for subsequent molecular studies. I performed real time-polymerase chain reaction (RT-PCR) (Abi,Taqman) to quantitate endogenous inhibitors of uterine contractility (cGRPR, EP2,NOS), cytokines (interleukins- IL6, IL8, IL1ß), uterine stimulants COX-2 and gap junction components (connexin 26 and connexin 43) expressed relative to housekeeping gene 18s.

Lastly, I analysed the STOPPIT cohort of myometrial samples for to determine the in vivo effect of progesterone. We carried out RT-PCR (Abi,Taqman) to quantitate endogenous inhibitors of uterine contractility (cGRPR, EP2,NOS, PGDH), cytokines (IL6, IL8, IL1ß) and gap junction components (connexin 26 and 43).I performed immunohistochemistry, staining for localisation of pro-inflammatory cytokines. I then carried out protein expression analysis using Western blot for contraction associate protein, connexin 43.

The project was approved by North Glasgow University Hospitals Research Ethics Committee ref no. 05/S0705/18. All patients gave written informed consent to participate.

Results: I found that progesterone, exerted consistent, rapid and sustained inhibition of the amplitude of spontaneous myometrial contractions *in vitro* at high concentrations however, this affect was not achieved through the principal

potassium channels. Levcromakalim, a K_{ATP} channel opener, produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous contractions. These effects were antagonised by the K_{ATP} channel blocker, glibenclamide. In contrast, glibenclamide had no effect on the progesterone-induced inhibition of myometrial contractility. Charybdotoxin 10 nM (which blocks IK_{Ca}, BK_{Ca} and K_v channels), iberiotoxin 100 nM (which blocks BK_{Ca} channels) and apamin 100 nM (which blocks SK_{Ca} channels) failed to affect the ability of progesterone to inhibit myometrial contractility.

In contrast, 17OHPC did not exert any inhibitory effect on myometrial activity *in vitro*. Results indicated, at the selected pharmacological doses used *in vitro* that progesterone did not increase sensitivity to tocolytic agents tested. There was no decrease in sensitivity to the uterotonin oxytocin. Lastly, from our STOPPIT patient cohort I demonstrated no difference between the progesterone and placebo groups in either spontaneous contractility, response to tocolytics as above or response to oxytocin. One main conclusion of this arm of the study is that *in vivo* progesterone therapy to prevent pre-term labour does not appear to modify contractility *ex vivo*.

I demonstrated that administration of progesterone but not 17OHPC for up to 24 hours *in vitro* does not appear to modify mRNA expression of uterine stimulants such as cytokines, COX-2 or endogenous uterine relaxants such as NOS and PGDH. Progesterone but not 17OHPC inhibited production of gap junction component connexin 43. This modification of contraction associated protein is in agreement with other literature presented on human myometrial data *in vitro* (4) .

I used STOPPIT patients as a potential example of the myometrial effects of progesterone *in vivo* with a placebo treated control group. Prolonged maternal administration of progesterone appeared to inhibit expression of gap junction components connexin 26 and 43 in myometrium. Connexin 43 importantly, was also modified *in vitro* within the progesterone treated arm. However, *ex-vivo* assessment of the functional impact on human myometrium does not demonstrate a long-term inhibitory impact on myometrial function with down regulation of endogenous contractile inhibitors such as eNOS and EP2. The connexins play an essential role in regulating synchronous myometrial

contractions. If progesterone has been of benefit in those at risk of preterm labour with a history of spontaneous preterm birth, it is possible therefore that this is by reducing connexin expression, which prevents the development of these synchronous contractions whilst on progesterone therapy.

In summary, I have demonstrated putative mechanisms by which progesterone (and its analogue 17OHPC) might prevent preterm birth. Further studies characterising these pathways might inform the design of other agents which could provide additional efficacy in preventing preterm delivery.

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"The best laid schemes o' mice an' men gang aft agley"

Robert Burns

This thesis is dedicated to my "old" family, Dad, Mum and Robert, and my "new" family to be, Graham and our baby, Imogen Alexandra Robertson born 24/09/09.

Declaration

The contents of this thesis have not been submitted elsewhere for any other degree, diploma or professional qualification.

This thesis has been composed by me, and I have been responsible for patient recruitment, tissue collection and laboratory studies unless otherwise acknowledged.

Laurie Anderson, June 2009

Definitions

AKAP	membrane associated A kinase anchoring protein
ANOVA	analysis of variance
ATP	adenosine triphosphate
BK ca	large conductance calcium activated potassium channel
BMI	body mass index
bNOS	neuronal (brain) nitric oxide synthase
Ca	calcium
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
cGRP	calcitonin gene related peptide
cGRPR	calcitonin gene related peptide receptor
CNS	central nervous system
COX	cyclooxygenase
CRH	corticotrophin releasing hormone
Cx	Connexin
Da	Daltons
DAG	diacylglycerol
DEPC	diethyl procarbonate

DES	diethylstilboestrol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC50	half maximal effective concentration
ELISA	enzyme linked immunoassay
ELLUCS	elective lower uterine segment caesarean section
eNOS	endothelial nitric oxide synthase
EP2	endothelial phosphate prostaglandin 2 receptor
ETOH	ethanol
GABA	gamma amino butyric acid
HBSS	Hank's buffered salt solution
HCl	hydrochloric acid
IFNγ	interferon gamma
IK ca	intermediate conductance calcium activated potassium channel
IL	interleukin
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
IU	international Units
K	potassium

K ATP	ATP sensitive potassium channel
kDa	kilo Daltons
Kv	voltage operated potassium channel
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M	moles
MCP-1	monocyte chemoattractant protein-1
MLCK	myosin light chain kinase
mM	milliMoles
mN	millinewtons
mPR	membrane progesterone receptor
mRNA	messenger ribonucleic acid
N	newtons
NAD	nicotinamide adenine dinucleotide
NFκB	nuclear factor kappa B
nM	nanomoles
NO	nitric oxide
nPR	nuclear progesterone receptor
PGDH	15-Hydroxyprostaglandin dehydrogenase

PGF-2α	prostaglandin F-2 alpha
PIP2	phosphatidylinositol biphosphate
PKA	protein kinase A
PPROM	preterm premature rupture of membranes
PRA	progesterone receptor A
PRB	progesterone receptor B
PRC	progesterone receptor C
PRR	pattern recognition receptor
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	randomised controlled trial
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	real time-polymerase chain reaction
SEM	standard error of the mean
SK ca	small conductance calcium activated potassium channel
StAR	steroidgenic acute regulatory protein
STOPPIT	study of the prevention for preterm labour in twins
TLR	toll like receptor
TNFα	tumour necrosis factor alpha

◦C	degrees centigrade
μM	micromoles
17OHPC	17 α-hydroxyprogesterone caproate
AKAP	membrane associated A kinase anchoring protein

Chapter 1

Introduction and Literature Review

Introduction

Adaptive change of the uterus and myometrium during pregnancy is vital – initially this incorporates alteration of structure and size to accommodate the growing fetus, but then a change in phenotype to facilitate synchronous contractions to allow labour and delivery to occur. The mechanisms of this are still not fully understood in humans, and consequently the predicting and prevention of preterm labour remains one of the greatest obstetric challenges. Progesterone is thought to play a pivotal role in maintaining uterine quiescence. We have tried to expand our knowledge of the myometrial effects of progesterone and their role in labour both at term and preterm.

Preterm Birth and preterm labour

Preterm birth is the largest cause of perinatal morbidity and mortality; with rates of preterm birth rising. In the USA, the preterm delivery rate is 12–13% and in Europe and other developed countries, reported rates are generally 5–9% (5). The UK now has the highest rate of premature birth in Europe with 7.8% of overall births in Scotland occurring before 37 weeks gestation (6). Preterm birth is defined as birth before 37 completed weeks gestation by the World Health Organisation and has a multifactorial aetiology (7). Preterm birth is one of the largest contributors to mortality in the neonatal period and infancy and to morbidity later in life (8) and it accounts for 5–10% of all births in developed countries (9). In longer term, the morbidity amongst survivors of preterm birth has been noted to lead to poorer health and reduced achievement in school and beyond into adulthood. There are also significant economic costs and implications to both the health service and individuals affected as well as there families(10).

There is a degree of overlap within the definitions of preterm birth and labour. Preterm birth can be spontaneous or induced and medical intervention may determine a preterm delivery in indicated cases. Preterm labour is responsible for the majority, approximately 70% of preterm births (11). It may be appropriate to deliver a patient by caesarean section at 35 weeks with fulminating pre eclampsia due to the increased risk of maternal and fetal complications if no action is taken. Preterm labour however is when parturition

occurs prior to the 37 completed weeks of pregnancy. Our understanding of parturition as a normal physiological process is not yet complete and how this then becomes a pathological process prior to the 37 completed weeks of pregnancy is even less well understood. It is possible that preterm labour represents idiopathic activation of normal parturition process however the same triggers may not always be the initiating factor and it has been referred to as the “preterm parturition syndrome” in view of multiple aetiologies (12) .

The obstetric precursors which lead to preterm birth are 1) delivery for maternal or fetal indications, in which labour is either induced or the infant is delivered by prelabour caesarean section; 2) spontaneous preterm labour with intact membranes; and 3) preterm premature rupture of the membranes (PPROM), irrespective of whether delivery is vaginal or by caesarean section (5). Approximately 30–35% of preterm births are indicated, 40–45% follows spontaneous preterm labour, and 25–30% follows PPROM.

In general terms infants delivering at less than 24 weeks are considered pre-viable, under 28 weeks is extremely preterm, 28–31 weeks very preterm and 32–36 weeks moderately preterm (13).The percentage of deliveries according to the relative classifications of preterm birth are approximately 5% born less than 28 weeks, 15% between 28 and 31 weeks, 20% between 32 and 33 weeks and 60-70% at 34-36 weeks gestation (5). Preterm babies are at risk of multiple conditions including respiratory distress syndrome, central nervous system abnormalities, necrotising enterocolitis and sepsis. The prognosis for the preterm infant varies with presence and severity of complications, but usually mortality and likelihood of complications decrease greatly with increasing gestational age and birth weight. Preterm labour is associated with preterm rupture of membranes, cervical incompetence, polyhydramnios, fetal and uterine anomalies, infections, social factors, stress, smoking, heavy work and other risk factors. There is also a genetic link as women who experienced an early preterm birth less than 32 completed weeks in their first pregnancy have the highest rate of recurrent preterm birth in subsequent pregnancies (14).The diagnosis is made on the patients presenting symptoms, clinical findings and of progressive effacement and dilatation of the cervix (9).

There are multiple risk factors for preterm labour including African-American race, low body mass index (BMI), previous preterm birth or previous miscarriage, cervical abnormalities after colposcopic procedures as well as cervical or uterine anomalies can increase the potential for preterm labour. Fetal risk factors include fetal abnormalities, multiple pregnancy and polyhydramnios. Infection is often linked to preterm labour however many cases are simply idiopathic with no determining factors. Clinically once preterm labour is diagnosed by confirmation of regular uterine contractions and evidence of cervical change; it is managed with tocolytics thereby providing time for maternal corticosteroid administration which is known to reduce preterm morbidity and mortality (15).Notably, there is no effective long term management option to stop uterine contractions once they have established. This may however only be part of the preterm parturition problem as once the parturition process has commenced it may not be possible to stop the cascade of events that follow such as inflammation. Uterine contractions may therefore be a late event of parturition and we need to intervene at a far earlier point to succeed in stopping or preventing this process in the preterm. This suggests that to avoid preterm labour we must establish an effective mechanism to avoid the initial trigger and cause where appropriate.

Romero et al categorise several pathological processes that may well be implicated in preterm labour. These include intrauterine infection, inflammation as a mechanism or preterm labour, uterine ischemia, uterine over distension, cervical disease and hormonal disorders (12). I will utilise these subheadings to assess underlying mechanisms of preterm labour however our main focus will be on hormonal disorders and in particular progesterone and its mechanism of action.

Intrauterine Infection

The association between infection and preterm birth is well established and thought to be the underlying cause of preterm birth in up to 40% of all cases(16). Importantly, there is a gestational link between preterm labour and infection effect as around 24 weeks, nearly all spontaneous deliveries show evidence of intrauterine infection, however between 34 and 36 weeks, only about 15% show this relationship (17). Consistent with this amniotic fluid is normally sterile and

isolation of bacteria within the amniotic fluid is a pathological finding. The majority of these preterm births are caused by bacterial infections of the amnion and chorion, with organisms likely originating in the vagina. Bacterial infections within the uterus can occur between the maternal tissues and the fetal membranes (i.e. within the choriodecidual space), within the fetal membranes (the amnion and chorion), within the placenta, within the amniotic fluid, or within the umbilical cord or the fetus (18).The most common organisms present are a common pathogens implicated in the infection of the lower female genital tract, caused by heavy concentrations of a mixed group of organisms (19). The most commonly identified bacteria are Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, peptostreptococci, and bacteroides species (18). Many of these organisms are present in small numbers in the vagina normally as commensals, and although there is a strong association between the presence of bacterial vaginosis and preterm delivery it is not absolute (20). The mechanisms that convert a subclinical infection to actual preterm labour and rupture of membranes are thought to involve an inflammatory response including increased production of cytokines, prostaglandins, and metalloproteases – which can all have a direct effect on the myometrium and cervix.

Extrauterine Infection

There is also evidence that infection from extrauterine sites is linked to causality of preterm birth. Specifically, urinary tract infections (21) as well as periodontal disease may have implications as extrauterine sources of infection leading to preterm birth. It has been hypothesized that in the presence of severe periodontal disease, oral organisms can spread hematogenously to target the placenta, membranes, and fetus (22).

Inflammation

Human parturition is increasingly recognised to be an inflammatory event. It is therefore possible therefore that preterm labour may be due to a prematurely triggered inflammatory response. There is evidence to a suggesting that labour involves an inflammatory process and this involves myometrium, cervix, fetal membranes and placenta via a cascade of events (23-25). Broadly inflammation can either be acute or chronic. Acute inflammation is characterised by its short

duration, blood vessel dilatation and leakiness, exudation of fluid and plasma proteins and emigration of leucocytes (26). Many of these features have been seen in conjunction with human labour. Pro-inflammatory cytokines are thought to be closely involved in the parturition process and these cytokines may contribute to the onset of labour by stimulating interleukin-8 (IL8) production and prostaglandins (27). Cytokines such as IL1ß, IL6 and IL8 can induce cervical remodelling and weakening and rupture of membranes via synthesis of inducible nitric oxide synthase, cyclo-oxygenase-2 (COX-2) and matrix metalloproteinases (28, 29). In both term and preterm labour there is evidence to show increased levels of IL1ß, IL6 and tumour necrosis factor-alpha (TNFα) within amniotic fluid (30-32).

Previous research carried out within our laboratory was able to demonstrate a massive influx of inflammatory cells (neutrophils and macrophages) into both lower and upper segment of myometrium in association with labour (23). They found that parturition was associated with a significant increase in IL1-ß, IL-6 and IL-8 messenger ribonucleic acid (mRNA) expression in cervix and myometrium, IL-6 and IL-8 mRNA expression in chorio-decidua and IL1-ß and IL-8 mRNA expression in amnion. Histological analysis demonstrated that leukocytes (predominantly neutrophils and macrophages) infiltrate the uterine cervix coincident with the onset of labour. (24).These results suggested that inflammatory cell infiltration was part of the underlying physiological mechanism that occurred in myometrium during parturition.

Nuclear factor-kappa B (NF-κB) is a transcription factor which is classically associated with inflammation. Importantly, many pro-inflammatory and labour-associated genes are regulated by NF-κB and there is suggestion that it plays a central role in parturition both at term and preterm. TNFα has been shown to stimulate NF-kB activity in uterine tissues. In addition, many genes which encode pro-inflammatory cytokines, such as TNFα , IL1-ß, IL-8 and IL-6, contain NF-kB recognition elements within their promoters and NF-kB is known to promote the formation of cytokines in many cell types (27).

Pattern recognition Receptors (PRRs) is a group of molecules which detect the presence of microorganisms and can trigger a host response to limit tissue invasion. One subclassification of these are transmembrane PRRs which include

toll like receptors (TLRs).These TLRs are important components of the innate immune system and initiate an immune response to bacterial, viral and fungal pathogens by activating signal transduction pathways that induce expression of pro-inflammatory cytokines and chemokines (12) . TLR2 and TLR4 expression has also been shown to increase in human decidua and amnion in preterm labour (33). TLR activation therefore may be important in inflammation and infection associated preterm birth.

Data from a Swedish study by Hagberg et al shows evidence of infection related to PPROM before 34 weeks gestation. They found microorganisms detected within the amniotic fluid in 25% of women with PPROM and in 16% of those in preterm labour. Nearly half of these women had intra-amniotic inflammation defined as elevated IL6 and IL8, and there was a high degree of correlation between cytokine levels and preterm birth or the presence of microbial colonisation (34).

The current view is that during the course of ascending intrauterine infection, microorganisms may reach the decidua, where they can stimulate a local inflammatory reaction and the production of proinflammatory cytokines and inflammatory mediators (platelet-activating factor, prostaglandins, leucotrienes, reactive oxygen species, nitric oxide etc). If these pro-inflammatory cytokines are implicated in labour and preterm labour this offers one possible target site for treatment or prevention of preterm labour. To date however the use of antibiotics to treat infection related preterm birth has shown inconsistent results and they are of little benefit. This may be related to the different outcome parameters, antibiotic regimes as well as the dose and duration of administration and the route, whether vaginal or systemic (16).

Uterine Ischaemia

Maternal vascular lesions could lead to preterm labour by causing uteroplacental ischaemia. There is evidence that changes in the maternal vascular compartment of the placenta in patients with preterm labour and premature rupture of membranes are similar to those found in patients with preeclampsia (35). Abruptio placenta is also implicated in preterm delivery (36). The precise mechanisms responsible for premature labour in women with evidence of utero

uteroplacental ischaemia remains unclear however, the poor survival of the fetus in such a hostile environment may initiate parturition in such cases. One potential mechanism is via thrombin, a coagulation protein which possesses potent uterotonic activity *in vitro* and *in vivo*. This activity has been proposed to play a role stimulating uterine contractions which occur after delivery and also in preterm birth related to intrauterine haemorrhage (37, 38).

Uterine Overdistension

It has been established that multiple pregnancy and polyhydramnios are classical risk factors for preterm birth (5). The size of the uterus itself can be considered as a predisposing factor for preterm labour and in addition, PPROM is observed more frequently in twin pregnancies than in singletons (39). Uterine stretch can initiate myometrial contractions and lead to expression of gap junction protein connexin 43 (40) and increased oxytocin receptor (41) in pregnant myometrium. The hypertrophy of uterine myocytes is one of the earliest responses to steroid hormones and may modify the cellular responses to stretch and pressure (42). Within smooth muscle tissues, including myometrium, stretch has been shown to induce the depolarization of the cell membrane and increase action potential frequency and contractions.

Within cell culture, myometrium has shown increased IL8 mRNA expression, COX-2 activation and an increase in Prostacyclin (PGI2), a potent smooth muscle relaxant production in response to stretch (43, 44). Similar cell culture experiments demonstrated an increase in oxytocin receptor mRNA expression in response to stretch (41). It is possible that myometrium may respond and adapt to gradual stretch to accommodate a growing fetus whilst maintaining quiescence, however as labour approaches the uterus may become primed and is more responsive to lesser stimuli such as inflammatory cytokines, allowing a contractile phenotype to take over.

Cervical Disorders

Cervical insufficiency is usually linked to midtrimester miscarriage however some forms of preterm labour present with bulging membranes in the absence of uterine contractions, a form of precipitous labour at term or preterm (12) .

Measuring cervical length by transvaginal ultrasonography has been postulated as a predictor of preterm labour. Cervical studies may be useful in the prediction of preterm delivery, both a shortened cervical length identified on transvaginal ultrasound examination and an increased level of fetal fibronectin in cervico-vaginal secretions are associated with an increased risk of preterm delivery (45). These investigations may be useful to identify women at high risk in order to take appropriate action prior to the event.

The cervix plays a key role in normal and abnormal labour and nitric oxide (NO) is believed to be the final mediator in the mechanisms that allow ripening of the cervix (46).

A recognised congenital abnormality in cervical function is seen in women exposed in utero to the synthetic oestrogen diethylstilboestrol (DES). More commonly, women may have a potentially weakened cervix because of previous medical or surgical treatment. The commonest reasons for this are previous mechanical cervical dilation (commonly at termination of pregnancy or surgical evacuation at the time of miscarriage) or cervical biopsy or destruction during treatment for cervical neoplasia. It is these women that may well be identified as high risk of preterm labour and be suitable for further investigation.

Hormonal Changes

Progesterone maintains the pregnant state and promotes myometrial relaxation, but levels remain constant unlike many animal models in which a fall in progesterone pre-empts labour (47) . Parturition is also associated with oestrogen activation and a combination of progesterone withdrawal and oestrogen activation allows the myometrium to change from a quiescent state to a contractile state in order to trigger labour. Progesterone is considered vital for pregnancy maintenance in humans because inhibition of progesterone via administration of progesterone receptors antagonists such as RU486 (Mifepristone) given to pregnant women can induce labour (48). This will be discussed in more detail later within the introduction.

Current identification and treatment strategies for preterm labour

Labour is characterised by changes in the myometrium, cervix and other gestational tissues both at term and preterm.

Detection and diagnosis of preterm labour

Unfortunately, clinical detection of these early changes is limited and subsequently uterine contractions and cervical dilatation are used as indicators of labour. So far measuring biochemical markers to predict preterm labour is limited, although measuring fetal fibronectin, an extracellular matrix glycoprotein, in cervicovaginal fluid has shown some promising data towards improving the diagnostic accuracy of preterm labour (49).

Treatment of Preterm labour

Current treatment strategies tend to focus on the basis that a regularly contracting uterus before 37 weeks gestation is the most recognisable indicator of preterm labour. Uterine contractions therefore have become the most common area to commence treatment and thus by inhibition of contractions with tocolysis. There are several tocolytic agents used clinically but since their introduction to clinical practice they have not been shown to improve perinatal or neonatal outcomes and have adverse effects on women in preterm labour. They usually maintain the pregnancy for a further 48 hours allowing corticosteroid administration within a therapeutic window (50). Current tocolysis is likely to have limited success as the available drugs do not alter fundamental processes leading to the initial myometrial activation, instead they target one aspect of contractions , which due to inherent redundancy of the process accommodates this and preterm birth still occurs (51).

β- adrenergic-receptor agonists

The ß-adrenergic-receptor agonists cause myometrial relaxation by binding to $ß_2$- adrenoreceptors which subsequently increase the levels of intracellular cAMP. This in turn activates protein kinase which inactivates myosin-light chain kinase and reduces myometrial contractility (52). All 4 main types of adrenergic receptor appear to be present on the uterus. This will effect neuronal

modulation and therefore uterine smooth muscle contraction. Ritodrine had been used for many years but is unpleasant and has infrequent but potentially life threatening maternal side effects. In general high progesterone levels increase the numbers of ß-receptors and produce the formation of high affinity state ß-adrenoreceptors. It does produce a short term delay in delivery however should not be used as a first line treatment for preterm labour. A review of $ß_2$ agonists (53) looked at 11 RCTs involving over a thousand women. There was a significant decrease in the number of women giving birth within 48 hours of administration (RR 0.63, 95% confidence intervals 0.53 to 0.75) but no reduction on deliveries before 37 weeks (RR 0.95, 95% confidence intervals 0.88 to 1.03). There were significant increases in maternal adverse effects but no significant effects on perinatal deaths, respiratory distress, cerebral palsy, neonatal death and necrotising enterocolitis.

Calcium channel blocker

Drugs that target calcium channels do so by directly inhibiting the influx of calcium ions through the cell membrane and the release of intracellular calcium from the sarcoplasmic reticulum. The reduction on intracellular calcium leads to the inhibition of MLCK mediated phosphorylation which is calcium dependent and results in myometrial relaxation (54). Nifedipine is a smooth muscle relaxant which inhibits the intracellular influx of calcium ions. It acts via L-type voltage sensitive gated channels. It is currently recommended first line use as a tocolytic by the RCOG green top guidelines, despite not being licensed for this use in the UK (55). King et al carried out a systematic review of 12 randomized, controlled trials involving over a thousand women (55). In this meta-analysis, compared with other tocolytic agents, calcium-channel blockers reduced the number of women giving birth within 7 days after receiving treatment (relative risk, 0.76; 95% confidence intervals, 0.60 to 0.97) and before 34 weeks of gestation (relative risk, 0.83; 95% confidence intervals, 0.69 to 0.99). They also noted that calcium-channel blockers appeared to reduce the frequency of the neonatal respiratory distress syndrome, necrotizing enterocolitis, intraventricular haemorrhage, and neonatal jaundice.

Magnesium sulphate

Magnesium sulphate is the most commonly used first-line tocolytic in North America (56). It inhibits the contractile response and decreases the intracellular concentration of calcium in myometrial strips obtained from pregnant women that is consistent with both extracellular and intracellular mechanisms of action. This agent hyperpolarises the plasma membrane and inhibits myosin light-chain kinase activity by competing with intracellular calcium, which in turn reduces myometrial contractility (51). A Cochrane review of the world's randomized controlled trials, encompassing more than 2,000 women in 23 trials, concluded that magnesium sulphate tocolysis is ineffective at delaying birth or preventing preterm birth, and its use is associated with an increased mortality for the infant (57). Grimes et al suggest further use of this agent is inappropriate unless in the context of a formal clinical trial with informed consent for participants (58).

Oxytocin-receptor antagonists

Atosiban is a synthetic analogue of oxytocin and therefore a competitive antagonist. It competes with oxytocin for binding to the receptors in myometrium and decidua and prevents the increase in intracellular free calcium. Within a Cochrane review of oxytocin receptor antagonists for inhibiting preterm labour six trials (1695 women) were included. Compared with placebo, atosiban did not reduce the incidence of preterm birth or improve neonatal outcome and failed to demonstrate the superiority of atosiban over ß-agonists or placebo in terms of tocolytic efficacy or infant outcomes (59).

Nitric Oxide Donors

Nitric oxide is a vasodilator essential for the maintenance of normal smooth-muscle tone. Nitric oxide is synthesized during the oxidation of L-arginine (an essential amino acid) to L-citrulline. This reaction is catalyzed by the enzyme nitric oxide synthase, which exists in several isoforms. For all three nitric oxide synthase isoforms, nitric oxide synthesis depends upon the enzyme's binding of the calcium regulatory protein, calmodulin. For eNOS and bNOS , increases in intracellular calcium concentrations are required for their binding to calmodulin, and consequently for their full activation. By contrast, iNOS appears able to bind

calmodulin with extremely high affinity, even at the low intracellular calcium concentrations. In contrast to iNOS, the activity of eNOS and bNOS may be closely regulated by changes in intracellular calcium concentration (60).Both inducible (type 2) or iNOS and brain (type 1) nitric oxide synthases or bNOS are expressed in myometrial cells and blood-vessel endothelial cells, whereas endothelial (type 3) nitric oxide synthase (eNOS) is expressed exclusively in blood vessel endothelial cells. A Cochrane review of nitric oxide donors for the treatment of preterm labour included five randomised controlled trials (466 women) .Nitroglycerine was the nitric oxide donor used in all these trials. Nitric oxide donors did not delay delivery nor improve neonatal outcome when compared with placebo, no treatment or alternative tocolytics. There was, however, a reduction in number of deliveries less than 37 weeks (relative risk 0.69, 95% confidence interval 0.53-0.88).when compared with alternative tocolytics but the numbers of deliveries before 32 and 34 weeks were not influenced. Women were significantly more likely to experience headache when NO donors had been used (61).

Cyclooxygenase Inhibitors

Cyclooxygenase (COX, or prostaglandin synthase), which exists in two isoforms, COX-1 and COX-2, converts arachidonic acid to prostaglandin H2 (PGH2). PGH2 serves as a substrate for the production to the alternative prostaglandins and prostacyclins. COX-1 is expressed in human decidua, myometrium, and fetal membranes, whereas COX-2 dramatically increases in the decidua and myometrium during term and preterm labour (51). Pharmacologic blockade of prostaglandin production with COX inhibitors using nonsteroidal anti-inflammatory drugs such as aspirin is widespread within medicine (62). Indomethacin was the most commonly used tocolytic agent in this group. A Cochrane review of COX inhibitors for treating preterm labour (indomethacin only) demonstrated a reduction in birth before 37 weeks gestation. Numbers were small however within the clinical trials and the review suggested the information available was insufficient to form reliable conclusions (63).

Potassium Channel Openers

Myocytes maintain an electrochemical potential gradient across the plasma membrane, with the interior of the cell negative to the exterior, through the

action of the sodium–potassium exchange pump. A component of this process is a potassium channel, which is calcium and voltage regulated and allows efflux of potassium, thereby increasing the potential difference across the cell membrane and making it less likely to depolarize. Repolarisation of the cell is brought about by inactivation of the calcium current and a 4-aminorpyridine sensitive potassium efflux. The smooth muscle relaxation is caused by opening potassium channels and hyperpolarising the cells. Levcromakalim, a potassium channel opener, causes relaxation of smooth muscle cells. Although this drug is not used directly in obstetrics it has been used in the treatment of hypertension and asthma. It is widely used for smooth muscle relaxation in pharmacological experiments and has in the past been suggested as a potential tocolytic agent (64).

Parturition

Human parturition is still not fully comprehended and although some areas have been determined there are still missing pieces of the jigsaw. Within research animal models are often used however, human parturition is unique and therefore animal models cannot answer all the remaining questions. Some of the main differences in gene analysis between humans and animals are in the genes related to reproduction. The study of pregnant women therefore is paramount to further developments in our understanding of parturition.

Timing of birth

Corticotrophin releasing hormone (CRH) of placental origin levels increase as pregnancy gestation advances. They peak at the time of delivery suggesting that a placental clock determines the timing of delivery. In women with preterm labour the levels of CRH rise rapidly and also peak at the time of delivery (65). The levels of placental CRH are also modified by steroid hormones such as progesterone (66). Not all cases of preterm birth however, are related to changes in placental CRH production. In particular, intrauterine infection is not associated with elevated placental CRH production. Low levels of maternal plasma CRH does not therefore, rule out preterm birth. A single CRH measurement has relatively low sensitivity for predicting preterm birth, although

in an individual woman, a high CRH level has a relatively specific association with a greatly increased risk of preterm birth (67).

Placental CRH is also released into the fetus, and although the concentrations are lower in the fetal than in the maternal circulation, they still rise with increasing gestation. Stimulation of the fetal pituitary by CRH increases corticotropin production and, consequently, the synthesis of cortisol by the fetal adrenal gland and maturation of the fetal lungs. The maturation of the fetal lungs as a result of increasing cortisol concentrations is associated with increased production of surfactant protein A and phospholipids. These are critical for lung function.

There appears to be coordination between rising CRH production, fetal lung maturation and potential myometrial activation which initiates labour.

Fetal membranes

It is becoming more evident that rupture of the fetal membranes, term or preterm, is not solely due to stretch and forces of uterine contractions, but may also be the consequence of a programmed weakening process. In the majority of patients that labour at term contractions are initiated prior to the rupture of membranes. In general, artificially rupturing membranes in women will augment labour once it has started.

Labour associated pro-inflammatory cytokines, TNFα and IL-1ß, normally increase towards the end of pregnancy and during labour and can cause significant fetal membrane weakening. This mechanism is via a process involving collagen remodelling and apoptosis (programmed cell death) (68). Rises in cortisol, CRH can stimulate COX-2 within the amniotic fluid and in turn increase prostaglandin E2 production with other inflammatory mediators in the amnion (27). PGDH, a potent inactivator of prostaglandins produced from the underlying chorion becomes less active towards term and underlying decidua, cervix and myometrium are in turn, exposed to rising levels of prostaglandin E2 (67).These prostaglandins will promote release of matrix metalloproteases (which can degrade extracellular matrix proteins) and weaken the membranes leading to membrane rupture .

Oestrogen

Oestrogens (mainly estradiol) oppose the relaxatory actions of progesterone and augment myometrial contractility and excitability. The balance between the relaxatory actions of progesterone and the stimulatory actions of oestrogen is may well determine the contractile state of the myometrium and the timing and process of parturition (48).

The role of oestrogens in human parturition was studied by Pinto et al in the sixties and they found by administrating a large amount of 17β-estradiol to non-labouring pregnant women at term that estradiol treatment increased uterine contractility and the response to oxytocin. Those findings were consistent with the stimulatory actions of estrogens on myometrial contractility and showed that the progesterone block was not absolute and could be overcome by estrogenic drive (69).

There is consistent data which demonstrates the role of oestrogen as the active hormone required for myometrial transformation into a contractile state. In humans the circulating oestrogen levels increase around mid gestation and rise up until birth. There is also the developing concept that as for the functional progesterone withdrawal, oestrogens and the parturition process involve functional oestrogen activation. The term gestation myometrium is thereby more oestrogen responsive (48). Mesiano also showed that oestrogen receptor-α (ERα) mRNA levels correlated positively with cyclooxygenase type 2 and oxytocin receptor mRNA levels in non-labouring myometrium, indicating that the increase in ERα expression is directly associated with the activation of contraction-associated genes and estrogen responsiveness (70).

The current functional progesterone withdrawal theory (discussed within next section) that progesterone via its interaction with progesterone receptor B inhibits myometrial (ERα) expression and causes the myometrium to be unresponsive to circulating estrogens. This subsequently will increase contraction associated proteins and enable myometrial transformation into a contractile state which precedes labour.

Oxytocin

Oxytocin is a small peptide hormone with multiple sites of action in human body and in particular it is able to stimulate uterine contractions. This is achieved by multiple mechanisms involving sarcoplasmic reticulum calcium release and sensitization of the contractile apparatus to calcium. Its role within the parturition process is certainly established and it is evident within the second stage of labour allowing forceful uterine contractions to aid delivery of the baby. The role within the lead up and initiation of labour is less comprehensive.

Towards term it is thought that the uterus and therefore myometrium, transforms into an active contracting form from a quiescent one and this is via the action of various contraction associated proteins. It is now thought that the nonapeptide hormone oxytocin has a fundamental role in the stimulation process of converting myometrial tissue to an active from and stimulates co-ordinated powerful contractions of labour (71). Oxytocin has been shown to be vital for the expulsive contractile part of labour and, although there is less evidence a role for oxytocin in the initiation of labour is likely.

The concentration of oxytocin receptors increased in the myometrium of pregnant women and reach maximum levels in early labour. In vitro, prostaglandin production by the decidua, but not by the myometrium, was increased by the addition of oxytocin. Oxytocin may therefore stimulate uterine contractions by acting both directly on the myometrium and indirectly on decidual prostaglandin production (72).

The effects of oxytocin are mediated by tissue-specific oxytocin receptor expression, which leads directly to contraction in the myometrium and prostaglandin formation in the decidua. There is a significant increase in oxytocin receptor expression in these tissues in late pregnancy and pharmacological inhibition with oxytocin receptor antagonist delays delivery. This would suggest that, in contrast to oxytocin, the oxytocin receptor is essential for normal labour (73).

Progesterone

This is essential within normal pregnancy and parturition and a functional progesterone withdrawal is now thought to instigate labour in humans. I will discuss this in much greater detail as this forms the basis of this thesis project.

Progesterone and its structure

Progesterone (4-pregnene-3, 20-Dione) (Figure 1) is a C-21 carbon steroid that plays a vital role in life as well as an integral role in pregnancy. It is often referred to as the hormone of pregnancy and its name derived from “pro gestational steroid hormone” encouraging pregnancy to develop with the corpus luteum at the early stages of pregnancy until the placenta takes over this role at 7 to 9 weeks gestation. Progesterone like all other steroid hormones is converted from pregnenolone, a cholesterol derivative and is produced in the adrenal glands, brain, corpus luteum and the placenta. Like other steroids, progesterone consists of four interconnected cyclic hydrocarbons. Progesterone contains ketone and oxygenated functional groups, as well as two methyl branches. Like all steroid hormones, it is hydrophobic. Ultimately, it is an essential hormone for reproduction, involved in the menstrual cycle, implantation and pregnancy maintenance. During the menstrual cycle the corpus luteum produces progesterone which stimulates secretory activity from the endometrium. If fertilisation occurs, then implantation, the corpus luteum will continue to produce progesterone under the tropic stimulation of human chorionic gonadotrophin (hCG) produced by the synctiotrophoblasts of the conceptus (74).Placental trophoblasts take over production of progesterone at around 6-8 weeks gestation and luteal progesterone production decreases as the amount of hCG produced by the placenta diminishes (75). Progesterone production in pregnancy continues to increase until the placenta is delivered at parturition and at term the placenta can produce up to 250mg of progesterone each day. Placental progesterone synthesis starts with the conversion of cholesterol to pregneneolone by cytochrome P450scc in the placenta. Progesterone synthesis by the human placenta however differs from steroid synthesis in other steroid producing tissues such as the adrenal cortex and corpus luteum. The human placenta is distinct from other steroid producing tissues in that it does not express the steroidgenic acute regulatory (StAR) protein. Within the placenta

pregneneolone is then converted to progesterone by type 1 3β-hydroxysteroid dehydrogenase located in the mitochondrion. Progesterone synthesis is stimulated by cAMP in the human placenta but uncertainty remains regarding the key hormones that control cyclic AMP levels (74).

17-Hydroxyprogesterone caproate (17-α-Hydroxypregn-4-ene-3, 20-dione hexanoate) (Figure 2) is a synthetic hormone that is similar in structure to medroxyprogesterone acetate. This however, is not the same progestin as 17-Hydroxyprogesterone (a metabolite of progesterone). There is supporting evidence that 17OHPC is no better than progesterone at binding to PR-A or PR-B isoforms and is no better than progesterone in eliciting gene expression of progestin-responsive genes (76).As well as progesterone, 17OHPC is being used within clinical trials as a potential progestin to prevent preterm labour.

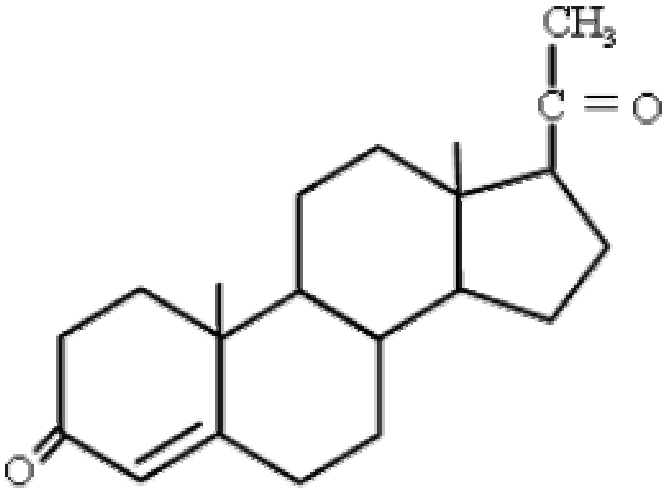

Figure 1 : Structure of progesterone (4-pregnene-3, 20-Dione)

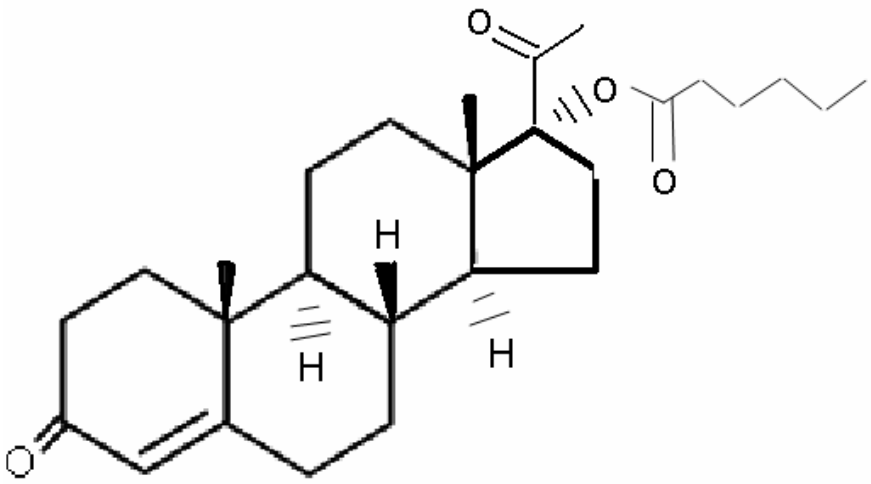

Figure 2 : Structure of 17 hydroxyprogesterone caproate (17-α-Hydroxypregn-4-ene-3, 20-dione hexanoate)

Progesterone and the functional progesterone withdrawal

Progesterone exerts its effects through both genomic and non genomic pathways, genomic pathways being mediated through nuclear progesterone receptors nPRs (77).The progesterone receptor isoforms are PR-A, PR-B, and PR-C. It has long been established that progesterone is a pre-requisite for a successful pregnancy outcome, with functional withdrawal of progesterone now thought to underlie human labour. This is regulated via the progesterone receptors, the full length PR-B and truncated (164 N-terminal amino acids) PR-A under the control of separate promoters. PR-B is suggested to be the principal mediator of progesterone actions and PR-A represses the activity of PR-B. As PR-A and PR-B have opposing actions the progesterone response is thought to be related to the ratio of PR-A to PR-B. At parturition PR-A expression increases until the PR-A/PR-B ratio reaches a point whereby the relaxatory actions mediated through PR-B are inhibited i.e., functional progesterone withdrawal and this may preclude labour in humans (48, 77, 78). The increase in myometrial PR-A expression with advancing gestation decreases PR-B and eventually eliminates the PR-B-mediated inhibition of ERα expression. The third progesterone receptor isoforms PR-C does not have the ability to bind DNA but can bind progesterone. There is some evidence to suggest that PR-C can bind to the PR-B isoforms thereby reducing the ability of PR-B to bind to progesterone receptor response elements and inducing loss of the progesterone quiescence. There is also a proposed association with upregulation of PR-B and PR-C expression associated with activation of the transcription factor nuclear factor-kappa B(NF-κB) in fundal myometrial samples (79). NF-κB is classically associated with inflammation and thought to be a key step within the parturition pathway (80). The role and presence of PR-C however, is somewhat controversial and there is disagreement between research groups regarding its existence as a naturally occurring isoform (81, 82). Further clarity on the progesterone receptor C and its role within parturition is still required.

Progesterone may inhibit myometrial contractility via a number of mechanisms and this forms part of the main focus of this thesis.

Genomic actions of progesterone

These genomic pathways alter the expression of specific contraction associated genes in order to modify the contractile phenotype. The contraction associated proteins which are of importance within the parturition process include the oxytocin receptor, prostaglandin F-2 alpha receptor ($PGF_{2\alpha}$), the gap junction component connexin 43(Cx43) and the prostaglandin-metabolising enzyme,15-hydroxy-prostaglandin-dehydrogenase (PGDH).

Progesterone can be inhibited by administering RU486 in rat myometrium and as a result, an increase in expression of myometrial oxytocin receptor was observed (83). Myometrial $PGF_{2\alpha}$ receptor mRNA levels significantly increased during labour at term and during ovariectomy-induced preterm labour in rats and this increase was blocked by progesterone (84). Progesterone can also promote inactivation of prostaglandins via increased PGDH (85).

Synchronous activity of myometrial cells in normal labour culminates in uterine contractions which are necessary to expel the fetus. Equally important are the periods of relaxation between contractions, which permit blood flow to the fetus. The uterus lacks a pacemaker that regulates these contractions however there is clearly evidence of synchronous activity in labour. The synchrony is achieved by electrical conduction through connected myofibrils, which transmit the electrical activity to nearby muscle fibres. The activated myocytes produce prostaglandins, which act in a paracrine fashion to depolarise their neighbouring myocytes. This process progresses as more myocytes are recruited into the contraction (67). Gap junctions are transmembrane proteins that serve to synchronise contractions within the uterus and a major component of myometrial gap junctions is connexin 43 (Cx43) (Figure 3).

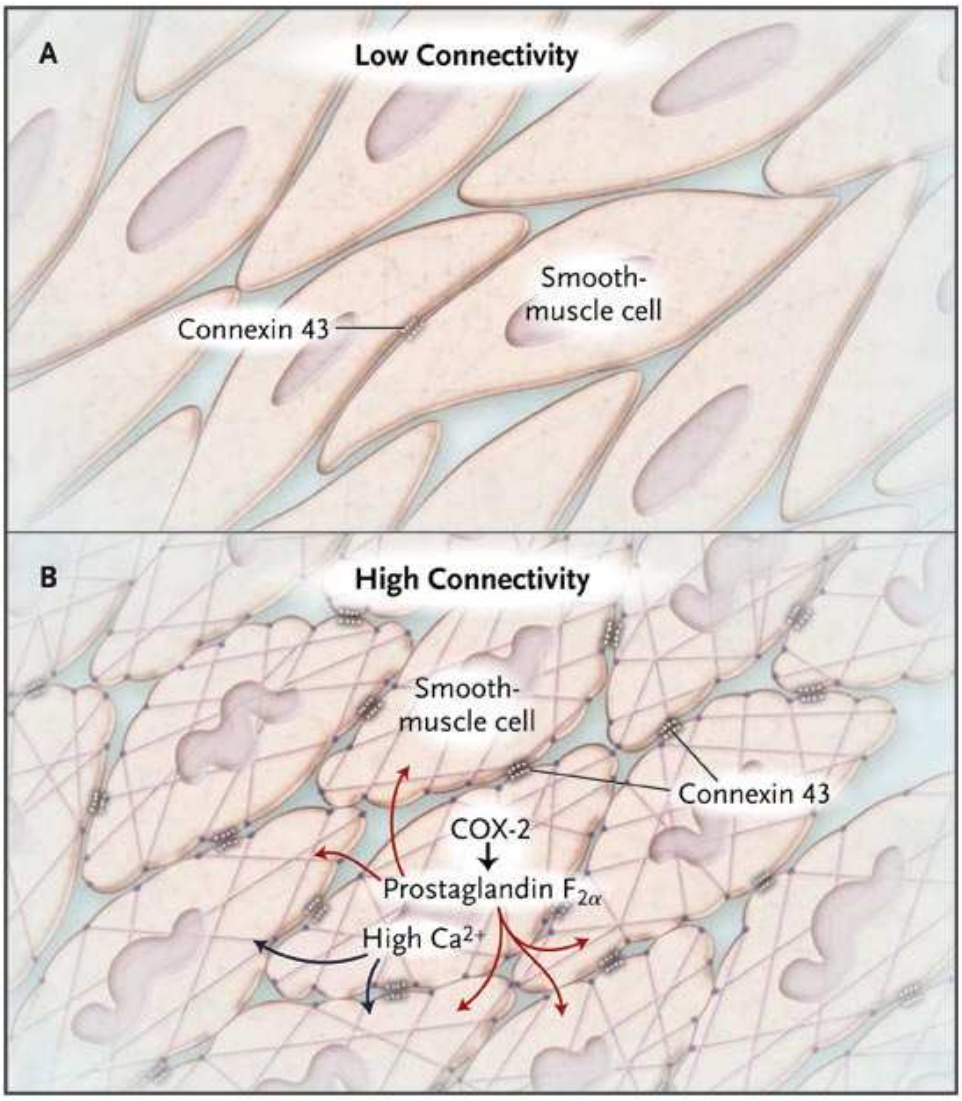

Figure 3 : Myocytes in Labour. During labour the myometrium converts from a low connectivity state (A) to a high connectivity state (B). The connections occur through gap junctions formed from s in particular Cx 43. Connections are also formed from the paracrine release of prostaglandins and local calcium concentrations. (Figure reproduced from Smith et al review on Parturition, with permission (67)).

These are upregulated with the onset of labour (86, 87). Progesterone represses the expression of Cx 43 within cultured human myometrial cells (4) and estrogen can upregulate expression (88). It is thought that Cx 43 is synthesised days prior to parturition but accumulates within the cytoplasm until labour when it is transported to the plasma membrane and forms part of the gap junction plaques present at the cell surface (89).

The assembly of gap junction protein into functional gap junction plaques is the last point in a stepwise process that begins with oestrogen-dependent expression of the Cx 43 gene and continues with synthesis of Cx 43 in the rough endoplasmic reticulum and transport to the Golgi, followed by its trafficking to the plasma membrane and its assembly into functional gap junctions. Hendrix et al found that the trafficking of myometrial Cx 43 from the Golgi and assembly into gap junctions at the plasma membrane was suppressed in progesterone treated rats but suggested that it may be the trafficking of to the plasma membrane and its assembly into functioning gap junctions which is important to the synchronous activity rather than just its synthesis (90).

Progesterone is known to up regulate endogenous inhibitors of uterine contractility such as calcitonin gene related peptide (91) and nitric oxide synthase (92) as well as inhibition of contraction associated proteins.

Receptor-coupled pathways that promote uterine relaxation involve activation of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) that phosphorylate proteins regulating calcium homeostasis. cGMP is the second intracellular messenger of several hormones whose main effect is relaxation of smooth muscle. cAMP is the second intracellular mediator of a significant number of hormone substances such as prostaglandins and caltitonin gene related peptide. The end result of these hormones is smooth muscle relaxation and therefore suggests a possible role in myometrial quiescence (93).cAMP has inhibitory effects on myometrial contractile activity and some of these effects require association of protein kinase A (PKA) with a plasma membrane-associated A-kinase-anchoring-protein (AKAP)(94) . Sanborn et al also demonstrated that PKA was associated with the myometrial plasma membrane and declined towards the end of pregnancy and that this decline was attenuated by progesterone and promoted by an antiprogestin (95).

Non-genomic actions of progesterone

More rapid non-genomic pathways are less well understood and act via a variety of ways including membrane progesterone receptors (48).The non genomic actions are characterised by a rapid time course , i.e. minutes rather than hours, no nuclear progesterone receptor activity and no requirement for RNA or protein synthesis (77) .

The non genomic effects of progesterone have been identified in other organs and systems including inducing the acrosome reaction in sperm (96) as well as its effects on the central nervous system, reducing CNS excitability by its neuroprotective properties (97).

Myometrial strips can be examined *in vitro* in order to ascertain the effects of progesterone. Generally studies have shown progesterone and its metabolites exert rapid inhibitory and concentration-dependent effects on spontaneous myometrial contractions, excluding 17α-hydroxyprogesterone caproate(17OHPC) which had no effect *in vitro* (98-100). Some however, have reported an increase in contractile frequency (101, 102). There is some variation between studies regarding the specific progestin, the doses used and the original contraction method used whether oxytocin induced or spontaneous. The data however is consistent that the affects seen are rapid, and sustained, suggesting a non genomic mechanism.

The myometrium is not highly innervated and does not contain pacemaker cells but myometrial function is influenced by both steroid and polypeptide hormones as well as cytokines and other signalling molecules(103) . One hypothesised mechanism is that progesterone inhibits myometrial contractility directly and or sensitises the uterus to other tocolytics. Several lines of evidence support this. For example, a recent clinical trial using vaginal progesterone *in vivo* reported that patients in the progesterone arm of the study responded more favourably to tocolysis with ß-mimetics than the placebo group (104). *In vitro* studies have also shown that progesterone sensitises the uterus to tocolytic agents such as ritodrine a ß-2 agonist (99). This has been suggested to operate via a non-genomic pathway. *In vivo* animal studies suggest that this may be via increased myometrial ß-adrenoreceptor density. They also reported a concentration

dependant relaxant effect of myometrial contractility using progesterone (105, 106).

Another suggested non-genomic pathway is by interacting with other receptors such as gamma amino butyric acid A ($GABA_A$). In rat myometrial models, using $GABA_A$ antagonists they were able to demonstrate a blocked inhibition of contractility with progesterone, suggesting that progesterone mediated its action via this receptor (107). More recently however, Perusqia et al showed that the relaxation produced by progesterone and its 5-reduced metabolites was not blocked by the $GABA_A$ receptor antagonists but was reversed by calcium. They suggest that the relaxant action of the sex steroids is not mediated by an interaction with $GABA_A$ receptors and instead a blockade of calcium influx appears to be responsible (108).

The non-genomic pathways by which progesterone itself inhibits myometrial contractility in vitro may operate through the cell membrane progesterone receptors α, ß and γ, which are similar to G-protein coupled receptors (109). Mesiano et al details explanations of these membrane receptors and their role within progesterone's mechanism of action and the functional withdrawal, this is summarised below. These membrane receptors have been identified recently and their exact role within human pregnant myometrium is not yet known. Several studies have shown that mPRα and -ß are expressed in the human pregnancy myometrium and activation of mPRα and mPRß in cultures of human myometrial cells decreased cAMP levels and increased phosphorylation of myosin. They proposed that these effects (decreased cAMP and activation of myosin) augment contractility. It is also evident that mPRα and mPRß increased the transcriptional activity of PR-B, which would be expected to decrease contractility via the genomic pathway. This proposed that for most of pregnancy the genomic actions of PR-B dominate to relax the myometrium and that mPRα and mPRß augment this pathway by augmenting PR-B activity. At parturition, functional progesterone withdrawal decreases PR-B actions, allowing non-genomic actions mediated by mPRα and -ß to become dominant, and therefore increase contractility by decreasing cAMP and increasing myosin phosphorylation. This suggests during pregnancy non-genomic and genomic actions of progesterone relax the myometrium, whereas after the functional progesterone withdrawal occurs prior to labour non-genomic pathways mediated

by mPRα and mPRß may prevail and promote contraction (48, 77). This may explain why in myometrial tissues the response to progesterone can sometimes vary and an increase in frequency observed. If the tissues tested were sampled at a time after the functional progesterone withdrawal and converted to a contractile phenotype then these may be less responsive to progesterone and still able to contract.

Progesterone and the prevention of Preterm labour

It has long been established that progesterone is a pre-requisite for a successful pregnancy outcome with functional withdrawal of progesterone now thought to underlie human labour as described above. In the 1956 Csapo published new data from rabbit models which proposed his “progesterone block hypothesis”. He suggested that progesterone maintained pregnancy by actively blocking labour and that removal of this block or a fall in progesterone preceded parturition (110). In the majority of animals this theory is upheld however in humans, parturition occurs without a fall in systemic progesterone levels. This has proven the subject of extensive research amongst many groups since the initial proposed hypothesis by Csapo. More recently progesterone and other progestins have been advocated as a supplementary treatment in order to prevent preterm birth. Consistent with this theory recent clinical trials have demonstrated a beneficial effect of supplementation with either progesterone or the synthetic progestin 17 α-hydroxyprogesterone caproate (17OHPC) in the prevention of preterm labour (104, 111). However, progesterone is not uniformly effective in preventing preterm labour and at present its principal mode of action on myometrium is unknown (112).

The clinical trials carried out recently have differed in the form of progesterone used, its method of administration, the gestation at commencement of treatment and the order of pregnancy, whether singleton or multiple. Although no teratogenic effects have been described with most progestins, there is little data regarding its long term safety. Maternal side-effects from progesterone therapy include headache, breast tenderness, nausea, cough and local irritation if administered intramuscularly. Current data regarding the optimal dose of progesterone, mode of administration, gestation to commence therapy, or duration of therapy is limited and continually under scrutiny (113, 114).

Dodd et al (114) published a meta-analysis of randomised controlled trials reviewing progesterone for the prevention preterm birth. In summary the primary outcome measures were perinatal mortality, preterm birth less than 34 weeks gestation and major neurodevelopmental handicap at childhood follow up. They also considered secondary outcomes such as threatened preterm labour

and PPROM. Their analysis of the following six publications (104, 111, 115-118) involved 988 women. The routes of administration varied with da Fonseca et al using vaginal progesterone 100 milligrams (mg) daily and the other 5 trials using intramuscular (IM) 17OHPC, administered doses varying from 250mg once weekly to a maximal 1000mg once weekly. The gestational age for commencement of progestin started at 20 weeks and finished at 32-37 weeks depending on the trial. Hatikainen et al was a twin study and the remaining were singleton patients identified at high risk of preterm labour. The results of the meta-analysis indicated that progesterone administration is associated with a reduction in the risk of preterm birth less than 37 weeks' gestation (six studies, 988 participants, relative risk (RR) 0.66, 95% confidence interval 0.54 to 0.79), and in infant birthweight less than 2500 grams (four studies, 763 infants, RR 0.63, 95% confidence interval 0.49 to 0.81).They concluded that intramuscular progesterone was associated with a reduction in the risk of preterm birth less than 37 weeks' gestation, and infant birthweight less than 2500 grams. They suggest however that further information was required about the use of vaginal progesterone and the potential long term effects of progestogens on maternal and infant health.

More recently data published on triplet pregnancies reported no reduction in the rate of preterm birth with IM 17OHPC (119). Furthermore in 2007, a randomised controlled trial reported no reduction in the frequency of recurrent preterm birth under 32 weeks, with prophylactic progesterone in women with a history of spontaneous preterm birth. They used a 90mg vaginal progesterone preparation from 18-22 weeks until 37 weeks gestation. They suggest that patients with a history of previous spontaneous preterm delivery constitute a heterogenous population and that a subset of these patients may benefit from progestin prophylaxis (responders) while others would not (non-responders) (120). This may explain the conflicting results amongst studies of progesterone to prevent preterm labour. In contrast , a similar regime of progesterone to O'Brien et al used in women with a short ultrasonographic cervical length les than 28 millimetres(mm) concluded that the rate of preterm birth under 32 weeks was significantly lower for those receiving progesterone than it was for those receiving the placebo (121). Another paper which examined progesterone use in women with a short cervix demonstrated that spontaneous delivery before 34 weeks of gestation was less frequent in the progesterone group than in the

placebo group. The criteria differed from the above study with cervical length of 15 mm or less and vaginal progesterone, 200 mg daily or placebo from 24 to 34 weeks of gestation (122).

Progesterone and the prevention of Preterm labour is a continually developing and evolving field of research. The most recent meta-analysis available at the time of writing this chapter was published in December 2008 by Tita et al. This review focuses on data from 2000 onwards and identified 8 randomised controlled trials, 6 meta-analyses and 3 national guidelines using the search terms “preterm” and “progesterone”. Overall, they suggest the data indicates that prophylactic use of progesterone does lead to a reduction in preterm birth and low birth weight. However, the data are less conclusive that progesterone improves neonatal morbidity and mortality. They make the following suggestions for guidelines in the use of progesterone for the prevention of preterm labour. In high risk women with a history of spontaneous preterm birth, weekly IM 17OHPC (250 mg) commenced at 16-20 weeks, or daily vaginal progesterone (at least 100 mg) beginning before 24 weeks should be given. In twin pregnancy, progesterone is not routinely indicated, although its use may be considered in women with a history of spontaneous preterm birth or short cervix of 15mm or less. Finally, in women with a singleton pregnancy and a short cervix of 15 mm or less, 200 mg of vaginal progesterone suppositories may be appropriate (3).

Progesterone and the prevention of Preterm labour: STOPPIT

The clinical trial upon which this study was partly based, STOPPIT (A randomised, double blind placebo controlled STudy of Progesterone for the Prevention of Preterm Birth In Twins) was carried out in the UK and submitted for publication in April 2009 (123). Five hundred women with twin pregnancy were recruited from UK NHS clinics specialising in the management of twin pregnancy. Women were randomised either to daily vaginal progesterone 90mg (Crinone®) or to placebo, given double blind for ten weeks from 24 weeks gestation. The primary outcome was delivery (or intrauterine death) prior to 34 weeks gestation (ISRCTN 35782581).

The findings indicated that the combined proportion of intrauterine death or delivery prior to 34 weeks of pregnancy was 24.7% in the progesterone and 19.4% in the placebo group, odds ratio [95% confidence intervals] of 1.36 [0.89 to 2.09]. Norman et al concluded that progesterone, administered vaginally, does not prevent preterm birth in women with twin pregnancy. The data are in agreement with those of Rouse (124) who showed that 17OHPC, administered intramuscularly, failed to prevent preterm birth in twin pregnancy. In conclusion different pathophysiological mechanisms may account for the apparent difference in efficacy of progesterone in high risk singleton and twin pregnancy and progestogens are not indicated in twin pregnancy to prevent preterm birth.

Progesterone potential mechanisms of action

Although there are some data which suggest that progesterone may not be of benefit to prevent preterm labour, due to the multifactorial background of preterm labour it appears likely that there are subgroups of patients for which a supplementary progestin may be beneficial. The outcomes of further clinical trials are awaited and evolving clinical data may support current trial guidelines. The consensus statement arising from the 46th Study Group of preterm birth within the Royal College of Obstetricians and Gynaecologists (RCOG) state that the role for progesterone in the management of preterm birth needs to be further critically evaluated. This research should address the dose, mode of administration and potential benefits and or risks of using progesterone in pregnancy. Its current use should be restricted to randomised controlled trials and further clinical trials need to be adequately powered to address neonatal and infant outcome (125).

This study, in particular focuses on the myometrial effects of progesterone. We aimed in particular to look at both *in vivo* and *in vitro* effects of progesterone upon pregnant human myometrium. The data on myometrial effects of progesterone in pregnancy are limited and further understanding of its mechanism of action is required in order to enable the efficacy of progesterone in the prevention of preterm delivery to be maximised. We aimed to examine the effects of progesterone on myometrium *in vitro*. We were also uniquely able to examine progesterone effects *in vivo* with access to the cohort of women participating within the STOPPIT clinical trial. We then aimed to compare the

effects of progesterone *in vivo* and *in vitro*. We therefore examined the following hypotheses.

Progesterone has a direct inhibitory effect on spontaneous myometrial contractility

Prolonged *in vivo* treatment with progesterone may directly inhibit myometrial contractions and *in vitro* treatment with progesterone or 17OHPC may inhibit contractions. Progesterone weakly inhibited myometrial contractions *in vitro* and other metabolites such as 5-ß dihydroprogesterone were more efficacious in reducing myometrial contractile activity (98, 126). The study of myometrial strips under organ bath settings within progesterone conditions either added *in vitro* or from STOPPIT participants aims to determine any direct effects of progestins on myometrial contractility.

Progesterone induces increased sensitivity to tocolytic agents

Prolonged *in vivo* administration of progesterone may sensitise the uterus to tocolysis. *In vitro*, incubation with progesterone increased the efficacy of the tocolytic agent, ritodrine (99). Within one clinical trial of women receiving vaginal progesterone versus placebo that presented in preterm labour, the progesterone treatment group appeared to respond more favourably to tocolytic treatment with ß- mimetics (104). We aimed to look at *in vivo* progesterone compared to placebo within the STOPPIT group and the efficacy of tocolytics examined *ex vivo* within organ baths. We also hypothesised this increased sensitivity would be seen with *in vitro* progesterone.

Progesterone induces decreased sensitivity to contractile agonist oxytocin

In a similar manner as above, we hypothesised that progesterone may decrease the sensitivity of myometrium to oxytocin, a potent uterotonin. A limited number of patients given a large bolus of progesterone in vivo into amniotic fluid decreased the frequency of contractions and weakened the response to oxytocin (127).

Progesterone utilises the potassium channel

Progesterone exerts its effects through both genomic and non genomic pathways, genomic pathways being mediated through nuclear progesterone receptors nPR's(77). Potassium also has a major role in maintaining uterine quiescence (128). Potassium channels are widely expressed on the myocyte membrane surface (129); specifically adenosine triphosphate sensitive-potassium channels ($K_{ATP)}$, BK_{Ca} (large conductance channel), IK_{Ca} (intermediate conductance channel), K_V (voltage-operated channel) and SK_{Ca} (small conductance channel) are all present in pregnant myometrium (128). Additionally, levcromakalim, a K_{ATP} opener, has been shown to exert a concentration-dependent inhibition of spontaneous myometrial activity *in vitro* (64, 92). The opening of potassium channels or closure of calcium channels by progesterone could either hyperpolarise or prevent depolarisation of the cell respectively and would thereby potentially directly inhibit contractile activity. It remains unclear, however, by which mechanism progesterone causes rapid myometrial relaxation. It has an instant non genomic effect and we hypothesised that this may have some impact by acting through potassium channels as these also maintain uterine quiescence. By using specific potassium channel blocking agents such as glibenclamide (blocks voltage gated potassium channels K_{ATP}), apamin (blocks small channels SK_{CA}), charybdotoxin (blocks intermediate channels IK_{CA}, BK_{CA}, Kv) and Iberiotoxin (large conductance channels BK_{CA}) we aimed to answer this hypothesis.

Progesterone has inhibitory effects on endogenous uterine stimulants and stimulatory effects on endogenous inhibitors of uterine contractions

The onset of parturition, both term and preterm is associated with increased myometrial prostaglandin concentrations via both increased synthesis and decreased breakdown (130, 131).There is also good evidence of increased pro-inflammatory cytokine expression (24). Prostaglandins and cytokines both are able to stimulate myometrial contractions (129, 132). We hypothesised that the mechanism of action of progesterone may include the suppression of endogenous myometrial prostaglandin and cytokine production. In addition to these inhibitory effects of progesterone on uterine stimulants, progesterone may

potentially upregulate endogenous inhibitors of uterine contractility such as calcitonin gene related peptide (91) and nitric oxide synthase (92).

Progesterone induces upregulation of endogenous receptors that inhibit uterine contractions

In addition to the direct effects on stimulators and inhibitors of uterine contractility, we hypothesised that progesterone may alter receptor concentration. In rat models, progesterone has upregulated expression of the prostaglandin EP2 receptor (133) and calcitonin gene related peptide receptor, both of which promote uterine quiescence (134).

Progesterone inhibits contraction associated proteins

Connexins 26 and 43 are major components of gap junctions within the myometrium. They serve to electrically couple adjacent cells and allow the uterus to function synchronously. Progesterone inhibits both hormonal and stretch induced expression in rat myometrium *in vivo* and *in vitro* (40, 49). We hypothesised that the mechanism of action of treatment with progesterone may include the inhibition of gap junction formation *in vivo* and *in vitro*.

Myometrial contractility

In order to understand the mechanism of preterm labour it is also important to consider the underlying mechanism of uterine contractility. Uterine contraction and its physiological mechanisms of modulation highlight the importance of control of the smooth muscle, myometrium, during pregnancy and parturition. (135)

The myometrium is composed of interlacing bundles of long, spindle shaped smooth muscle fibres, they are surrounded by collagen fibres, fibroblasts and bone marrow derived cells. In pregnancy myometrium grows with hypertrophy and there is an increase in the overall number of smooth muscle cells. Contractions occur through sliding of actin and myosin filaments.

Smooth muscle can develop spontaneous activity with or without hormonal and neuronal control. An isolated piece of pregnant of non pregnant myometrium will produce regular spontaneous contractions. These must be preceded by action potentials and the basis of this follows spontaneous depolarisation of pacemaker cells in the myometrium. These pacemaker cells are not in a specific anatomical site as in cardiac muscle. Changes in the permeability of the cell membrane to potassium (increase) and sodium (decrease) occur to give slow depolarisation of the membrane potential preceding the action potential. In human myometrium we know that resting membrane potential is between -45 and -50mV (136). In general the upstroke of the action potential is related to calcium entry and repolarisation due to inactivation of calcium channels and potassium efflux. Calcium entry is via voltage gated channels which are opened by spontaneous pacemaker activity, hormonal and neuronal control. The contractile activity is highly dependant on extracellular calcium concentration. This can be blocked by dihydropyridine derivatives, such as nifedipine, suggestive therefore that these are L-type channels. Repolarisation is brought about by inactivation of the calcium current and a potassium efflux. Levcromakalim, a potassium channel opener, causes relaxation in smooth muscles including the uterus and in a dose dependant manner. In other smooth muscles the drug exerts this effect by opening potassium channels and hyperpolarising the cell. In the uterus relaxation is usually only accompanies by a small hyperpolarisation i.e. their mechanisms may differ. Different hormones

and neurotransmitters may change electrical activity within these cells. Agonists can bring about uterine contraction by membrane potential-independent mechanisms such as the release of calcium from the sarcoplasmic reticulum by a chemical messenger, 1, 4, 5-triphosphate (IP3) .This is well known to be initiated not by depolarisation, but by agonist–receptor interaction which and is described as pharmacomechanical coupling. Another such pathway involving calcium entry via voltage operated channels after depolarisation can arise spontaneously within the myometrial membrane. The entry of calcium then leads to further depolarisation. Another such mechanism is an increase in intracellular calcium concentration produced by the agonist binding to its receptor, stimulating receptor operated channels, causing depolarisation which then activates voltage operated channels allowing more calcium to enter. An increase in the intracellular calcium concentration produced by an agonist induced calcium release from internal stores is another method by which activity can be enhanced. The agonist stimulates a G protein (secondary messenger) which inactivates phosphoinositidase C which produces 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) from phosphatidyl 4, 5-inositol biphosphate (PIP2) in membrane. IP3 causes release of calcium from the sarcoplasmic reticulum. DAG will stimulate protein kinase C which is further hydrolysed to phosphatidic acid and arachidonic acid. This can in turn be used for prostaglandin synthesis. The rise in intracellular calcium results in the formation of an active compound between calcium, calmodulin and myosin light chain kinase (MLCK). Calcium binds to the calmodulin forming a calcium-calmodulin complex, which allows the activation of enzyme myosin light chain kinase. This phosphorylates light chains on myosin allows actin binding and increases actin-activated myosin MG-ATPase concentration, a contraction may then occur with the hydrolysis of ATP. The phosphorylation of the myosin light chain causes the generation of ATPase activity, which promotes the sliding of myosin over the actin filaments and the movement that constitutes a contraction. Relaxation will occur as intracellular calcium lowers by uptake into internal stores and extrusion across the sarcolemma. The calcium calmodulin-MLCK complex is then inactivated.

There does appear to be a lack of overall understanding concerning uterine contractility and there are many modulators of control. The uterus has autonomic innervation via parasympathetic fibres. All 4 main types of adrenergic receptors are present on the uterus. In general α-activation stimulates

contraction and ß-activation mediates relaxation. ß-2 receptors are predominantly responsible for relaxation (137). Progesterone appears to produce the formation of high affinity state ß-adrenoreceptors. ß -adrenoreceptor agonists, such as ritodrine, have therefore been used to try to control preterm labour as this is linked with uterine relaxation.

Activation of the uterus and myometrium

The regulation of uterine activity during pregnancy and labour take place over several stages. During pregnancy, the uterus is maintained in a state of functional quiescence through the action of various mediators, including progesterone, prostacyclin, nitric oxide, parathyroid hormone–related peptide, corticotropin-releasing hormone, and calcitonin gene–related peptide. Before term, the uterus undergoes activation and stimulation perhaps as a result of the functional progesterone withdrawal. Activation then occurs in response to uterotonins, including estrogen, and is characterized by increased expression of a series of contraction-associated proteins (including myometrial receptors for prostaglandins and oxytocin), activation of certain ion channels, and an increase in connexin 43 (a key component of gap junctions). An increase in gap junctions between adjacent myometrial cells leads to electrical synchrony within the myometrium and allows effective coordination of contractions. Once activated, the "primed" uterus can be stimulated to contract by the actions of uterotonins such as oxytocin and the stimulatory prostaglandins. Preterm labour may represent an early activation of this normal term process due to various trigger factors.

Summary

Despite research efforts so far, the molecular mechanisms responsible for the onset of labour in humans, both at term and preterm, remain unclear. By utilising human myometrium from pregnant patients and focussing on its function and response to progesterone we aimed to elucidate some of these functional and molecular mechanisms. It is evident that progesterone plays a central role within this process however, the fundamental differences between animal models and humans have left unexplained questions.

There is much evidence about animal models and the role of progesterone *in vitro* however little evidence is available about the effects of progesterone on human myometrium *in vivo*. We aimed to try and ascertain some of the myometrial effects of progesterone by investigating women actively participating in a clinical trial of progesterone versus placebo to prevent preterm labour. It is this *in vivo* aspect that is unique to our study and an important aspect of the parturition pathway that has yet to be fully explained with appropriate supporting evidence.

Progesterone is integral to the parturition process. The prevention of preterm labour therefore, is a much more achievable research goal if its underlying mechanisms of action are clarified within human myometrium.

CHAPTER 2

Materials and methods

Patient Selection

Experiments were carried out on patients undergoing elective caesarean section at or greater than 37 weeks. These patients had clinical indications for an elective caesarean section such as previous caesarean, breech presentation, maternal request. All patients were consented prior to theatre and myometrial biopsies were collected by the operating obstetrician. I was able to perform all consents personally to give a full and detailed explanation to the patient allowing time to consider participation in the trial and also to fill out the appropriate paperwork. In order to maintain the same conditions each specimen was taken after delivery of the baby but prior to administration of 5 IU syntocinon. Anaesthetists administering the drug were informed prior to theatre to withhold syntocinon until the biopsy had been taken. This took approximately 5 minutes following delivery of the baby. At the time of caesarean section a myometrial biopsy (measuring 10mm wide by 10 deep by 20 mm length) was taken from the upper border of the incision into the lower uterine segment and closed routinely as required for caesarean section providing minimum risk to the patient. I was present in theatre at each biopsy in order to place the sample in the correct medium as soon as possible and to check each sample was biopsied correctly and from the same part of the lower uterine segment. The tissue for contractility, cell culture and molecular studies was immediately placed in separate buffers (Krebs Henseleit buffer, HBSS) prior to transportation to the laboratory. All tissue for functional contractility and tissue culture experiment data was placed immediately in Krebs or HBSS buffer respectively for use within 12 hours.

Women recruited to the *in vivo* study of placebo versus progesterone were patients included in the “STOPPIT” trial. This is a double blind randomised placebo controlled study of progesterone for the prevention of preterm birth in twins and was taking place within Scotland. Within the clinical trial, participants were randomised either to daily progesterone gel (90mg) (Crinone®) or placebo gel both administered vaginally by the participant and starting at 24 weeks + 0 days gestation. Drugs were supplied in a sealed opaque covering. Each cover contained a single use, one piece, white polyethylene applicator with a twist-off

top, designed for intravaginal insertion. Each applicator contained 1.45 g of gel and delivered 1.125g of gel, containing either 8% progesterone or excipients (glycerin, light liquid paraffin, hydrogenated palm oil glyceride, carbopol 974P, sorbic acid, polycarbophil, sodium hydroxide and purified water) only. Patients were required to administer the gel at the same time each day and also asked to keep a diary of any symptoms or added information. They generally attended the antenatal clinic between every two to four weeks and were seen by a designated STOPPIT midwife. They were also contacted by telephone at regular intervals to ensure compliance and general wellbeing. If patients were subsequently scheduled for caesarean section for obstetric clinical reasons such as twin 1 breech presentation, then they were selected and consented for this sub study. We collected samples form both elective and emergency caesarean section .All information, regarding STOPPIT placebo or progesterone treatment was blinded at the time of collection and the myometrial biopsy was taken as described above. Once the myometrial biopsy was obtained it was divided into 5 small sections. One placed in a Krebs buffer for functional contractility analysis and use within 12 hours. Another was placed in formalin for subsequent paraffin embedding and immunohistochemistry. The final 3 were placed in “RNA Later” (in view of multiple site location with trial based in several Scottish hospitals) for 4 weeks and then snap frozen in liquid nitrogen. These were then stored at -80°C for subsequent protein and molecular analysis.

Chemicals used in functional studies

All concentrations stated are final bath concentrations. All drugs were obtained from Sigma (Poole) except for iberiotoxin and charybdotoxin which were obtained from Latoxan (France). All drugs were dissolved in saline except levcromakalim (DMSO solvent), oxytocin (acetic acid), nifedipine (ethanol), non water soluble progesterone (70% ethanol), 17OHPCC (70% ethanol)and glibenclamide (DMSO).Water soluble progesterone, Sigma (Poole) is contained within a cylclodextrin compound. This enables hydrophobic molecules to be incorporated into the cavity of the cylclodextrin by displacing water, when the water soluble complex is dissolved in a larger volume of aqueous solvent the process is reversed thereby releasing the molecule into the solvent .Stock solutions of each chemical were made in advance and stored at -20ºC. Fresh solutions were made on the day of each experiment.

Lower uterine segment versus upper segment myometrium

Myometrium can be collected from the lower uterine segment at the incision site for caesarean section. Collection from this site involves the least risk to the patients since it is already the site of incision and could be performed by the operating obstetrician with competence in caesarean section. Upper segment biopsies require more senior obstetricians to obtain and are more likely to provoke extra bleeding and are therefore less acceptable to participants. Previous works within the group have directly compared upper and lower myometrium and have observed changes seen in labour in both upper and lower tissues (23). Other studies compared the contractile characteristics of myometrium taken from upper and lower uterine segments. Biopsies obtained from women undergoing classical caesarean section therefore upper and lower segment samples were available. Myometrial strips were dissected and mounted in an organ bath, and contractions recorded. There were no significant differences in the contractile rate and force produced by myometrium from upper and lower segments therefore demonstrating that for contractile studies, the use of lower segment is appropriate and there is little regional functional difference (138).

Lower segment tissues still give adequate results and that extra risks and difficulties of obtaining upper segment tissue was not justifiable. All biopsies therefore are from the lower uterine segment.

Contractility studies

Subject and preparation of tissue

Ethical approval for sample collection was obtained from the North Glasgow University Hospitals Research Ethics Committee Reference number 05/S0705/18(STOPPIT sub study) and Glasgow Royal Infirmary Ethics Committee number 08/S0704/41 .All patients gave written informed consent to participate.

Women recruited to the *in-vivo* study of placebo versus progesterone were patients included in the "STOPPIT" trial

Women recruited to all other aspects of the research were singleton patients undergoing elective caesarean section at 37 weeks or greater for the following clinical indications, previous caesarean section, breech presentation and maternal request. Patients were excluded if they were undergoing elective caesarean for placenta praevia, history of fibroids or had previously had a post partum haemorrhage greater than 1L.

Myometrial biopsies 2cm long, 1cm wide and 1cm thick were taken from the middle portion of the upper lip of the lower segment caesarean section incision. This was done following delivery of the baby and prior to administration of syntocinon 5IU. All biopsies were immediately placed in a buffered Krebs solution (NaCl 133nM, KCl 4.7nM, glu 11.1nM, $MgSO_4$ 1.2nM, KH_2PO_4 1.2nM, $CaCl_2$ 1.2nM, TES 10nM). Tissues were stored at 4°c and used within 12 hours of collection. Strips of myometrium 15mm long, 2mm wide and 2mm deep were cut under no tension. There was no stretch applied to tissue prior to its attachment to the rig for equilibration. The strips were secured with silk and placed under isometric conditions with a 20mN resting tension. Contractility data was recorded via a tension transducer (FT03, Grass Technologies, Slough, UK) and the signal amplified and stored in a commercial data acquisition system (Octal ML228 Bridge Amplifier; PowerLab ML870/P, Chart, version 3.6, all AD Instruments. Each tissue strip was suspended in a separate 10ml organ bath filled with Krebs-Henseleit solution, gassed with 95% O_2/5% CO_2, and maintained at 37 °C. Tissues were equilibrated for a minimum of 2 hours prior to adding any substances. When suspended in this manner strips developed rhythmic activity

which stabilised within 90 minutes to 3 hours. Once stable activity had developed with the amplitude and frequency having less than 5% variation between contractions then we were added drugs as relevant for each experiment. Each biopsy from a participant was divided into identical strips. One a strip from each participant was used to test each drug and a further strip was used as a control strip. Additionally multiple drugs were tested in parallel from each participant. No participant provided more than one strip for each drug (or control). All analysis of amplitude and frequency was read in real time and inserted into the CHART® datapad software. This was then transferred to Microsoft Excel for further analysis. If a strip did not develop stable activity then this arm of the experiment was excluded. If more than 1 strip failed to contract then the subsequent experimentation and analysis was not possible so this patient would be excluded.

Organ bath experiments

Oxytocin

At the end of the 2 hour equilibration period rhythmic contractile activity was assessed in each myometrial strip. 6 organ baths were available within this protocol and a control strip was used for each patient. Oxytocin was used to stimulate contractions. A concentration-response curve was carried out between 10 pM and 100μM. 20 minute time intervals were used for each concentration to allow drug interface interaction.

Tocolysis

Three separate tocolytic agents were tested, nifedipine a calcium channel blocker, levcromakalim a potassium ATP channel opener and ritodrine a $ß_2$-agonist. Concentration-response curves were obtained from nifedipine and levcromakalim 10 pM-1μM and 0.01μM to 100μM for ritodrine hydrochloride. Each drug was tested on separate myometrial strips in a cumulative fashion. 6 organ baths in total were available. This allowed a separate strip from the same patient to be treated with oxytocin, levcromakalim, nifedipine and ritodrine as well as 2 control strips.

STOPPIT trial

After stable activity had developed in myometrial tissues from the specific STOPPIT myometrial samples collected we added oxytocin 100 picoM -100 μM and derived a full concentration response curve. In separate baths each of the tocolytics nifedipine 1nM-100 μM, levcromakalim 10nM-10 μM and ritodrine 10nM-0.1mM were added in cumulative fashion to individual myometrial strips. A control strip was included for all experiments to examine the inherent amplitude and frequency of the placebo vs. progesterone group. We recorded amplitude and frequency as described before at the time of each experiment whilst still blinded to the groups to minimise bias.

The STOPPIT codes were unlocked for the specified patients after the experiments were completed, and analysis performed as comparison of *in-vivo* placebo vs. progesterone tested under *in-vitro* conditions.

Progesterone incubation *in vitro*

We carried out concentration-response curves with oxytocin (stimulates myometrial contractions 10 pM-10 μM), levcromakalim (a K_{ATP} channel opener, 100 pM -10 μM), nifedipine (a calcium channel blocker, 100 pM-10 μM) and ritodrine (a ß-2 agonist, 0.01 μM-1 mM) all final bath concentrations, on separate strips from patients, n≥6. We then carried out concentration-response curves to oxytocin, levcromakalim, nifedipine and ritodrine as described above, following an incubation of each myometrial strip with progesterone 1 μM from a separate group of patients n≥6. This concentration was selected as it became clear that ethanol had a tocolytic effect itself and therefore we wished to minimise this. Progesterone 1 μM (dissolved in 70% ethanol) with an appropriate ethanol vehicle control (0.07% final bath concentration) was added to the myometrial strips at the end of the equilibration period for a 60 minute time period before carrying out each concentration-response in the second group and we recorded amplitude and frequency as before.

Progesterone and 17-α hydroxyprogesterone caproate

Progesterone and 17OHPC were dissolved in 70% ethanol stock. Water soluble progesterone was dissolved in distilled water. All potassium channel blockers other than glibenclamide were made into saline solution. DMSO solution was used for gliblenclamide. Oxytocin was dissolved in 2% acetic acid solution and tocolytic substances were made in saline solution. All drugs were obtained from Sigma (Poole), iberiotoxin and charybdotoxin from Latoxan, France. Each drug was measured into stock concentrations and fresh solutions were made for each separate experiment. Progesterone (10nM-100μM) was added in a cumulative manner at 20-minute intervals, amplitude and frequency were recorded at the end of each time period. Similar experiments were then carried out with 17OHPC. Each of progesterone and 17OHPC were dissolved in a 70% ethanol vehicle, 10-3 M stock. Vehicle controls were included in all experiments. In view of ethanol having a significant tocolytic effect all progesterone experiments were repeated using water soluble progesterone. Both concentration-response curves and time point analyses were carried out for water soluble progesterone and the same concentrations. For time plot experiments a single dose of progesterone 10μM, 30μM and 100μM was added to organ baths after the equilibration period. The amplitude and frequency was recorded as before, after stable rhythmic activity achieved around 2 hours and then measured at 30 minute intervals noting both amplitude and frequency. This was recoded for 4 hours after addition of the progesterone.

Potassium channels blocking functional studies

Given the demonstrable inhibitory effect of ethanol at higher concentrations that we observed on contractions, all subsequent experiments were conducted with only water soluble progesterone. Each of the potassium channel blocking agents, glibenclamide 1.5 μM (blocks K_{ATP} channels), charybdotoxin 100 nM (blocks intermediate channels IK_{Ca}, BK_{Ca}, K_v), iberiotoxin 100 nM (blocks large conductance BK_{Ca} channels) or apamin 100 nM (blocks small SK_{Ca} channels) were added to myometrial strips at the end of the equilibration period. Concentration-response curves were then carried out adding water soluble progesterone as described above. Experiments were performed on separate strips with n≥6 for each agent. Time matched controls were included for all agents and a DMSO vehicle control was also included for glibenclamide. Time course experiments were also repeated with progesterone 100 μM after the addition of each potassium channel blocking agent. If no spontaneous activity developed in an organ bath then this arm of the experiment was excluded.

Potassium channel blocker	KATP	BKCa	IKCa	SKCa	Kv
Glibenclamide	+				
Charybdotoxin		+	+		+
Apamin				+	
Iberiotoxin		+			

Table 1: Potassium channel blocking agents

Contractile analysis

Spontaneous contraction amplitude was taken as the mean amplitude of three consecutive contractions recorded prior to the addition of drug. Stable activity is defined as a series of at least 3 contractions reaching the same amplitude and frequency after the 2 hour equilibration period. The response to drug was defined as the effect on a single contraction at the time point of interest and recorded in milliNewtons (mN). Frequency was recorded in Hertz (number of events occurring per second) by measuring the interval between the peaks of two consecutive contractions occurring at the time point of interest. This was subsequently converted to contractions/h^{-1}. For time point analysis, contraction amplitude and frequency were analysed following the development of stable rhythmic activity, immediately prior to addition of drug, time 0, and at every 30 minute periods thereafter. An integral measure of contractile activity was determined by the area under the tension curve for each contraction and recorded for 60 minutes after the addition of each substance.

Contractility statistical Analysis

The tension (amplitude of contraction) of the myometrial strips was recorded in millinewtons (mN). A reduction in the magnitude of the contractions was expressed as a percentage inhibition (mean ± SEM) of the contraction obtained immediately before the first addition of the drug. The frequency of contractions was defined as the number of contractions h^{-1}. Each data set from each myometrial strip was transferred from the Chart® datapad onto Excel spreadsheets. Statistical analysis was carried out using one-way analysis of variance with Bonferoni correction and Kruskall-wallis test for non parametric data as well as paired t test for parametric data (Graph Pad prism, Minitab). A value of $p< 0.05$ was considered to indicate statistical significance.

Cell Culture

Patients were selected and were asked for informed consent as described previously. Myometrial biopsies were obtained each morning from non-labouring elective caesarean section patients. These were placed into HBSS (Sigma) immediately after delivery. They were then transferred to the laboratory and in tissue culture hood washed 3 times in Dulbeccos PBS (Sigma) to remove any excess blood. The biopsy was then dissected into small pieces (10-30mg weight). Three myometrial explants of known weight were then placed into each of a 24 well plate containing 1ml of Isocoves medium supplemented with 100µl/ml penicillin,100µg/ml streptomycin(500µl in 50ml) and 250ng/ml fungizone(amphoterocin 50Vl in 50ml). After a pre-incubation at 37ºC for 45 minutes in a humidified chamber the explants were transferred to a new well containing fresh culture medium. Tissue samples were incubated with nil (control) , Progesterone(water soluble) at 10-6M, 10-5M, 10-4M concentration and 17-α hydroxyprogesterone caproate 10-6M,10-5M,10-4M . DMSO controls were carried out for 17 hydroxyprogesterone caproate as it was made in stock solution at 10-1M with 100% DMSO. Each well was carried out in duplicate and separate wells were used for the LDH assay to check tissue viability. After incubations periods of 1hour, 4hours and 24 hours supernatants were collected in duplicate and stored at -20 ºC. Myometrial explants were frozen in liquid nitrogen at the end of the 24 hour period and these were stored at -80 ºC for subsequent RT-PCR analysis. LDH assays were performed on the supernatants and a standard curve was performed using LDH to quantify results. This allowed us to confirm tissue viability for the 24 hours of the tissue culture experiment.

LDH Assay

The LDH (lactate dehydrogenase) assay was used as a means of determining membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The resulting coloured compound is measured spectrophotometrically. If cells are lysed prior to assaying the medium, an increase or decrease in cell numbers results in a change in the amount of substrate converted. This indicates the degree of inhibition of cell growth (cytotoxicity) caused by the test material. With myometrial explants we were unable to determine the actual cell number so weights were noted for each experiment. We also used LDH to make standards 1000, 750, 500, 250, 125, 62.5, 31.25, 15.625 mU/ml) in order to quantify the LDH in vitro toxicology assay kit (Sigma). We used both methods as described by the manufacturer, the first providing a measure of total cell LDH and the second assessing the membrane integrity of cells as a function of the amount of LDH leakage into the supernatant. We also left tissue culture explants for greater than 24 hours and measured LDH release into the supernatant over time. We prepared a tissue culture experiment as described above and collected the supernatants to measure LDH with the different conditions of progesterone, 17OHPC and DMSO. We also performed a separate group of cell cultures in order to ascertain LDH release. Using 4 time points 0, 1, 4 and 24 hours we wanted to clarify LDH release at time 0 with and without the lysis buffer. This gives us a comparison of levels of LDH spontaneously released from untreated cells and maximum LDH activity that can be released in response to lysis. At time 0 we incubated tissue in duplicate with Isocoves medium and lysis buffer and then froze samples at -20 ºC to perform LDH assay together with other time points. Using 3 separate 24 well plates for the 1, 4 and 24 hour time points we performed duplicate cell culture for myometrial explant tissue alone (in Isocoves medium) and also incubated tissues with a lysis buffer and isocoves medium. The lysis solution was added in a 1/10 volume and incubated for 45 minutes at the end of each time point. Supernatants were then collected at each time point and frozen at -20 ºC. For the LDH assay we used a 96 well plate, and carried out eight standards for LDH in combination to clarify the kit was detecting LDH correctly. We transferred the all supernatants and standards and centrifuged this at 1100rpm

for 4 minutes. Two wells containing medium (isocoves) only were included as blanks. Every sample was measured in duplicate neat and at a 1: 10 dilution. We prepared the LDH assay mixture by adding equal amounts of LDH substrate, enzyme and dye adding this in an amount equal to half of the volume of culture medium. This was then incubated (covered in foil to protect from light) for 30 minutes. 15µl (a 1/10 volume) of 1N HCl was added to each well to terminate the reaction. We then measured absorbance spectrophotometricallly at a wavelength of 490nM and measured the background absorbance of the plate at 690nM and subtracted from the primary wavelength measurement (Ascent software®). This allowed us to compare LDH release at time 0 from all other timepoints used within the cell culture experiments and indicating the degree of LDH release over time. The medium (blank) wavelength could then be subtracted from all of the results and points compared to our standards and also over time.

In another LDH assay we measured supernatant LDH release over time at 24, 48, 72, 96, 120, 144 hours to determine cell membrane integrity over time.

BIO-PLex

Cytokine quantification was carried out with Bio-Plex (BIO-RAD®) system and suspension array technology. Supernatant samples were defrosted and centrifuged at 10,000rpm for 1 minute. 100% beads and neat supernatants were selected from a test bioplex experiment with duplicate samples. A pre-designed selection of cytokines were analysed using the BIO-RAD system .This is a multiplexed, particle based, flow cytometric assay which utilises anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportion of two fluorescent dyes. Each of the 100 spectrally addressed bead sets can contain a captive antibody specific for a unique target protein. Fluorescent intensity of the bead identifies the reaction. Our assay was customised to detect and measure IL1β, IL6, IL8, IL10, IFN γ, MCP and TNF α. Cytokine assays were performed as per the manufacturer's protocol (BIO-RAD®) system. Following completion of the cell culture step we then stored supernatants at -20 ºC. The samples were then selected using each control, progesterone and 17OHPCC concentration as well as standards according to the Bio-Plex protocol. We used a labelled 96 well plate with corresponding key for each concentration

and drug. All weights of initial tissue were recorded and taken into account for the final analysis of results. The results were expressed as picograms per ml/1gm wet weight.

BIO-PLex cytokine assay from BIO-RAD®

Sample preparation

We centrifuged the supernatants at 10,000 rpm for 1 minute and then tested dilute samples up to 1:20 if required –test first. A neat solution was decided as optimum conditions. All samples were kept on ice for the duration of the experiment. With the Bio-Plex kit a standard is available; this was diluted in the same medium as cell culture supernatants. A 96 well plate was suitable for 2 patients using a total of 3 separate kits for 6 patients in total. Adding 500ul of appropriate matrix then vortexing gently and incubated on ice for 30 minutes. We prepared multiplex bead working solution from 25 x beads Bio-Plex (BIO-RAD®) and protected beads from light by wrapping in foil. We filled 96 wells of the filter plate with 100ul Bio-Plex assay buffer each time removing the buffer by vacuum filtration and drying the bottom of filter plate thoroughly with a clean paper towel (lint free). The multiplex beads are vortexed for 15-20 seconds at medium speed and 50ul pipetted into each well and again removing the buffer by vacuum filtration each time. 100ul of Bioplex wash buffer was added to each well and then removed by vacuum filtration and this step repeated. 50ul of each diluted standard and supernatant sample was then added into each well and covered with sealing tape. The plates were covered with foil and then shaken at 1,100rpm for the first 30 seconds then 300rpm for 30 minutes at room temperature. We removed the buffer by vacuum filtration and wash 3 times with 100ul Bio-Plex wash buffer blotting the bottom of filter plate with clean paper towel after every wash to prevent cross-contamination. The detection antibody solution 25ul was then added to each well. It was covered with sealing tape and with foil and shaken as described before. The 3 washes were repeated as. Streptavidin-PE 50ul was added to each well covered and shaken as before. The 3 washes were then repeated. We then resuspended beads in each well with 125ul Bio-Plex assay buffer covered with sealing tape and shaken at 1100rpm for 30secs and stored at 4 ºC overnight before analysing the plate the following day .

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded myometrial samples from STOPPIT patients using antibodies against IL- 1b, IL-6, IL-8, TNFa, and CD45. These were selected in view of previous works published on these antibodies within our laboratory ad their importance in the role of parturition. Sections 5 mm thick were cut from the paraffin-embedded tissues, mounted on silane-coated slides, heated to 60◦C for 35 min, depariffinized in xylene, and rehydrated in a graded alcohol series. Endogenous peroxidise activity was quenched using 0.5% hydrogen peroxide in methanol. Sections were washed in PBS, and antigen was retrieved by microwaving in a pressure cooker (Lakeland Plastics Ltd., Cumbria, UK) at full power for 5 min in citrate buffer (10 mM, pH 6.0).All sections to be incubated with antibodies against interleukins were washed in PBS with 0.1% saponin (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) then blocked with 20% rabbit/20% human serum. Sections to be incubated with CD45 were washed in PBS then blocked in 20% horse/20% human serum for 30 minutes at room temperature. Sections were then incubated overnight for 16 hours at 4◦C with the primary antibody diluted either in 2% rabbit serum with 0.1% saponin (cytokines) or in 2% horse serum (CD45). Sections were washed in either PBS/0.1% saponin or PBS alone and then incubated for 30 min with biotinylated rabbit anti-goat (Vector Laboratories, Peterborough, UK) (cytokines) or biotinylated horse anti-mouse (Vector) (CD45) diluted 1:200 in 2% rabbit serum in PBS/0.1% saponin or 2% horse serum in PBS both with 5% human serum added. Sections were washed in PBS/0.1% saponin or PBS alone and then incubated with avidin DH/biotinylated horseradish peroxidase H reagent (Vector) in PBS for 30 min before final washing. The antigen was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 0.2% hydrogen peroxide in 50 mM Tris HCl, pH 7.6, and appeared as a brown end product. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Negative controls included slides incubated without the primary antibody.

For analysis we looked at each stained section for all STOPPIT myometrial biopsies obtained. We stained the tissue for cytokine localisations however we did not attempt to analyse staining intensity semi-quantitatively for these. We described each slide and then grouped them according to their STOPPIT placebo or progesterone category for descriptive purposes. The leucocytes were

identified using histological analysis in ten randomly selected high powered fields (x 400 objective magnification) and were counted by two independent observers who were blinded to the specimen details. The area for each high-powered filed was 0.23mm^2. The means for each observer were calculated and an unpaired t-test used to perform statistical analysis, a p value of <0.05 was considered significant.

RNA extraction for RT-PCR

Total RNA was extracted and purified from the lower segment myometrial samples using the Tri-reagent method (Trizol, Invitrogen Ltd). This technique was employed for RNA isolation from both STOPPIT samples (kept in RNA later) and myometrial explants from cell culture experiments at the 24 hour timepoint. This was used for subsequent RT-PCR analysis using Taqman analysis. Tissues of known weight were ground and homogenized in Trizol (Invitrogen 15596-026) and 1ml of Trizol added for each 50-100mg of sample. A short 5 minute incubation at room temperature then allowed dissociation of nucleoprotein complexes. Chloroform was added at a 1:5 concentration. This mixture was shaken and incubated at room temperature for as further 3 minutes. Samples were then centrifuged at 13,000 rpm for 15 minutes at 4°C. The upper aqueous phase containing the RNA was removed and the precipitated with isopropyl 1:2 concentration of initial volume and incubated for 10 minutes at room temperature. After a further centrifuge at 10,000 for 5 minutes at 4°C the isopropyl alcohol was decanted and 1ml of 75% ethanol added to the small white pellet at the bottom of the tube (containing total RNA). At this stage samples could all be stored at -80 °C prior to the next stage.

Samples were centrifuged at 10,000 rpm for 5 minutes at 4°C and then the ethanol was removed taking care not to dislodge the pellet. The pellet was allowed to dry at room temperature for 10 minutes before adding approximately 20 µl of diethylprocarbonate treated water (DEPC). These were spun and incubated at 65°C for 5 minutes on a heating block twice and then samples combined into one tube if duplicates were required due to an initial weight >100mg. Quantification was carried out using a nanodrop to check RNA concentration ng/µl and ratio 260:280 for all samples which was used for subsequent step to DNase treat samples.

DNase treatment of RNA sample

After calculating the amount of RNA required (2.5µg) then making up each sample to total volume of 21.5µl with an individually calculated amount of DEPC water, we then added 2.5 µl of the 10X DNase buffer and 1 µl of DNase 1 (DNA Free kit, Ambion cat no. 1906) to each sample. This was mixed at 37°C for 30

minutes in the OMN-E thermacycler. The DNase inactivation reagent (microbeads) was resuspended and 2.5 µl added to each sample. This was incubated at room temperature for 2 minutes before centrifuging at 13,000 rpm for 1 minute. The supernatant containing the RNA was then transferred to a new microcentrifuge tube. Again, samples could be frozen at -80 ˚C at this point before the next stage of DNA preparation. After quantification (5 µl) was reverse transcribed using the High Capacity cDNA Reverse Transcriptase kit (ABI).This makes single stranded DNA. By using the reverse transcription kit we prepared enough mastermix for the number of samples plus one. We also prepared a No RT control (Table 2). By adding 5 µl of 2 X RT mastermix to 5 µl of Dnase treated RNA (up to 1µg) and then mixing and centrifuging, it is then incubated at 25˚C for 10 minutes followed by 37˚C for 120 minutes and finally 85˚C for 5 seconds on the PCR express. The resulting cDNA can be stored at -20˚C until further analysis.

Preparation	Sample mix with RT(µl)	No RT control(µl)
10X RT buffer	1	1
25 X dNTPs	0.4	0.4
10 X random primers	1	1
Mutiscribe reverse transcriptase	0.5	0
Superasin (1U/µl)	0.5	0.5
Nuclease free water	1.6	2.1
Total per reaction	5	5
	+ 5µl RNA	+ 5µl RNA
Total	10 µl	10 µl

Table 2: DNase treatment of RNA sample

Quantitative RT-PCR

Primers/Probes for pro labour genes, contraction associated proteins, endogenous uterine stimulants and endogenous inhibitors of uterine contractility were used from (ABI). cDNA was quantified using TaqMan technology on ABI Prism 7900HT (Applied Biosystems).Briefly, 1.25 µl of 20 X target assay or control assay mix was added to 12.5 µl of 2X Mastermix (Applied Biosystems), with 10.25 µl deionised distilled water and 1 µl cDNA. The thermal cycler conditions were 50°C for 2 minutes; 95°C for 10 minutes followed by the 40 cycles of 95°C 15 seconds and 60°C for 1 minute. The cycle at which the fluorescence reached a preset threshold (cycle threshold) was used for quantitative analyses. Data was analysed using the sequence detection software which calculated the threshold cycle (C_T) values .The cycle threshold in each assay was set at a level where the exponential increase in amplicon abundance was approximately parallel between all the samples. The expression target assays were normalised by subtracting the C_T value of the 18S control or beta-actin from the C_T value of the relevant target assay. The fold increase relative to the control was obtained by using the formula $2^{-\Delta CT}$.TaqMan® gene expression assays are listed in Table 3.Progesterone Receptor A+B was supplied by Applied Biosystems and is detailed in Table 4.

Title	TaqMan® Gene expression Assay	Assay ID	Source
Inos	Nitric oxide synthase (inducible)	Hs00167257_m1	Applied Biosystems
Enos	Nitric oxide synthase (endothelial)	Hs00167166_m1	Applied Biosystems
Bnos	Nitric oxide synthase (neuronal)	Hs00167223_m1	Applied Biosystems
cGRP	Calcitonin gene related peptide	Hs00266142_m1	Applied Biosystems
cGRP t	Calcitonin gene related peptide receptor	Hs00173787_m1	Applied Biosystems
EP2	Prostaglandin E receptor (subtype EP2)	Hs00168754_m1	Applied Biosystems
Cx 26	Gap junction protein connexin 26	Hs0095589_m1	Applied Biosystems
Cx 43	Gap junction protein connexin 43	Hs00748445_s1	Applied Biosystems
IL1-ß	Interleukin -1 Beta	Hs00174097_m1	Applied Biosystems
IL-6	Interleukin-6	Hs00174131_m1	Applied Biosystems
IL-8	Interleukin-8	Hs00174103_m1	Applied Biosystems
Cox-2	Cyclooxygenase-2	Hs00377721_m1	Applied Biosystems
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	Hs00168359_m1	Applied Biosystems
18S	Control	4310893E	Applied Biosystems
B-actin	Beta-actin control	4310881E	Applied Biosystems

Table 3: TaqMan® gene expression assays

Progesterone receptor	Title	Reference	Sequence
PR-A+B F	Forward primer	1817227	CAG TGG CGC TTC CAA ATG A
PR-A+B R	Reverse primer	1817228	GGT GGA ATC AAC TGT ATG TCT TGA
PR-A+B probe	Probe	1817229	AGC CAA GCC CTA AGC CAG AGA TTC ACT

Table 4 : Progesterone Receptor A+B gene expression assays

Cell lysis and Western Blot

Cells were lysed for western blotting using the cell lytic method (Sigma) and complete mini protease inhibitor cocktail (Roche). Around 50 mg of weighed tissue was homogenised with 0.5 ml of the working cell lysis solution (1 tablet to 10ml Cell lytic MT) and microcentrifuged at 6000 RPM for 10 minutes. The supernates were removed and microcentrifuged again at 13,000 RPM for a further 10 minutes. The final supernates were stored at -70◦C for subsequent analysis. After the protein concentration was determined in the supernatant by the Bradford technique (1976) all samples were diluted to 3 g/L with working cell lysis solution. The samples were diluted 2:1 with sample loading buffer (SDS, 35 mM: glycerol, 1.4 mM; 2-mercaptoethanol, 0.3 mM; Bromophenol Blue, 15 mM) and heat-treated at 90 ◦C for 5 min prior to electrophoresis. Gel electrophoresis was carried out using STOPPIT samples within a NuPAGE 4-12% Bis-Tris preset gel with NuPAGE MES SDS running buffer containing 1mM dithiothreitol and set at 50mA for approximately 120 minutes. Following electrophoresis, the gel was washed in 25 mM phosphate buffer containing 0.1%tween, pH 7.4 at room temperature, for 10 min before being transferred to nitrocellulose paper. Bands were detected with the use of a marker (Biorad precision plus protein standard). Transfer was carried out with NuPage transfer and 10% methanol, using an X Cell Sure Lock ™ Mini-cell system (Invitrogen Laboratories Ltd). The electrophoretic transfer of polypeptides to nitrocellulose paper was performed at 250mA for 90 minutes. The membrane was blocked overnight with in PBS containing 5% dried milk, washed with PBS, 0.1% tween and incubated for 1 h with the polyclonal rabbit antibody raised against connexin 43 (Sigma). The primary antibody dilution was 1:16,000 in PBS and 0.1% tween, containing 5% dried milk. Following sequential washing of the membrane with PBS plus 0.1% Tween 20 visualisation of the polypeptides which reacted with the primary antibody was achieved using a second (anti-rabbit IgG) antibody labelled with horseradish-peroxidase (1:20,000 dilution) followed by enhanced chemiluminescence (Supersignal Ultra, Pierce & Warriner, Chester, UK) according to the manufacturers instructions. The bands illuminated were detected on X-ray film (Kodak) for visualization for 30 seconds to 1 minute.

The nitrocellulose membrane was then stripped using a western blot stripping buffer (25mM glycine-HCL, pH2.5, 1% SDS in distilled water) and following

sequential washing was blocked overnight in a combination of 10% dried milk with 15% horse serum made up in PBS. This was incubated for 1 hour with the monoclonal mouse antibody raised against ß-tubulin (Santa-Cruz) at a 1:1000 dilution. After sequential washing of the membrane with PBS plus 0.1% Tween 20 visualisation of the polypeptides which reacted with the primary antibody was achieved using a second (anti-mouse IgG) antibody labelled with horseradish-peroxidase (1:20,000 dilution) followed by enhanced chemiluminescence as described before.

Analysis Western Blots

Using Adobe Photoshop 7.0 we scanned our images with the EPSON Perfection 3200 photo scanner. We then used an image analysis programme (Bio-Rad Quantity one) to assess the overall density ratio of the western blots. Bands of interest were highlighted and a global background subtraction was made for each film. We expressed the results as a ratio of density, measured in intensity units x mm^2 ($INTmm^2$) relative to the loading control gene ß-tubulin.

CHAPTER 3

The effects of progesterone, tocolytics and uterotonins on spontaneous myometrial contractility.

Contractility experiments

Introduction

Organ bath experiments are an effective tool for testing pharmacological agents. They allow for functional tests to be carried out on animal and human tissue which would not be possible in vivo. Muscles of all types are routinely excised and studied under isometric conditions using force transducers in standard organ baths. In such studies, the muscle is stretched or "preloaded," as the magnitudes of the contractions evoked by various stimuli can vary markedly depending on this baseline parameter and many studies refer to an optimal preload tension (139). Organ bath studies have often been used to assess the functional properties of both animal and human myometrium under various conditions and under influence of various pharmacological agents to advance knowledge within this area (108, 126, 138). The tissue (human myometrium) can be divided evenly and treated with different drugs under highly controlled conditions and this can be repeated or carried out in using replicates to try and minimise intra-patient variation. This allows for a quality control step as certain tissues which failed to spontaneously contract can be eliminated from any further experiment. We obtained concentration-response curves for several different drugs to examine their effects on spontaneous myometrial contractions.

Aim of chapter

This chapter therefore discusses spontaneous myometrial contractility and its response to uterotonins, tocolytics and whether progesterone has any impact on myometrial contractility.

Spontaneous myometrial Contractions

We collected data from a select group of experiments, before undertaking experiments using specific drugs we were able to ascertain the spontaneous contractile response from a larger number of strips. Following equilibration for 2 hours, myometrial strips contracted in a rhythmic manner (amplitude 46±1 mN, frequency 7.7±0.1 contractions h^{-1}), data obtained from 64 strips from 15

patients. Contractions were sustained at a regular rate and rhythm after equilibration for in excess of 6 hours allowing completion of experiments in full (Figure 4).

Oxytocin

Oxytocin has an endogenous role in the induction of labour and is frequently used in the labour ward to both stimulate contractions and prevent post partum haemorrhage(71). The aim of this experiment was to determine the effects oxytocin, a potent uterotonins, has on spontaneous myometrial contractions and obtain the concentration-response relationship for this action.

Oxytocin 10 pM-10 μM produced a concentration-dependent enhancement of spontaneous myometrial contractions. The maximum increase in amplitude was 38 ± 17 %, the log EC_{50} oxytocin measured -9.9 (-10.2,-9.5 95% confidence intervals) on overall amplitude of contractions (Figure 5, Figure 6).The frequency of contractions were also enhanced by oxytocin by a maximum of 383 ± 243 % ,the log EC_{50} oxytocin measured -9.1 (-9.8,-8.4 95% confidence intervals). These data were obtained from 16 patients.

Levcromakalim

Levcromakalim is K_{ATP} channel opener which is able to hyperpolarise smooth muscle cells and prevents action potential generation. It was recognised as a potential tocolytic in 1993 by its effect on pregnant human myometrium before and after the onset of labour (64). The aim of this experiment was therefore to determine the effect that levcromakalim as on spontaneous myometrial contractions and determine the concentration-response relationship for this.

Levcromakalim 100 pM -10 μM induced concentration-dependent inhibition of myometrial contraction amplitude and frequency. At concentrations of 100pM-0.01 μM it exerted no effects but higher concentrations (> 0.01 μM) led to a progressive fall in amplitude and frequency with the highest concentrations (>1 μM) suppressing activity completely. Log EC_{50} of the levcromakalim measured -7.1 (-7.3,-6.9 95% confidence intervals) for amplitude of contractions. The

frequency of spontaneous contractions log EC_{50} levcromakalim measured -7.2 (-7.5,-6.9 95% confidence intervals) (Figure 7, Figure 8). These data were obtained from 12 strips from 12 patients.

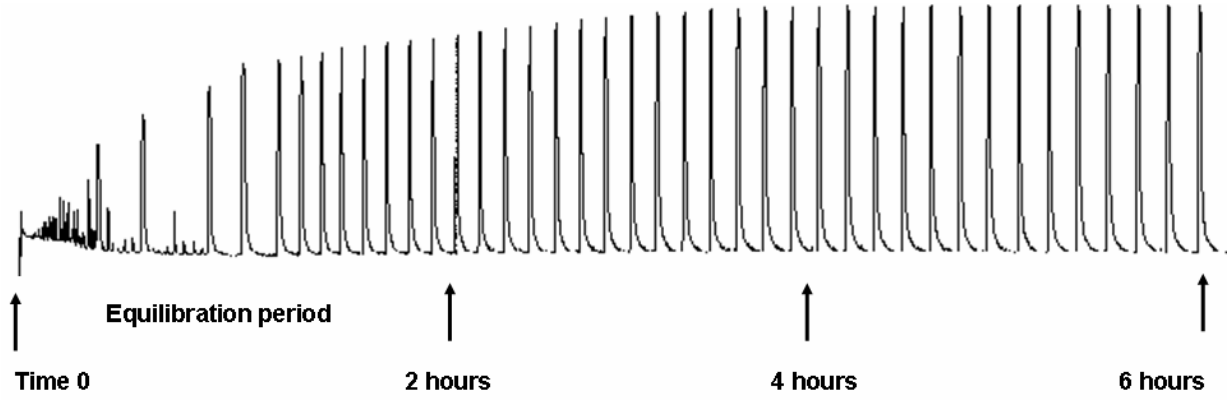

Figure 4 : Contractility trace showing the stability of rhythmic contractions of myometrial strip over time including the equilibration period for the first two hours.

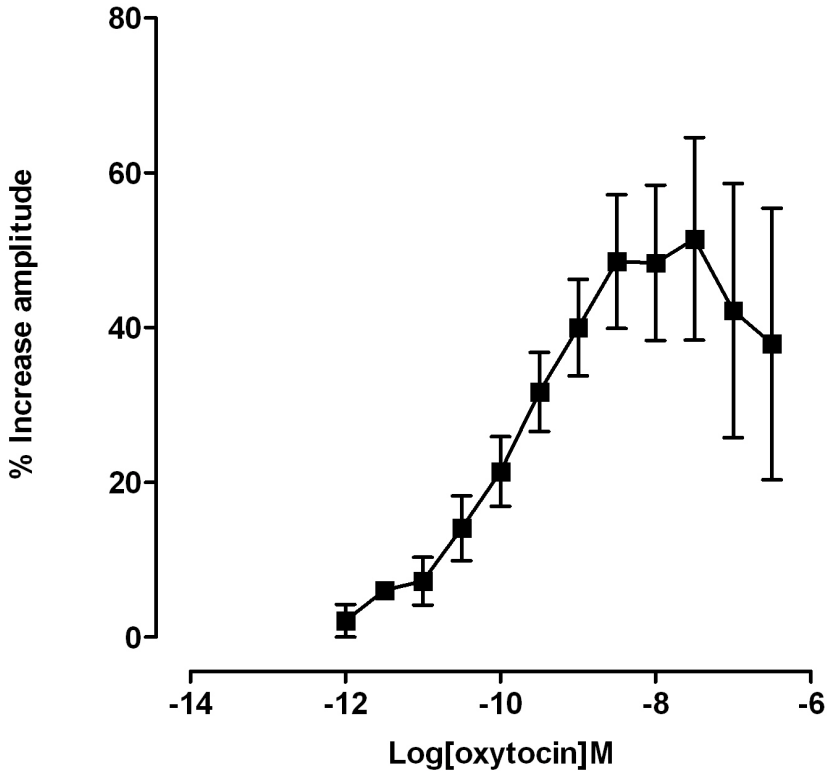

Figure 5 : Concentration-response curve showing the effect of oxytocin on the amplitude of spontaneous myometrial contractions.

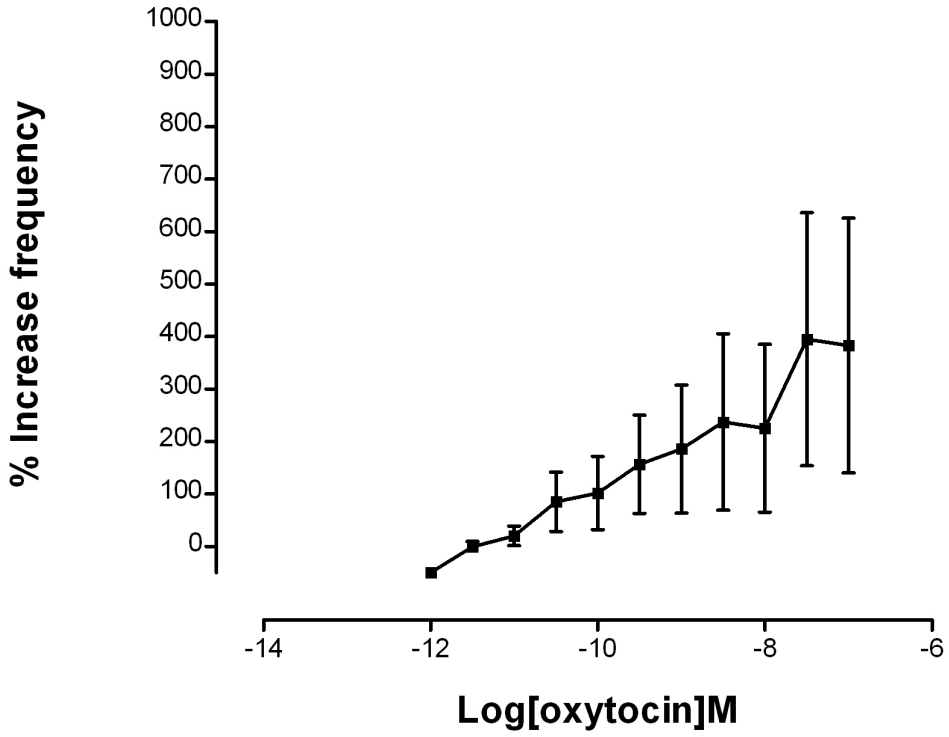

Figure 6 : Concentration-response curve showing the effect of oxytocin on the frequency of spontaneous myometrial contractions.

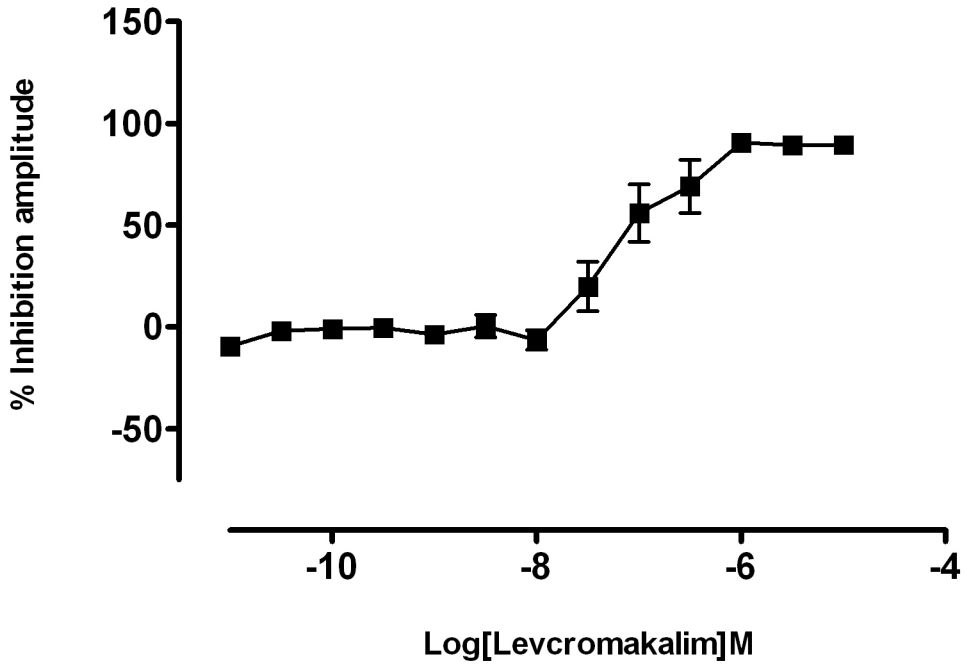

Figure 7 : Concentration-response curve showing the inhibitory effect of levcromakalim on the amplitude of spontaneous myometrial contractions.

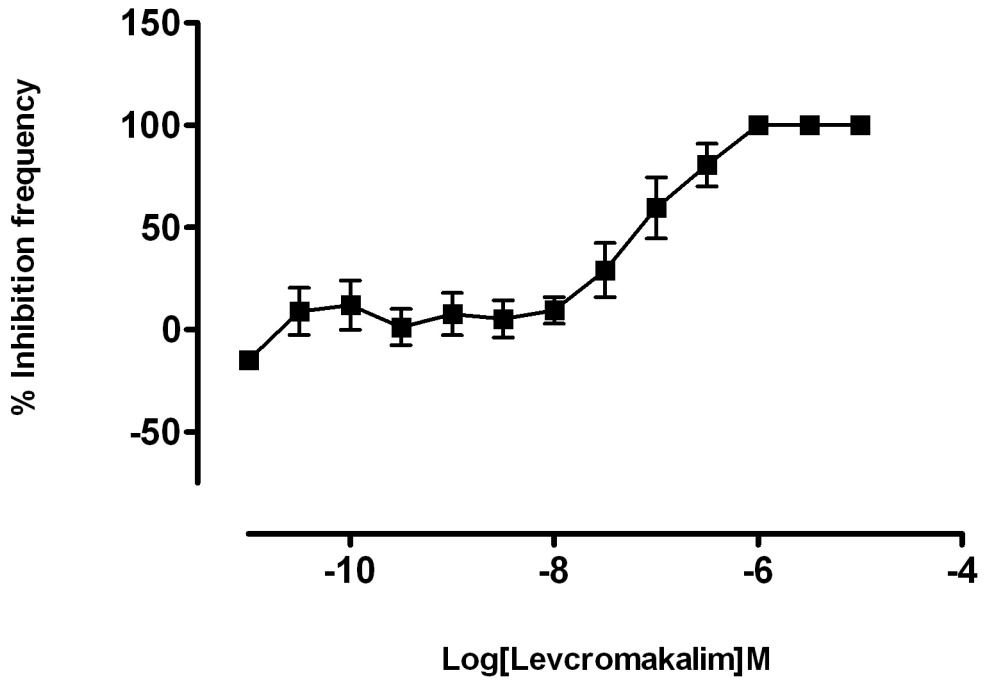

Figure 8 : Concentration-response curve showing the inhibitory effect of levcromakalim on the frequency of spontaneous myometrial contractions.

Nifedipine

Nifedipine is a calcium channel blocker which although usually used in treatment of hypertension and angina (140) is used clinically as a tocolytic treatment for the preventions of preterm labour as recommended by RCOG guidelines (141). The aim of this experiment was therefore to determine the effects nifedipine has on spontaneous myometrial contractions and establish a concentration-response relationship for this.

Nifedipine (100 pM-10 μM) produced concentration-dependent inhibition of spontaneous myometrial contractions amplitude and frequency (Figure 9, Figure 10). At low concentrations of 100pM- 0.01 μM it showed little or no effect on amplitude or frequency but at higher concentrations >0.003 μM led to a progressive fall in the amplitude and frequency of contractions with the highest concentrations >0.3 μM suppressing activity completely. The log EC_{50} of nifedipine measured -8.0 (-8.1,-7.8 95% confidence intervals) for amplitude and log EC_{50} nifedipine -7.1 (-7.6,-6.6 95% confidence intervals) for frequency.

Ritodrine

Ritodrine has previously been a recommended tocolytic according to RCOG guidelines but has since been superseded by atosiban and nifedipine (141). It is a Beta $_{2e}$-adrenergic agonist (142). The aim of this experiment was therefore to determine the effects ritodrine has on spontaneous myometrial contractions and establish the concentration-response relationship for this.

Ritodrine ($ß_2$-agonist, 0.01 μM-1 mM) produced a concentration–dependent inhibition on amplitude spontaneous myometrial concentrations (Figure 11). At concentrations of 0.3 mM it produced a tachyphylaxis and despite inhibiting amplitude of contractions in a concentration-dependent manner frequency increased by around 50% prior to complete inhibition at 1mM concentration within the organ bath (Figure 12). Lack of solubility prevented an examination at higher concentrations of ritodrine with any degree of accuracy. Log EC_{50} [ritodrine] measured -3.9 (-3.9,-3.0 95% confidence intervals) for amplitude of spontaneous activity.

Summary of tocolytic data

When compared as a group (ANOVA) nifedipine was the most potent inhibitor of the amplitude of myometrial smooth muscle, levcromakalim was next and ritodrine was the least potent.

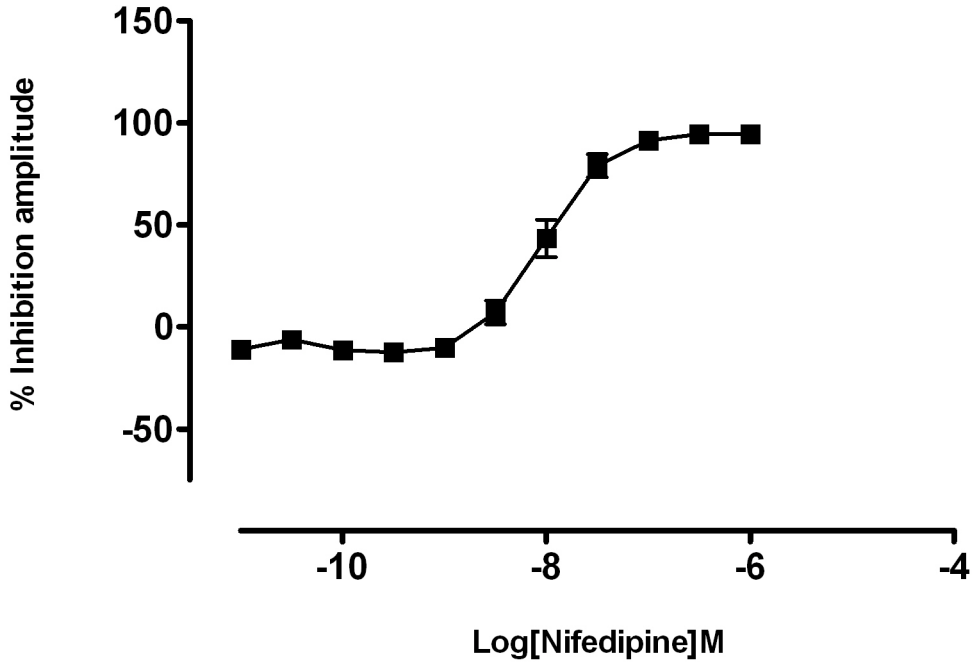

Figure 9 : Concentration-response curve showing the inhibitory effect of nifedipine on the amplitude of spontaneous myometrial contractions.

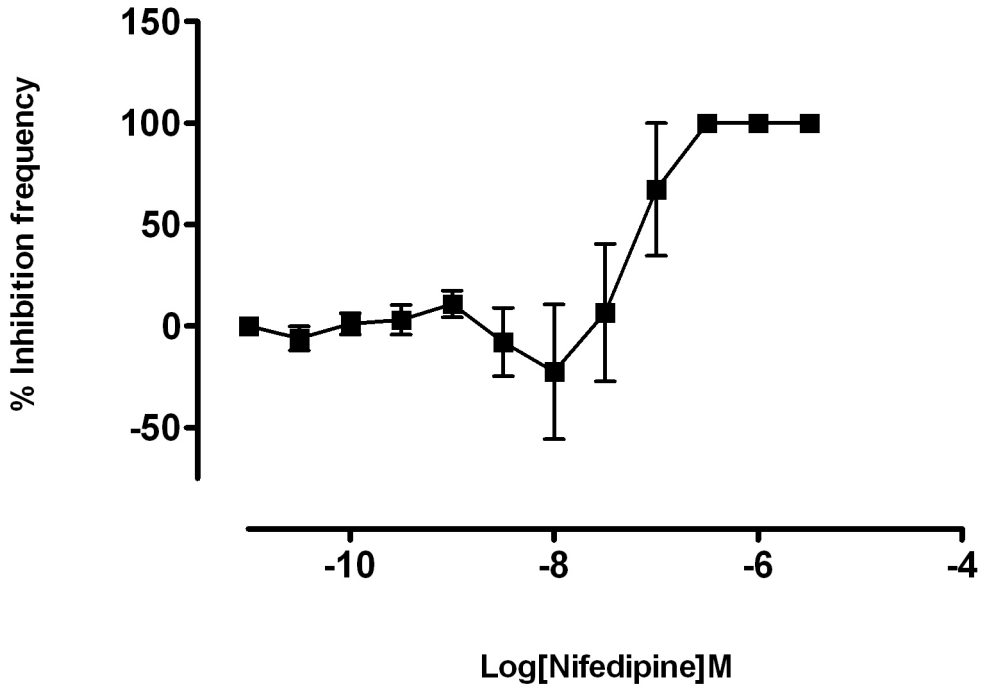

Figure 10 : Concentration-response curve showing the inhibitory effect of nifedipine on the frequency of spontaneous myometrial contractions.

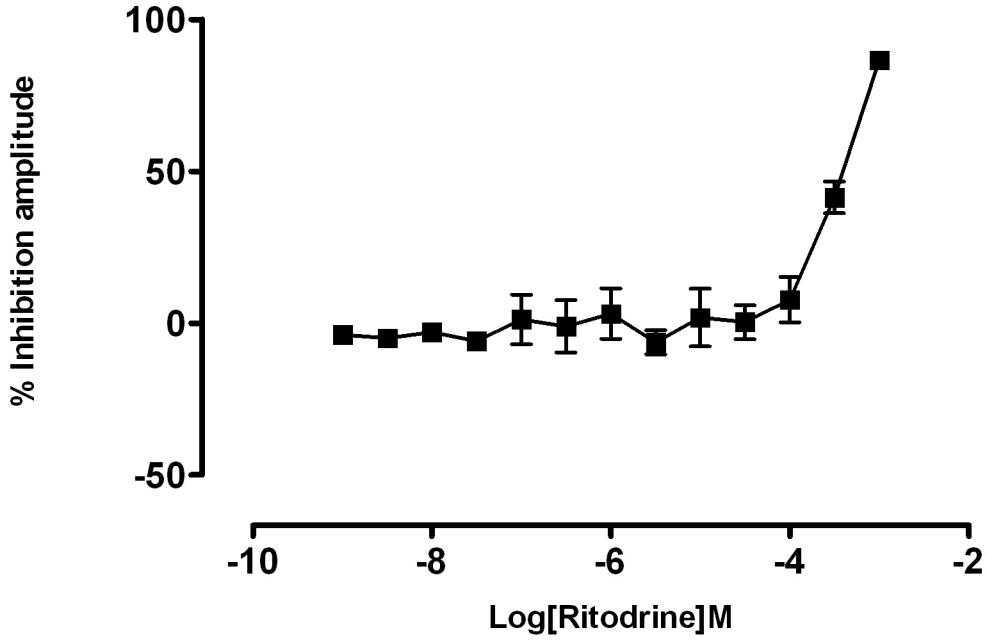

Figure 11 : Concentration-response curve showing the inhibitory effect of ritodrine on the amplitude of spontaneous myometrial contractions.

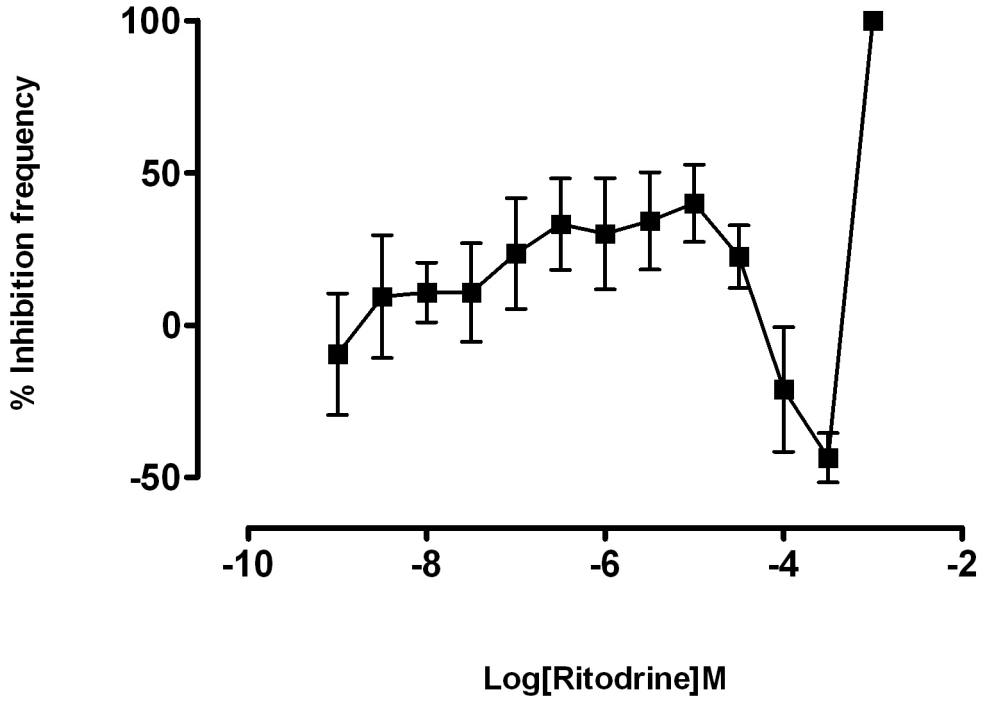

Figure 12: Concentration-response curve showing the inhibitory effect of ritodrine on the frequency of spontaneous myometrial contractions.

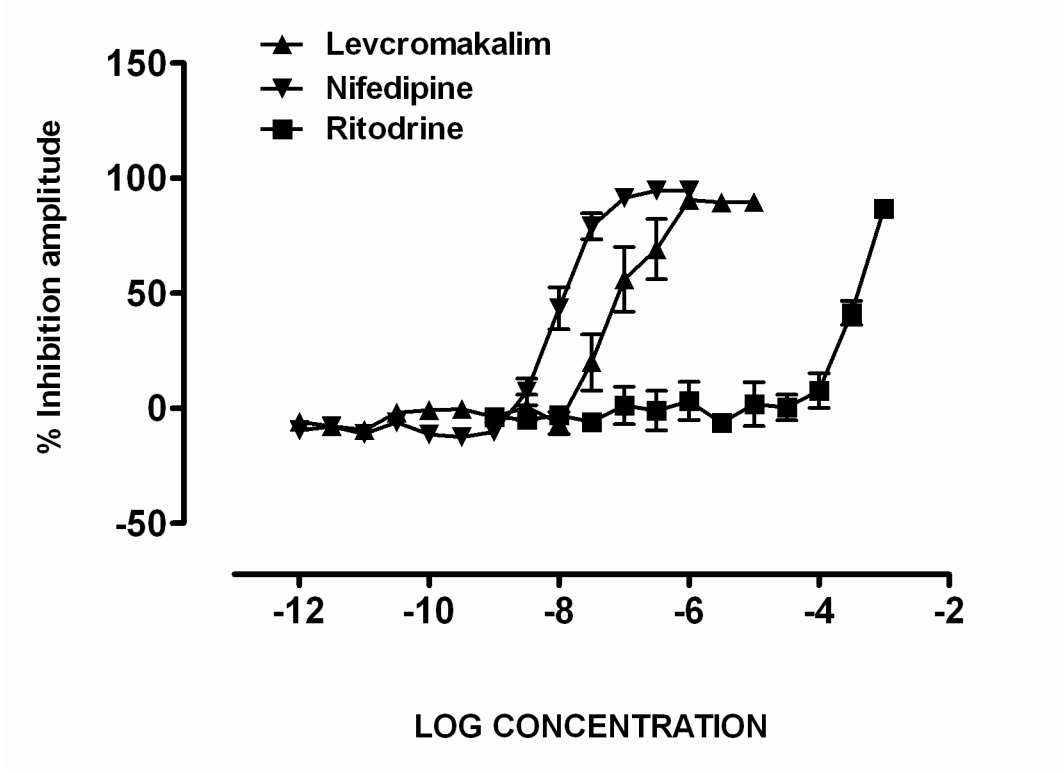

Figure 13 : Concentration-response curves showing the summary of tocolytic data. The amplitude of myometrial contractions and that both nifedipine and levcromakalim have an effect at lower concentrations than ritodrine.

Effects of progesterone and the synthetic progestin, 17α hydroxyprogesterone caproate

Having established the effects of oxytocin and a range of tocolytics, we aimed to determine the *in vitro* effects that progesterone exerted on spontaneous myometrial contractions as well as the synthetic progestin 17α-hydroxyprogesterone caproate (17OHPC).

Progesterone (10 nM- 30 μM) produced a concentration-dependent inhibitory effect on the amplitude of myometrial contractions (Figure 14, Figure 15). The vehicle, 70% ethanol, at appropriate dilutions, also exerted an inhibitory effect on the amplitude of contractions. Maximum inhibition of amplitude was 93±2% and 67±14% for progesterone 30 μM and vehicle respectively. 17OHPC also exerted an inhibitory effect on contraction amplitude but this was not significantly different from the vehicle (Figure 16).

Water Soluble Progesterone

The aim of this experiment was to establish a concentration-response relationship to progesterone *in vitro* without the added inhibitory effects of ethanol seen at higher concentrations. Water soluble progesterone was encapsulated with a cylclodextrin compound. This enables hydrophobic molecules to be incorporated into the cavity of the cylclodextrin by displacing water, and when the water soluble complex is dissolved in a larger volume of aqueous solvent the process is reversed thereby releasing the molecule into the solvent. We subsequently tested the effects of the cyclodextrin compound which was used to make the progesterone water soluble on myometrial contractions.

Water soluble progesterone also exerted a concentration-dependent inhibitory effect on amplitude (maximum inhibition of 82±10% at 100 μM, Figure 17). Time course experiments showed that this water soluble progesterone (100 μM) exerted a 42±5% inhibition of contractile amplitude 30 minutes after addition to the bath, and reached a maximal inhibition of 82±10% at 1 hour (Figure 18). We found an inconsistent effect of progesterone on the frequency of contractions (Figure 19); in some cases with higher frequency but low amplitude contractions prior to complete inhibition. The activity integral (area under the curve) showed an overall decrease with progesterone vs. control, $p<0.05$ (Figure 20). The

cylclodextrin compound exerted no effect on either the amplitude or frequency of myometrial contractions (Figure 21, Figure 22).

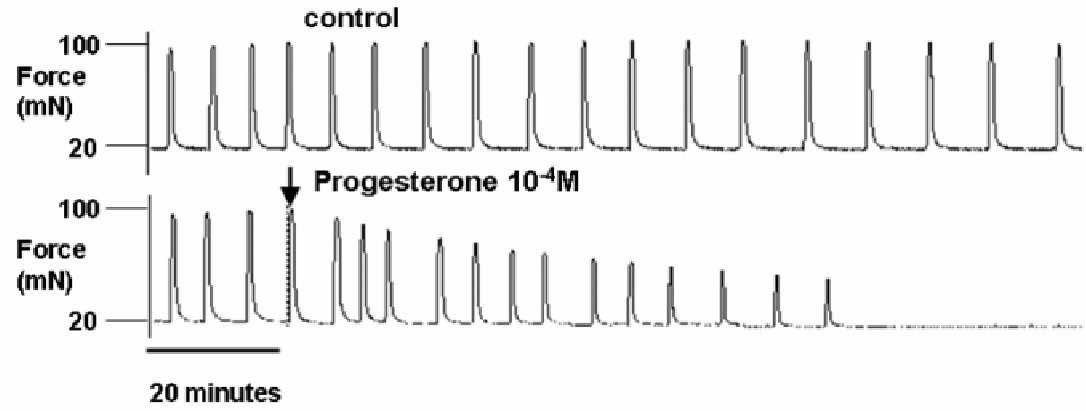

Figure 14 : Contractility trace showing the stability of rhythmic contraction of myometrial strips and inhibition of activity with progesterone 10^{-4}M.

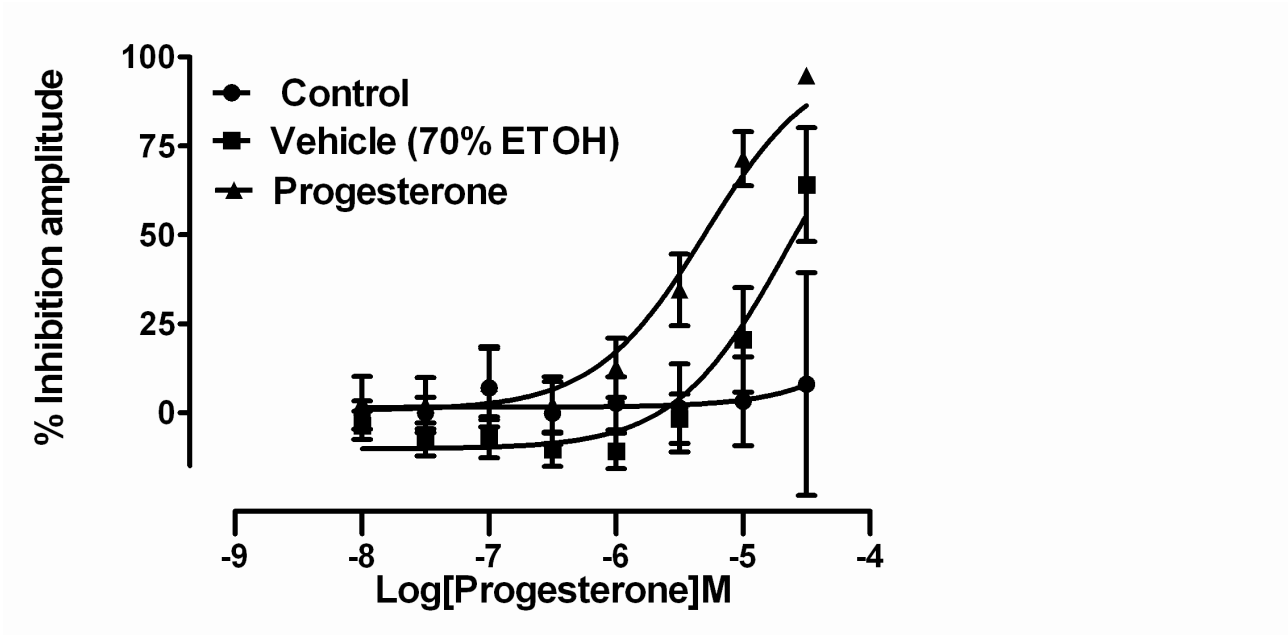

Figure 15 : Concentration-response curve showing the effects of progesterone and vehicle (70% ethanol) on the amplitude of myometrial contractions.

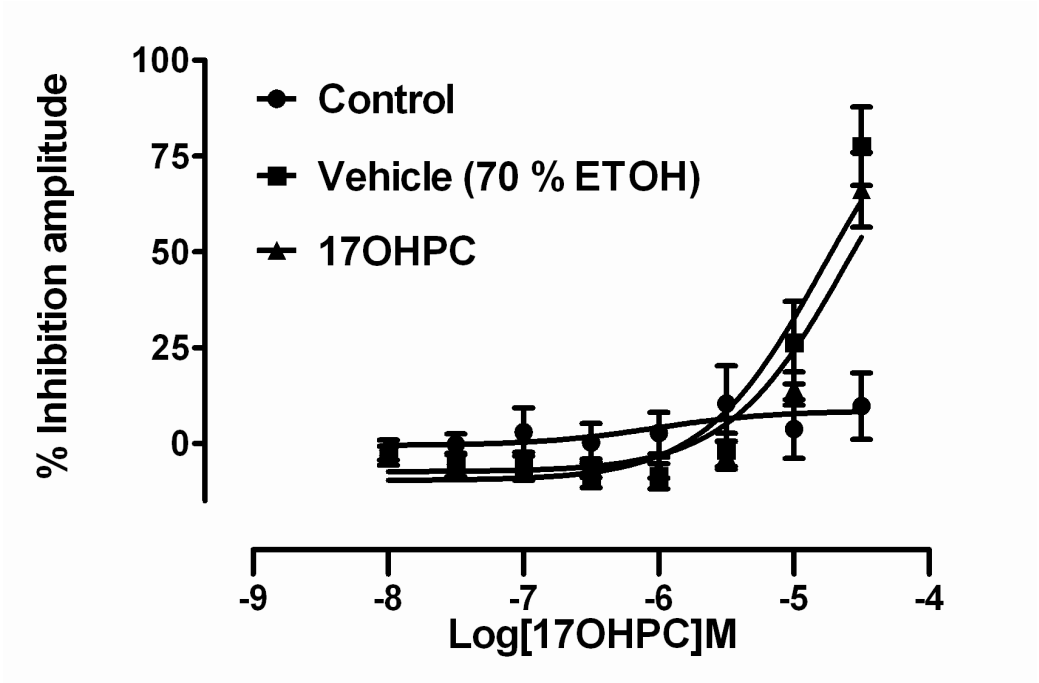

Figure 16 : Concentration-response curve showing the effects of 17OHPC and vehicle (70% ethanol) on the amplitude of myometrial contractions.

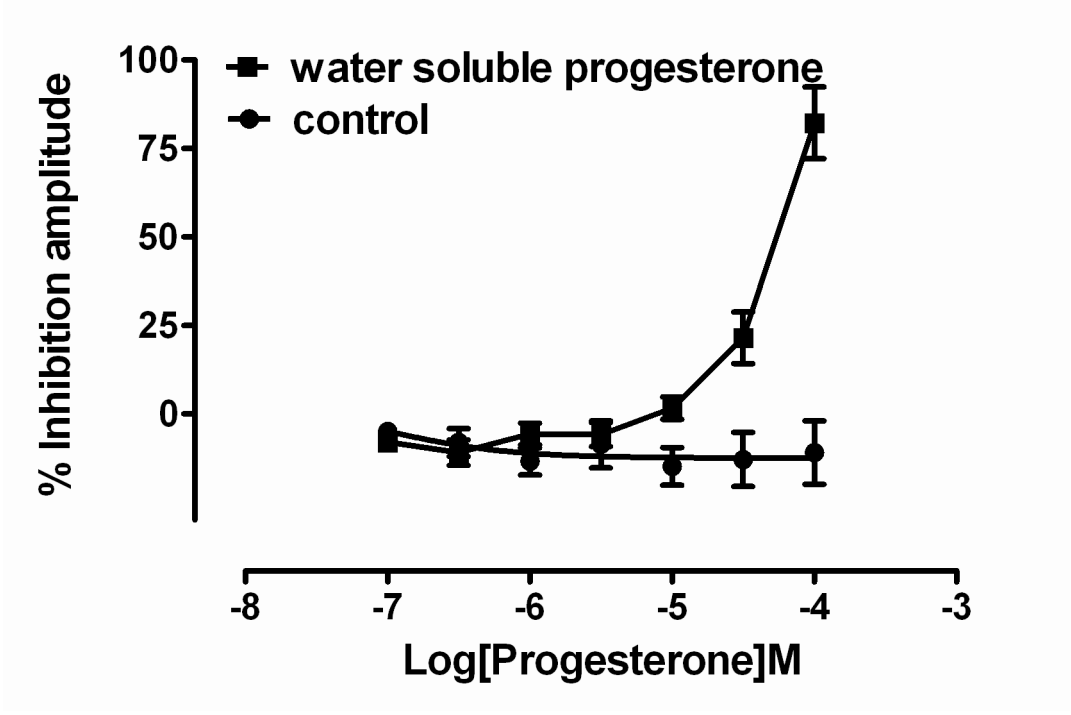

Figure 17 : Concentration-response curve for amplitude of contractions for water soluble progesterone

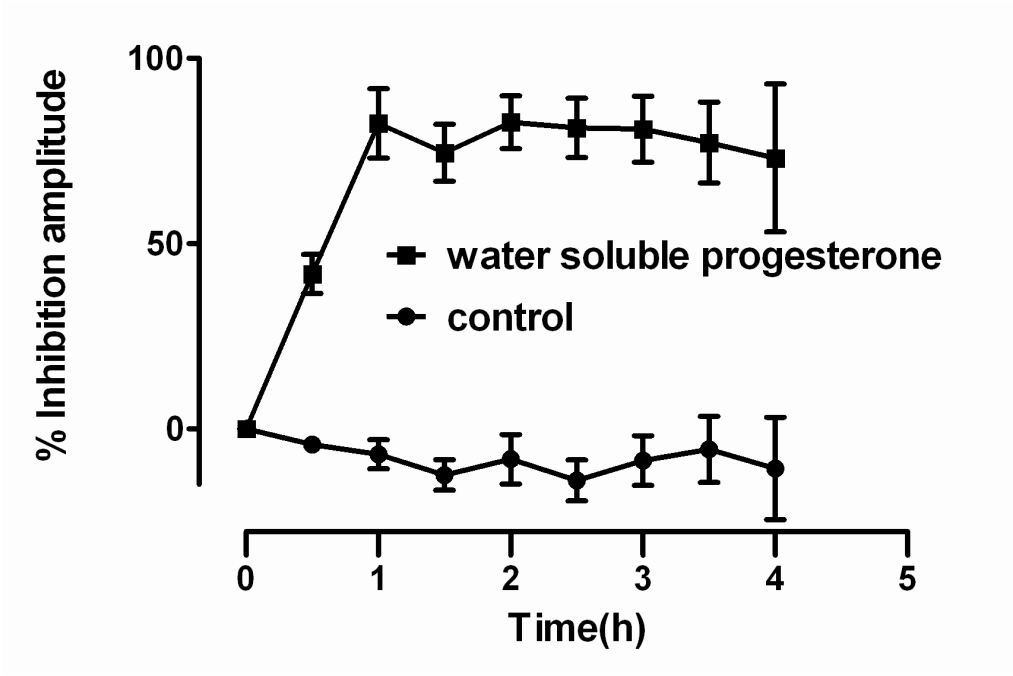

Figure 18 : Time course assessment of maximal concentration water soluble progesterone 10^{-4}M over 4 hours on the amplitude of contractions.

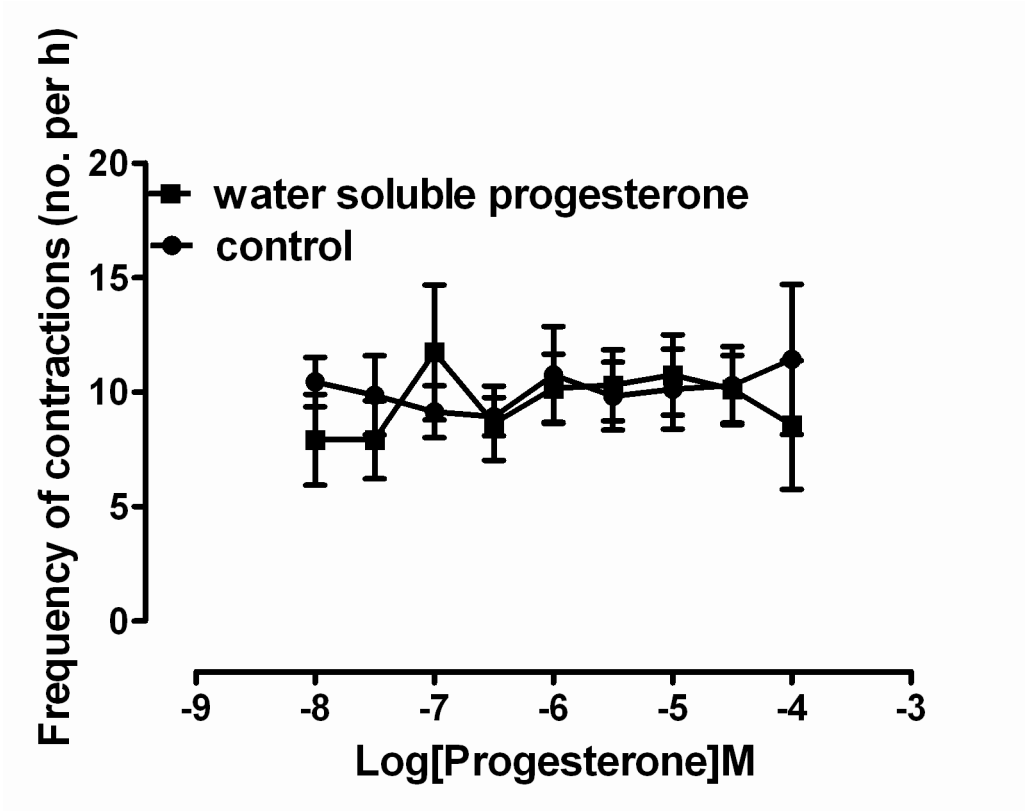

Figure 19 : Concentration-response curve for frequency of contractions with water soluble progesterone.

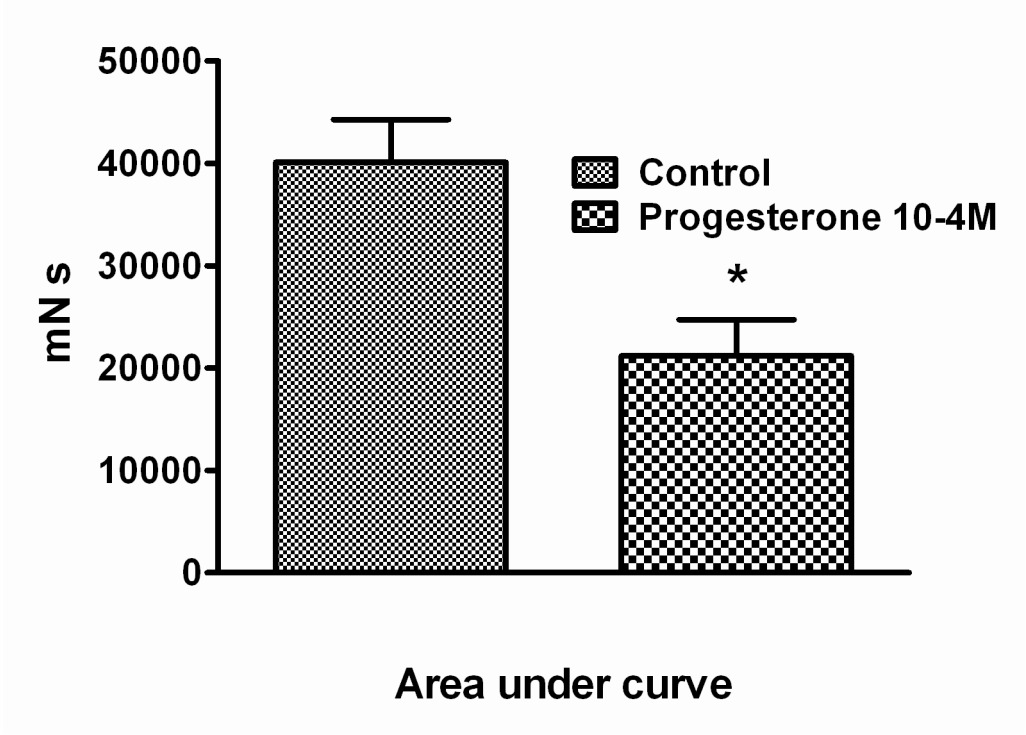

Figure 20 : Activity integral (area under the curve) assessment of control versus water soluble progesterone.

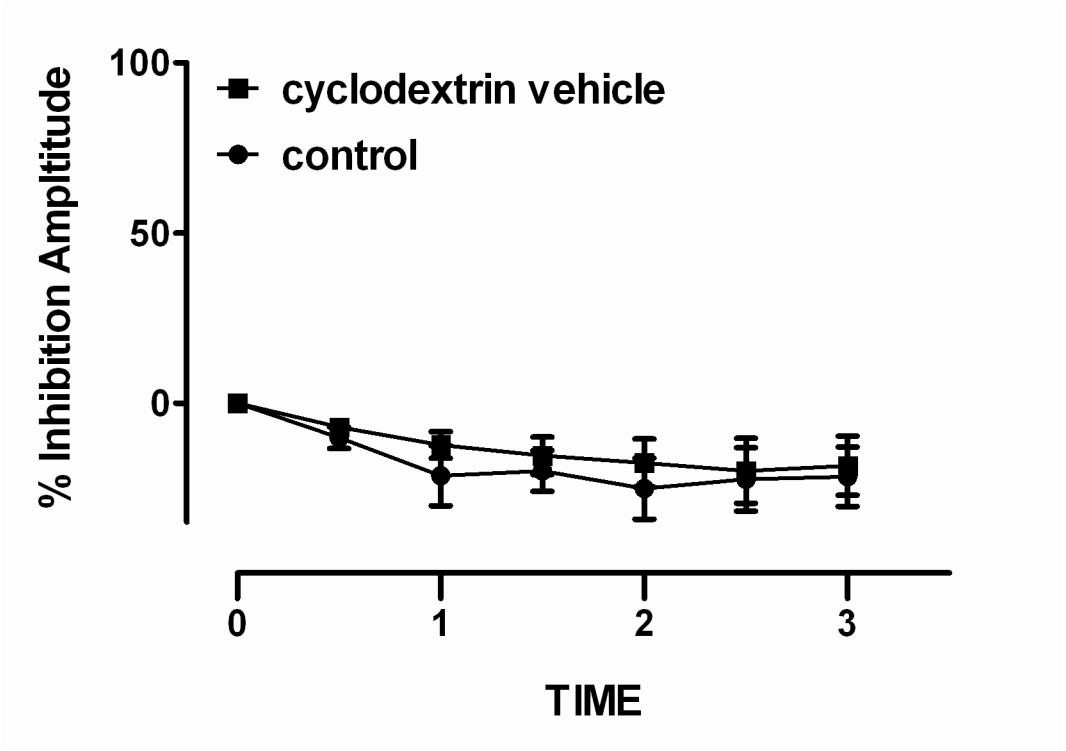

Figure 21 : Cyclodextrin compound contractility results. Time course assessment of maximal concentration of cyclodextrin equivalent to the water soluble progesterone 10-4M concentration over 3 hours on the amplitude of contractions.

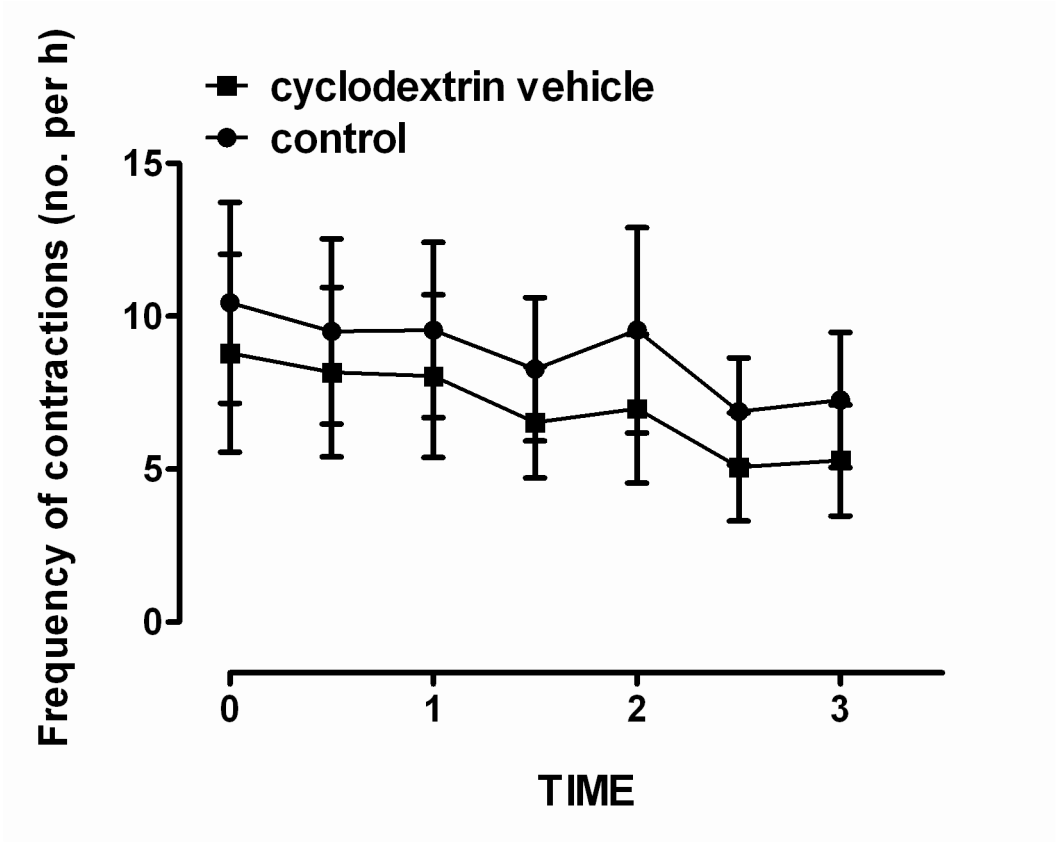

Figure 22 : Time course assessment of maximal concentration of cyclodextrin equivalent to the water soluble progesterone 10-4M concentration over 3 hours on the frequency of contractions.

Effects of potassium channel blockers on responses to progesterone

It became evident with contractility experiments that progesterone exerted a rapid inhibitory effect on myometrial contractions. This apparent non genomic effect is still not fully understood although there are suggestions of progesterone membrane receptors regulating this function (48). These are still poorly understood and a rapidly developing area of research. I therefore hypothesised that this membranous effect acted via potassium channels. Potassium channels are present on myoctyes and comprise a group of proteins that contribute significantly to uterine quiescence during pregnancy (128).

In myometrium the potassium channels allow for adaptation of the gravid uterus to stretch and pressure akin to our simplified model using stretch to initiate myometrial contractions within the organ bath set up.

As both progesterone (143) and potassium channels (128) are linked with maintaining uterine quiescence the aim of this experiment group was to determine if progesterone exerted its inhibitory effect on myometrial contractility via activation of potassium channels. In order to determine this we adopted the approach of selectively blocking individual or groups of potassium channels using pharmacological inhibitors.

Each of the potassium channel blocking agents, glibenclamide 1.5 μM (blocks K_{ATP} channels) (144), charybdotoxin 100 nM (blocks intermediate channels IK_{Ca}, BK_{Ca}, Kv) (144), iberiotoxin 100 nM (blocks large conductance BK_{Ca} channels) (145) or apamin 100 nM (blocks small SK_{Ca} channels) (146) were added to myometrial strips at the end of the equilibration period. Concentration-response curves were then carried out adding water soluble progesterone as described above. Experiments were performed on separate strips with n> 6 for each agent.

Table 1 details the demographics for the potassium channel series of experiments. Table 2 gives the new data for each potassium channel blocker with and without the presence of progesterone and the values for % inhibition of the amplitude of spontaneous contractions as well as the overall frequency in contractions per hour.

None of the selected potassium channel blockers had an independent effect on either the amplitude or frequency of spontaneous myometrial contractions prior to addition of progesterone.

Levcromakalim, a K_{ATP} channel opener, produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous contractions. These effects were antagonised by the K_{ATP} channel blocker glibenclamide (1.5 μM) and a right shift in the curve is evident $p<0.05$ (Figure 23, Figure 24). In contrast, glibenclamide had no effect on the progesterone induced inhibition of myometrial contractility (Figure 25, Figure 26).

Charybdotoxin 10 nM (blocks IK_{Ca}, BK_{Ca} and K_v channels) also had no effect on the progesterone-induced inhibition of contractility. We observed with the addition of progesterone, a significant increase in frequency of contractions when in the presence of charybdotoxin. This was only observed at 10nM and not a sustained effect throughout the concentration-response regime (Figure 27, Figure 28).

Iberiotoxin 100 nM (blocks BK_{Ca} channels) (Figure 29, Figure 30) and apamin 100 nM (blocks SK_{Ca} channels) also failed to affect the ability of progesterone to inhibit myometrial contractility (Figure 31, Figure 32).

	Progesterone & 17OHPC	Water soluble Progesterone	Potassium channel Series	Total
Number women	26	8	21	55
Total strips	90	27	184	301
Age (Years)	29.2 (±1.02)	33.4 (±1.4)	30.3(±1.4)	30.3(±0.7)
BMI (kg/m2)	25.0 (±0.9)	27.6 (±2.2)	26.9 (±1.3)	26.1(±0.7)
Gestation (days)	274 (±0.9)	274 (±0.3)	274 (±0.8)	274(±0.3)
Baby weight (Kg)	3.47 (±0.1)	3.75 (±0.2)	3.48 (±0.1)	3.52(±0.1)
Indication for LUSCS				
Previous LUSS	18 (69.2%)	8 (100%)	14 (66.7%)	40(72.7%)
Other	8 (30.8 %)	0 (0%)	7 (23.3%)	15(27.3%)
Parity				
0	4 (15.4%)	0(0%)	5 (23.8%)	9(16.4%)
1	16 (61.5%)	7(87.5%)	13 (61.9%)	36(65.5%)
2	3(11.5%)	1(12.5%)	3 (14.3%)	7(12.7%)
≥3	3 (11.5%)	0(0.0%)	0 (0.0%)	3(5.4%)

Table 5 : Patient demographics .Details of patients, number of strips and demographics for each group of progesterone experiments. There was no detectable difference in age, BMI, gestation or baby weights from each subgroup of patients.

		Glibenclamide	Charybdotoxin	Apamin	Iberiotoxin
% Inhibition magnitude	Prog (+)	58.4±10.4	68.5±10.4	40.7±6.2	43.4±5.9
	Prog (-)	74.9±6.9	74.3±8.2	54.8±7.6	54.8±7.6
Frequency of contractions per hour	Prog (+)	4.9±1.5	9.5±2.4	10.7±3.4	18.3±2.9
	Prog (-)	6.6±2.3	9.5±3.6	13.8±2.2	13.8±2.2

Table 6 : Potassium channel results. This shows the individual values of percentage inhibition (% ± SEM) of the amplitude of contractions and frequency per hour ± SEM. The selected potassium channel blocking agents as listed above are shown both in the presence of progesterone (prog+) and absence (prog-).

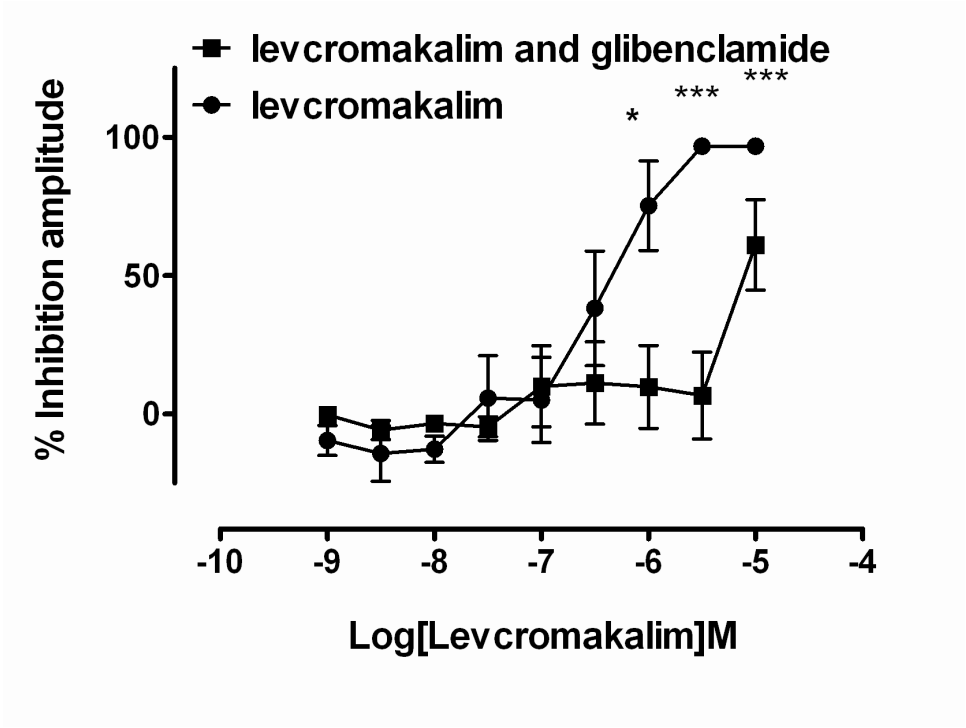

Figure 23 : Concentration-response curve showing the effects on contraction amplitude of levcromakalim alone and in the presence of its antagonist, glibenclamide. The right shift of the curve is seen with glibenclamide $p<0.05$ ANOVA.

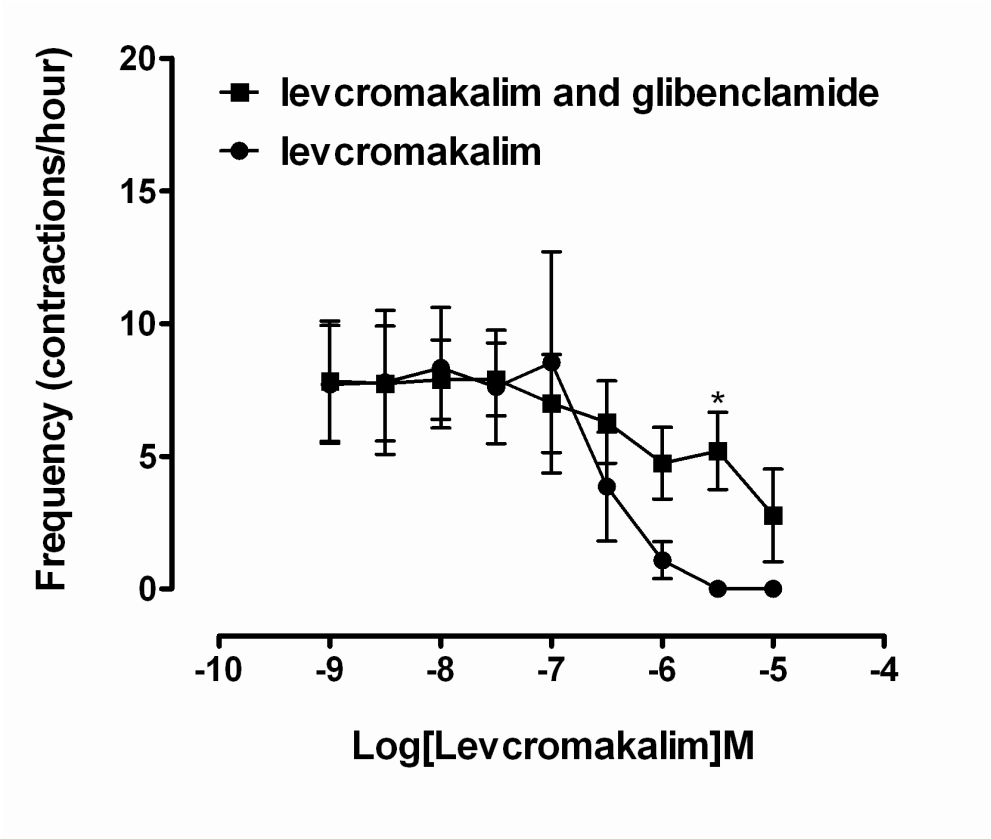

Figure 24 : Concentration-response curve showing the effects on contraction frequency of levcromakalim alone and in the presence of its antagonist, glibenclamide. The right shift of the curve is seen with glibenclamide $p<0.05$ ANOVA.

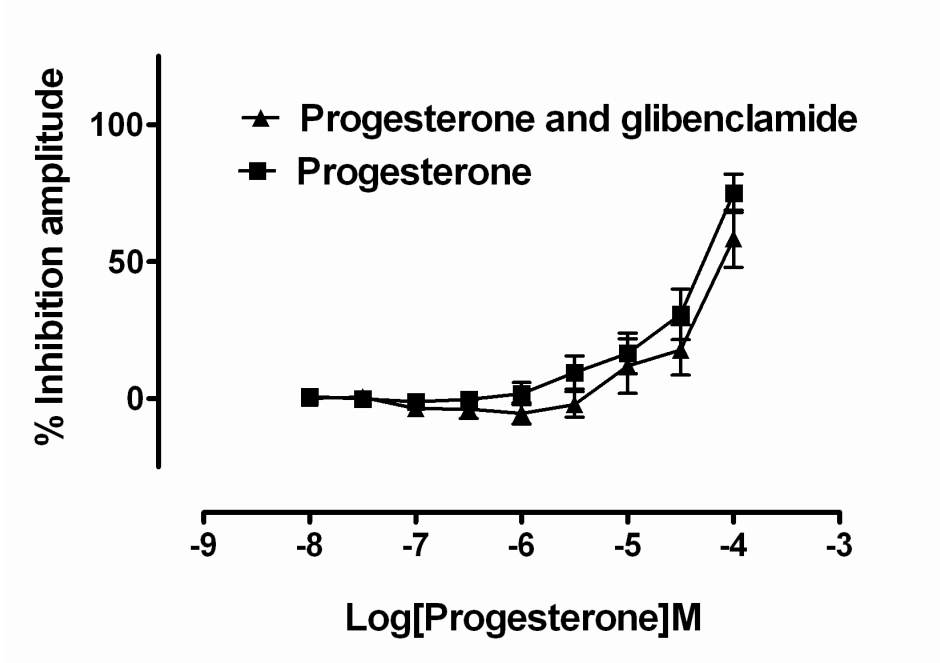

Figure 25 : Concentration-response curve showing the effects on contraction amplitude of progesterone alone and in the presence of glibenclamide. No right shift of the curve is seen with glibenclamide.

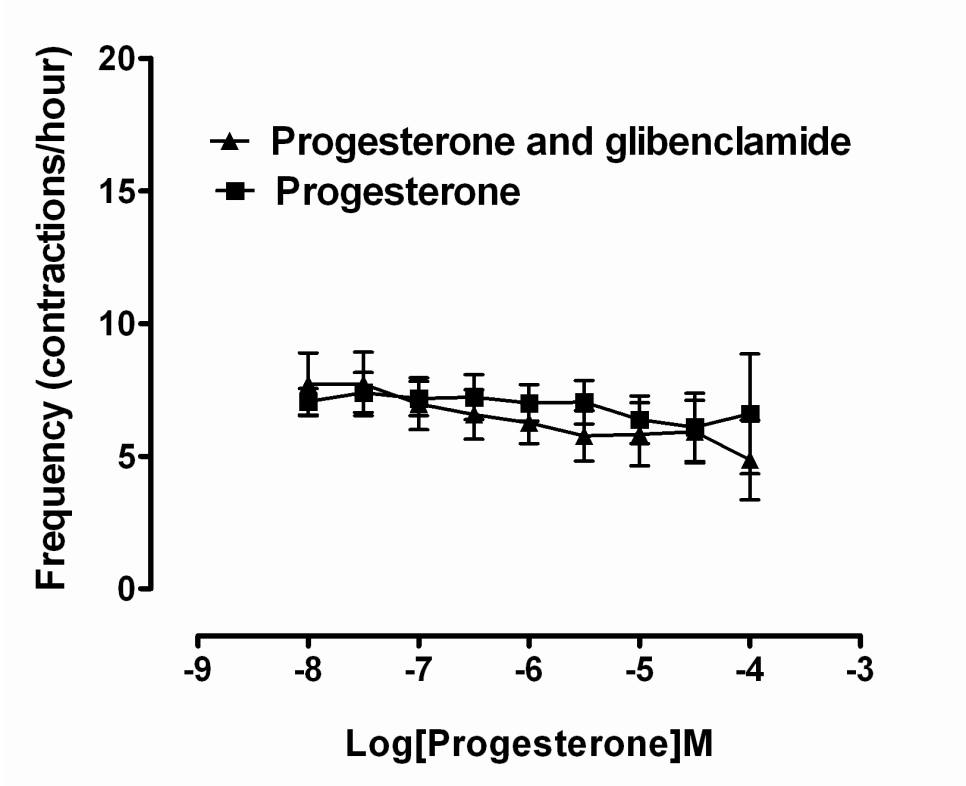

Figure 26 : Concentration-response curve showing the effects on contraction frequency of progesterone alone and in the presence of glibenclamide. No right shift of the curve is seen with glibenclamide.

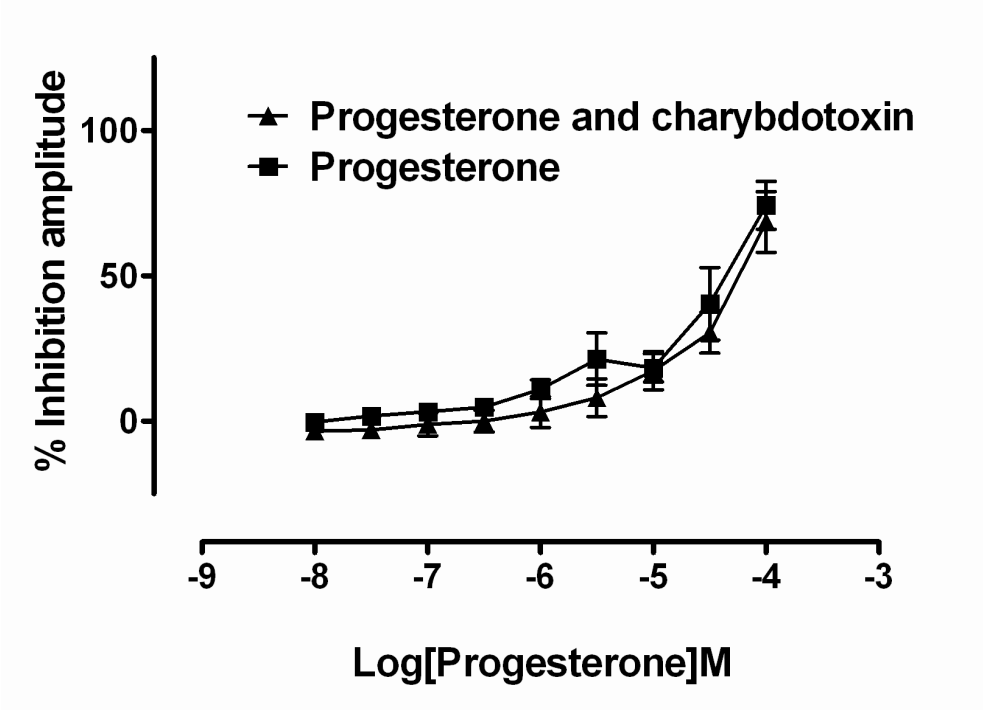

Figure 27 : Concentration-response curve showing the effects on contraction amplitude of progesterone alone and in the presence of charybdotoxin. No right shift of the curve is seen with charybdotoxin.

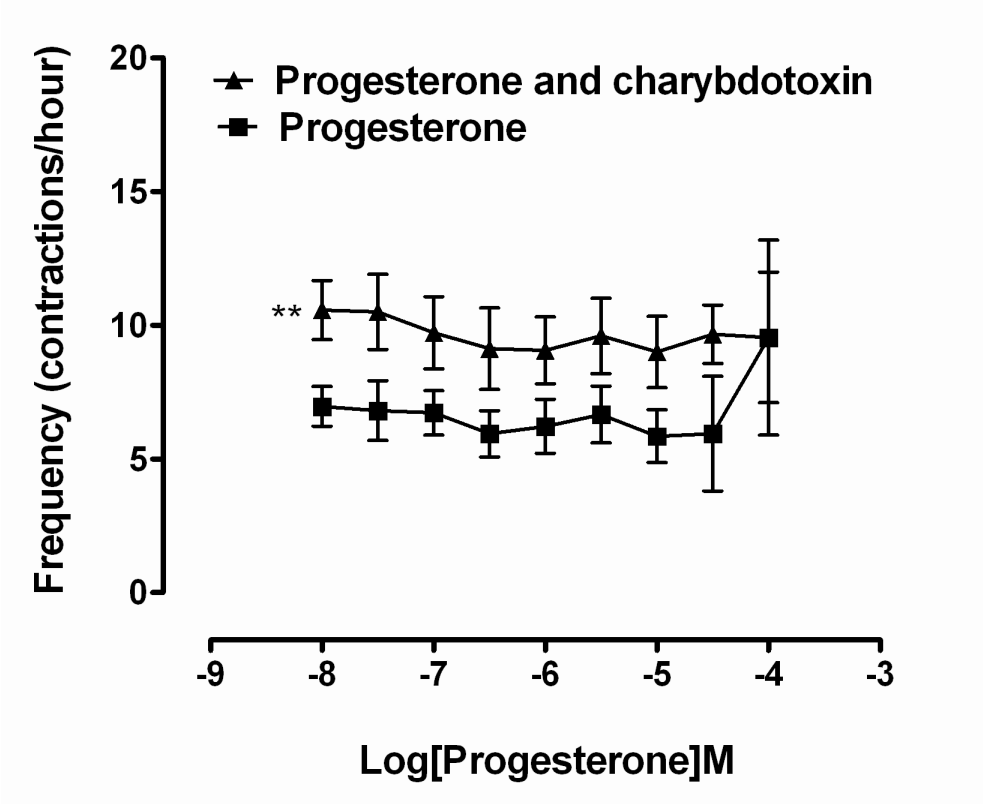

Figure 28 : Concentration-response curve showing the effects on contraction frequency of progesterone alone and in the presence of charybdotoxin. There is a significant increase in frequency of contractions when both progesterone and charybdotoxin are present at the lower concentration $p<0.05$ ANOVA.

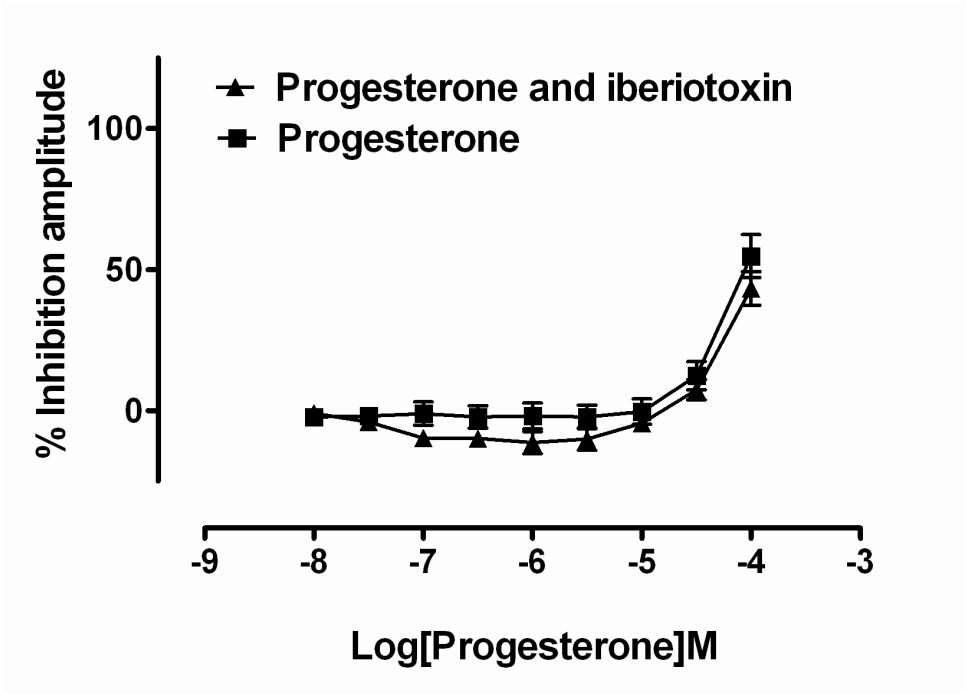

Figure 29 : Concentration-response curve showing the effects on contraction amplitude of progesterone alone and in the presence of iberiotoxin. No right shift of the curve is seen with iberiotoxin.

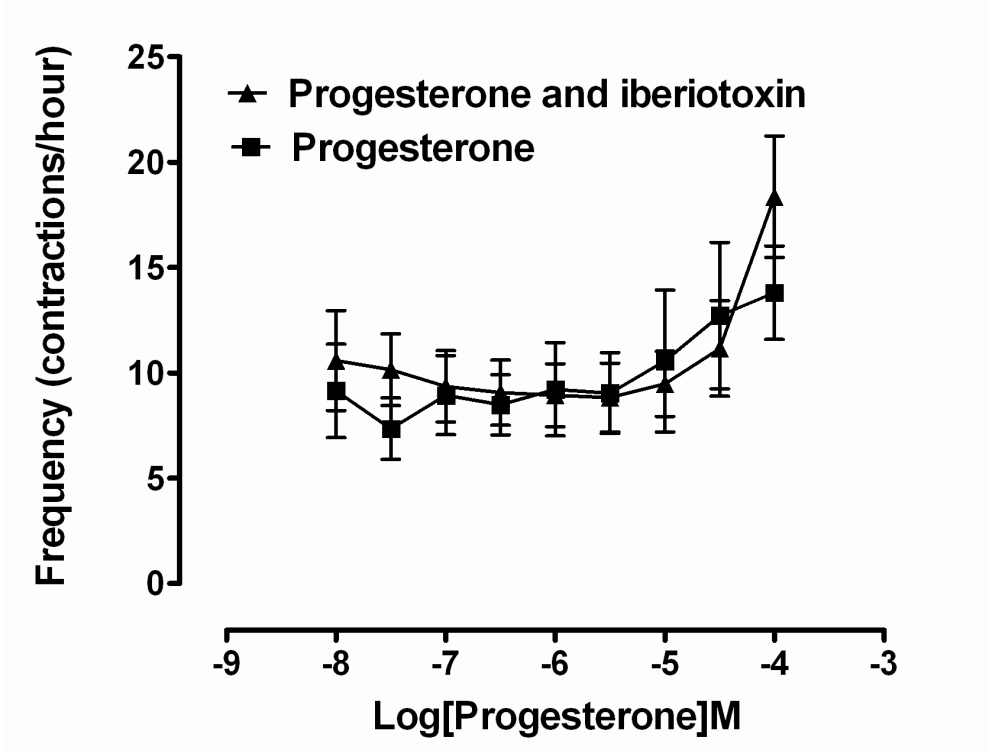

Figure 30 : Concentration-response curve showing the effects on contraction frequency of progesterone alone and in the presence of iberiotoxin. No right shift of the curve is seen with iberiotoxin.

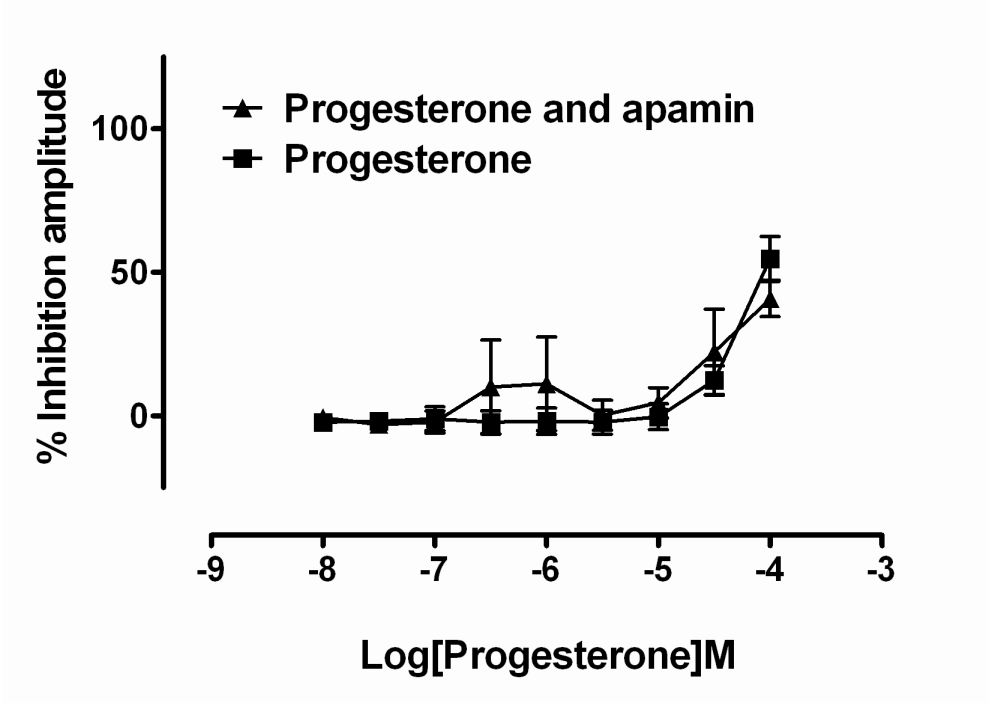

Figure 31 : Concentration-response curve showing the effects on contraction amplitude of progesterone alone and in the presence of apamin. No right shift of the curve is seen with apamin.

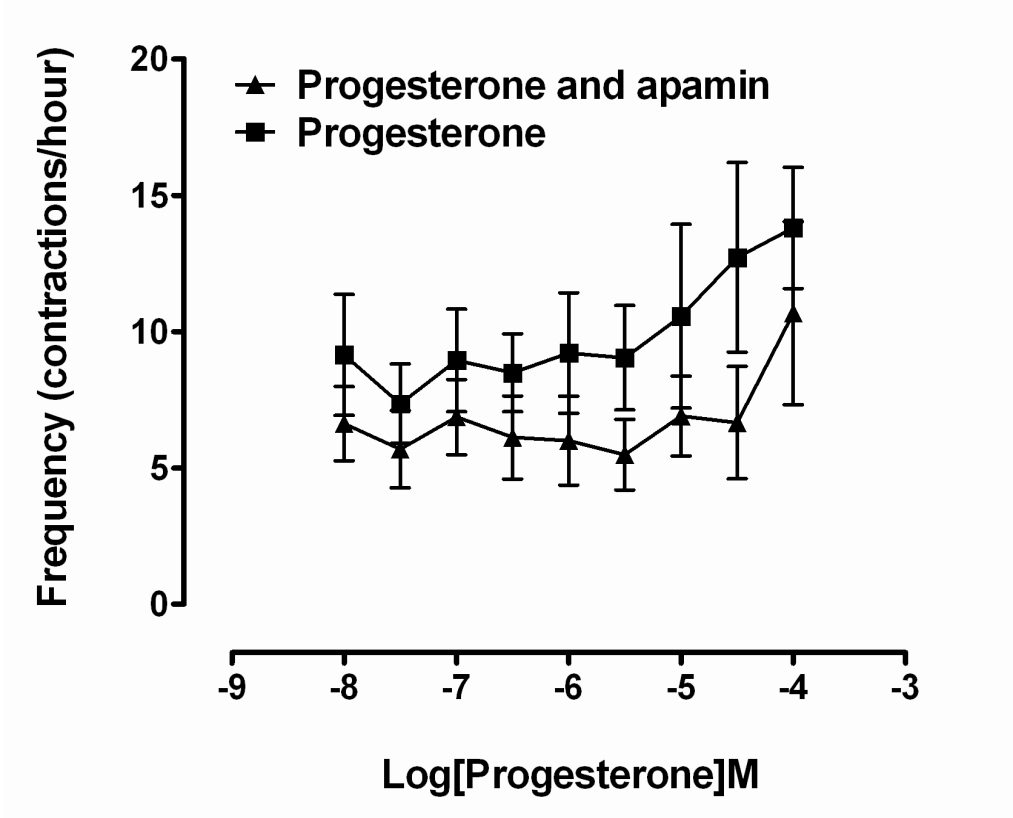

Figure 32 : Concentration-response curve showing the effects on contraction frequency of progesterone alone and in the presence of apamin. No right shift of the curve is seen with apamin.

Effects of progesterone 1 μM incubation in vitro on oxytocin and tocolytics

We added progesterone 1 μM, to the organ baths for 1 hour following the equilibration period, we then carried out the established concentration–response regimes for oxytocin and the three tocolytic drugs, levcromakalim, nifedipine and ritodrine. The aim was to establish if *in vitro* progesterone either enhanced the ability of tocolytics to function or inhibited the uterotonin, oxytocin.

We performed this group of experiments following the initial established protocols for concentration–response curves and therefore used progesterone that was dissolved in ethanol rather than water soluble progesterone at this stage. We selected a 1 μM concentration of progesterone to be added to the baths based on data (figure 11) that indicated progesterone was starting to have an inhibitory effect on myometrial contractions at this concentration but there was no significant vehicle effect. Oxytocin (10 pM-10 μM) produced a concentration-dependent increase in both amplitude and frequency of myometrial contractions. Levcromakalim (100 pM -10 μM), nifedipine (100 pM-10 μM) and ritodrine (0.01 μM-1 mM) each produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous contractions. A 60 minute progesterone (1 μM) incubation significantly reduced spontaneous amplitude of contractions compared with vehicle control $p<0.01$ ANOVA with Bonferoni correction (Figure 33). There was no significant difference in frequency of contractions with control, ethanol vehicle and 1 μM progesterone Figure 34. The vehicle control for the progesterone (0.07% ethanol) showed no effect on amplitude or frequency compared with the control. This 1 μM progesterone treatment however, failed to alter the concentration-dependent stimulatory effect of oxytocin (no shift seen in the concentration-response curve or inhibitory effects of levcromakalim, nifedipine or ritodrine, no shift seen in the concentration-response curves) $n\geq10$ (Figure 35, Figure 36, Figure 37, Figure 38, Figure 39, Figure 40, Figure 41, Figure 42).

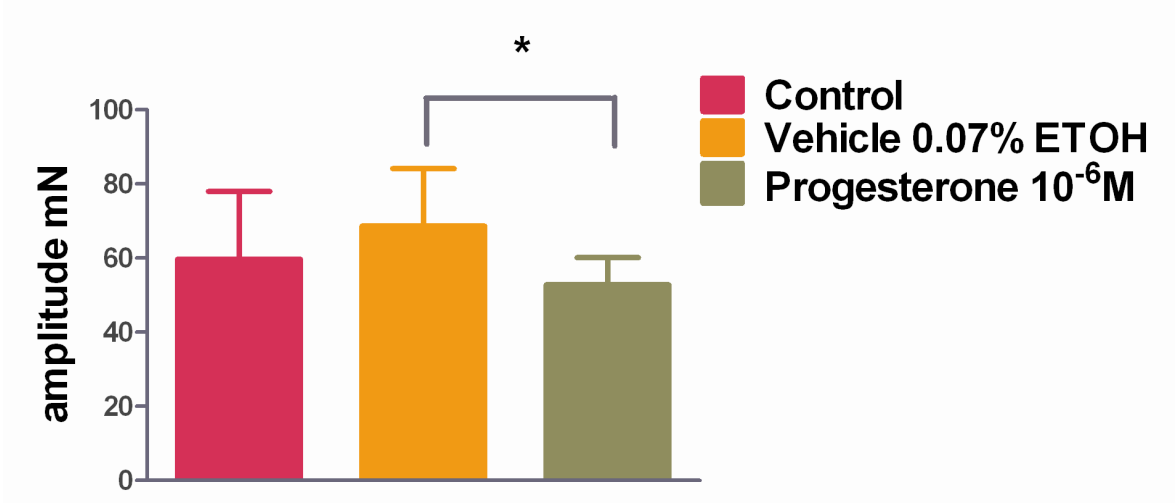

Figure 33 : This graph shows the contraction amplitude after one hour incubation with nil (control), ethanol 0.07% (vehicle) or progesterone 10-6M. There was a significant reduction in contraction amplitude with the progesterone compared to vehicle control $p<0.01$ ANOVA. This dosage regime was chosen from previous progesterone data (Figure 15) as there appeared to be no vehicle effect at this concentration.

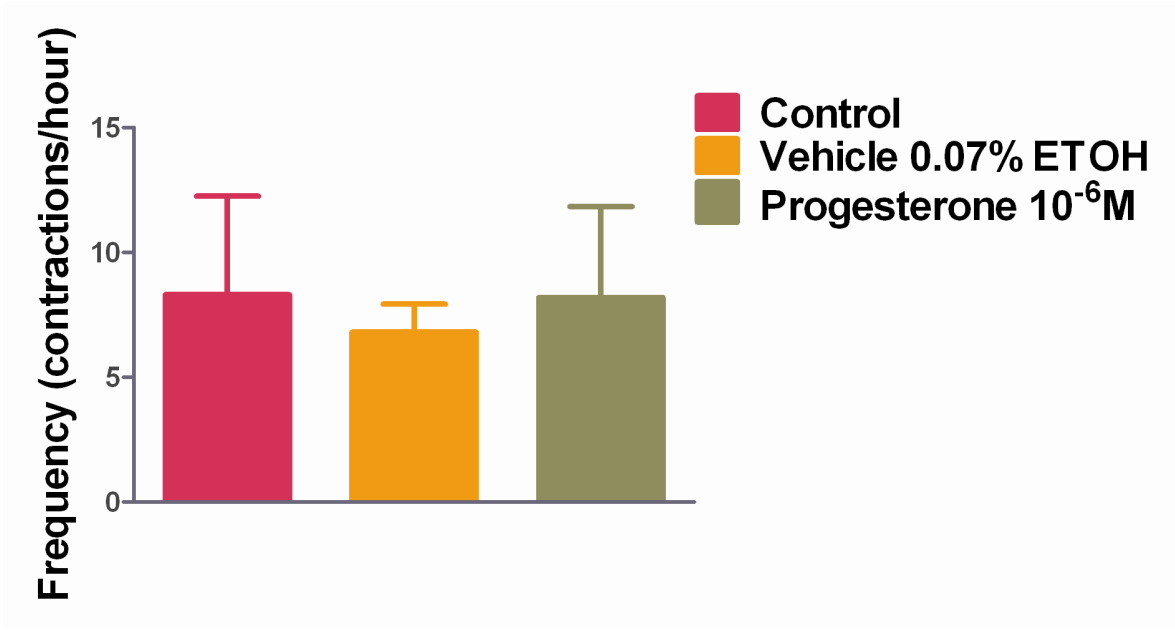

Figure 34 : This graph shows the contraction frequency after one hour incubation with nil (control), ethanol 0.07% (vehicle) or progesterone 10^{-6}M. There was no significant difference between the groups.

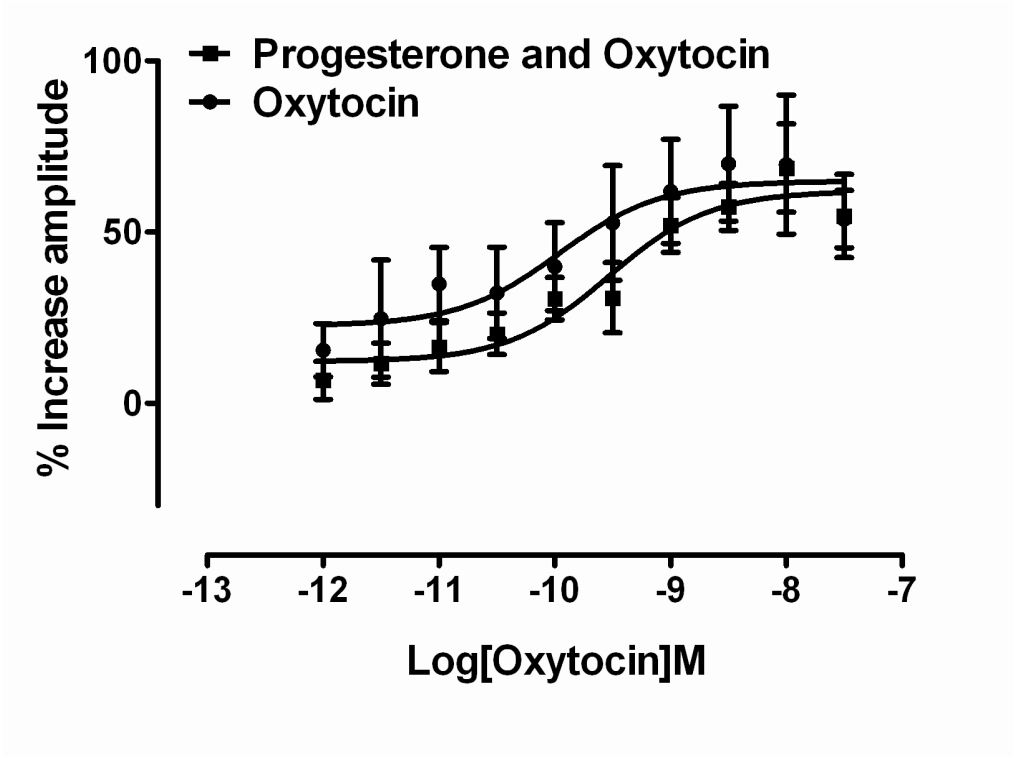

Figure 35 : Concentration-response curve showing the effects on contraction amplitude of oxytocin alone and in the presence of progesterone. No right shift of the curve is seen with progesterone.

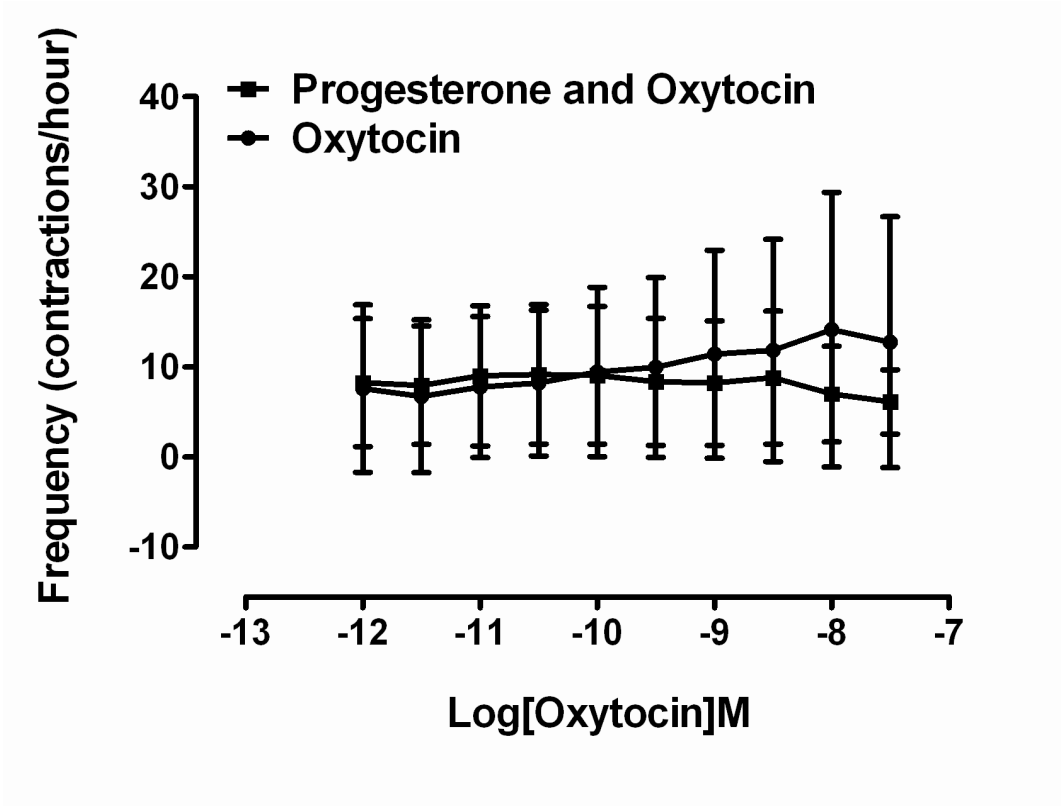

Figure 36 : Concentration-response curve showing the effects on contraction frequency of oxytocin alone and in the presence of progesterone. No right shift of the curve is seen with progesterone.

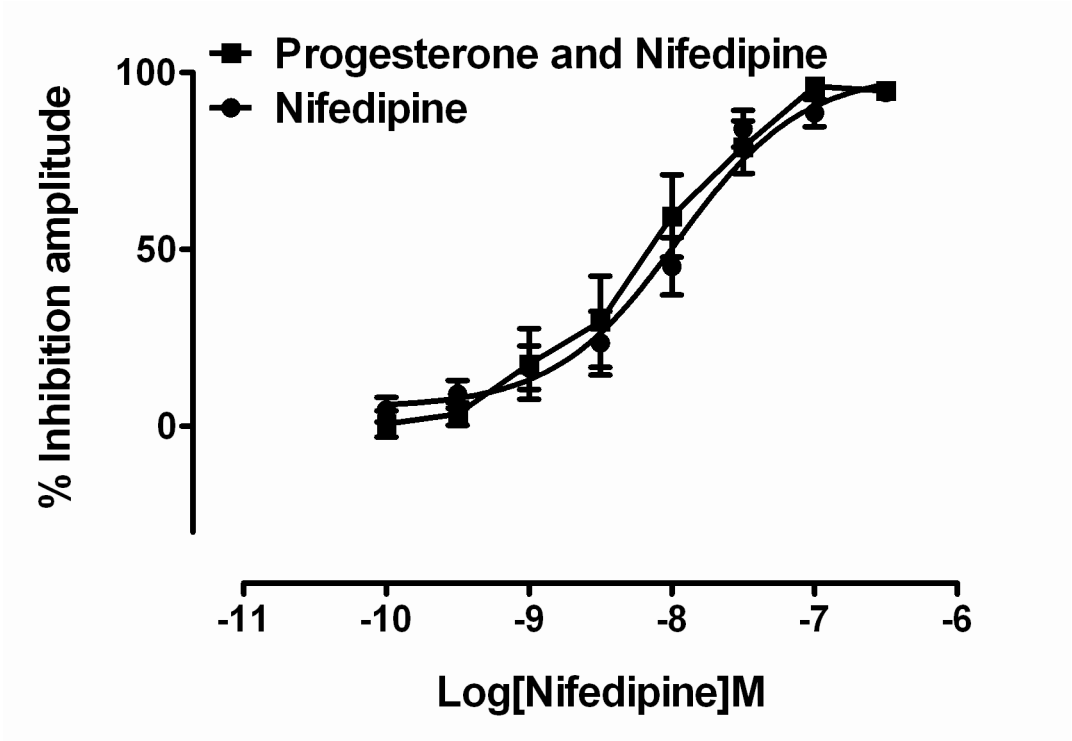

Figure 37 : Concentration-response curve showing the effects on contraction amplitude of nifedipine alone and in the presence of progesterone. No left shift of the curve is seen with progesterone.

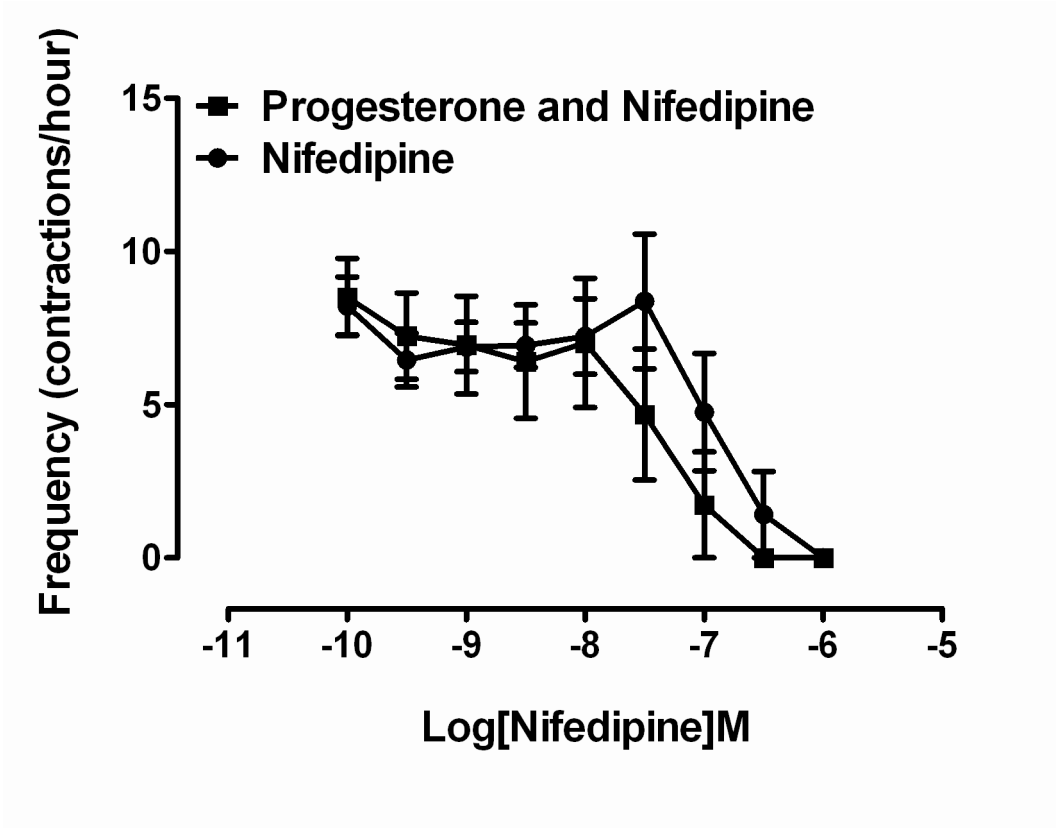

Figure 38 : Concentration-response curve showing the effects on contraction frequency of nifedipine alone and in the presence of progesterone. No left shift of the curve is seen with progesterone.

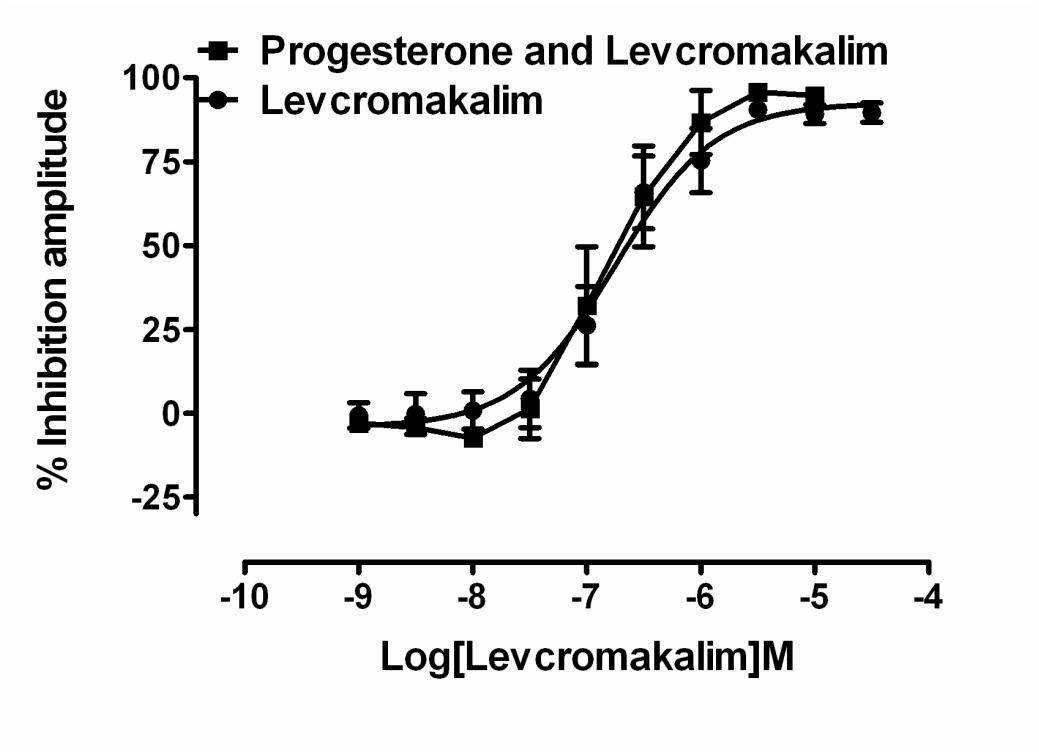

Figure 39 : Concentration-response curve showing the effects on contraction amplitude of levcromakalim alone and in the presence of progesterone. No left shift of the curve is seen with progesterone.

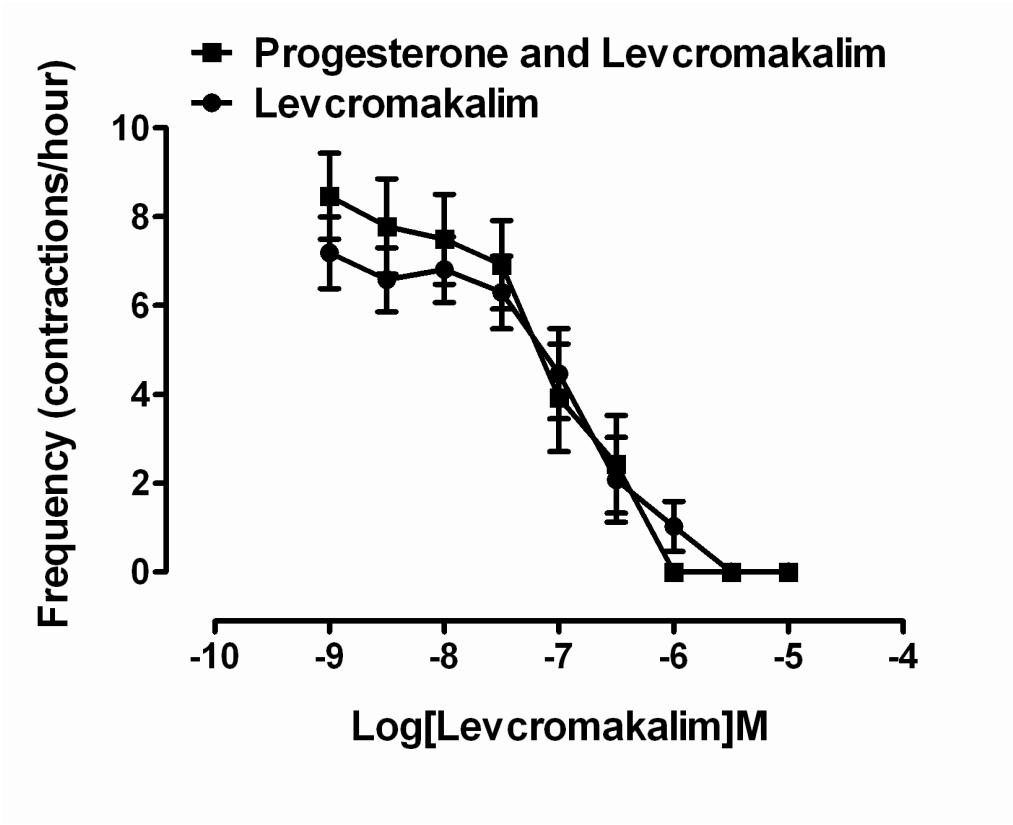

Figure 40 : Concentration-response curve showing the effects on contraction frequency of levcromakalim alone and in the presence of progesterone. No left shift of the curve is seen with progesterone.

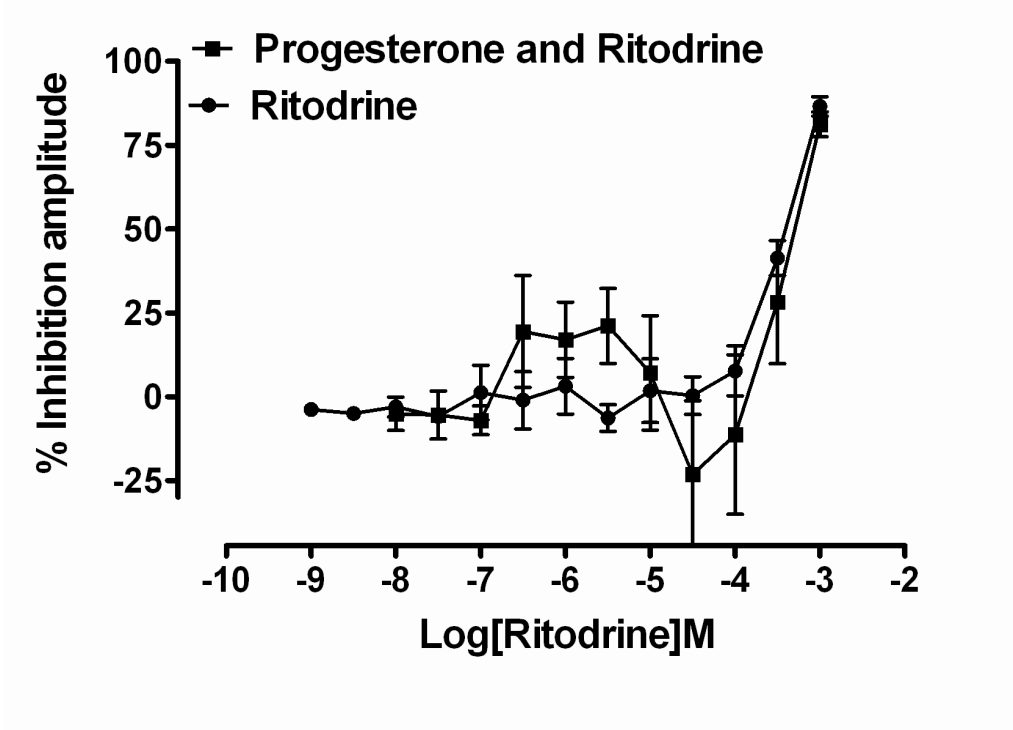

Figure 41 : Concentration-response curve showing the effects on contraction amplitude of ritodrine alone and in the presence of progesterone. No left shift of the curve is seen with progesterone.

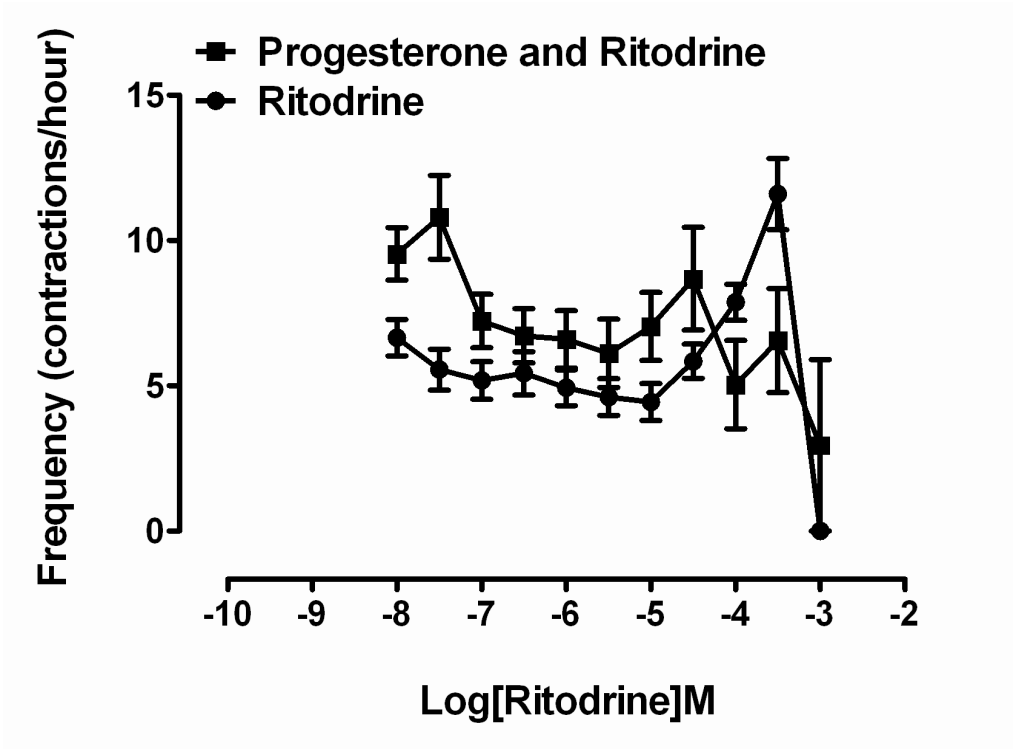

Figure 42: Concentration-response curve showing the effects on contraction frequency of ritodrine alone and in the presence of progesterone. No left shift of the curve is seen with progesterone. Ritodrine causes an increase in overall contraction frequency at higher doses 10-3M prior to inhibition.

Effects of *in vivo* progesterone and placebo

We aimed to determine if prolonged maternal progesterone *in vivo* had any effects on spontaneous myometrial contractility when tested *ex vivo* and if again tocolytic effects were enhanced or oxytocic effects diminished by the progesterone used within the STOPPIT clinical trial.

We collected 18 samples in total from the STOPPIT trial patients undergoing caesarean section for clinical reasons. 16 were used for contractility studies within 12 hours of collection. At the time of sampling and the experiments we remained blinded to the placebo. Progesterone status was unblinded after all samples had been taken and the trial was completed (Spring 2008).Ten patients were within the placebo group and six patients were from the progesterone group, two of which were taken at emergency caesarean section, not pre-labour. Table 3 shows the patient demographics of the STOPPIT placebo and progesterone groups. There were no statistical differences between the groups in any category including age, BMI, gestation at delivery and baby weights of twin one and two (unpaired t-test). The majority of specimens, 12 of 18 in total, were collected at elective caesarean section, pre labour. In view of small numbers and difficulty obtaining these specimens we also collected some in labour samples at emergency caesarean section. Only two patients remained on trial treatment at the time of sampling, both within the placebo group, thus there were no patients actively receiving progesterone therapy at time of sampling. Mean pregnancy gestation (and gestation of sampling) was 252 days in the placebo group and 262 within the progesterone group (p, 0.075). Thus, the interval between sampling and last progesterone administration was 14 ±14 days (mean, SD) in the placebo group and 24 ± 6 in the progesterone group. We obtained contractility data from 16 of the 18 samples collected. In total, 30 strips were examined from the placebo group versus 27 in the progesterone group.

Contraction amplitude measured 55±4mN, frequency 8.1±0.7 h^{-1}, for progesterone and 48±6 mN, 8.1±0.5 h^{-1}, for placebo respectively $p>0.05$. . There was no significant difference between placebo and progesterone groups when spontaneous amplitude or frequency of contractions was measured over time (Figure 43, Figure 44). There was no statistical difference following activation

with to oxytocin, 1 pM-100 nM, (Figure 45, Figure 46) $p>0.05$, or inhibition with levcromakalim, 1 nM-100 μM, (Figure 47, Figure 48), nifedipine, 0.1 nM-10 μM, (Figure 49, Figure 50) $p>0.05$ or ritodrine, 10 nM-1 mM $p>0.05$, (Figure 51, Figure 52) between the placebo and progesterone groups collected from STOPPIT patients (ANOVA).

	STOPPIT Placebo	**STOPPIT Progesterone**
Number women	12	6
Total strips	30	27
Age (Years)	29.3(1.2)	31.8(1.1)
BMI (kg/m2)	25.9(1.5)	25.0(3.1)
Gestation (days)	252(4.6)	262(2.6)
Baby weight (Kg)		
Twin 1	2.3(0.1)	2.6(0.1)
Twin 2	2.3(0.2)	2.6(0.1)
Previous LUSS	0	3
ELLUCS	9	3
Parity		
0	4	3
1	6	3

Table 7: STOPPIT patient demographics

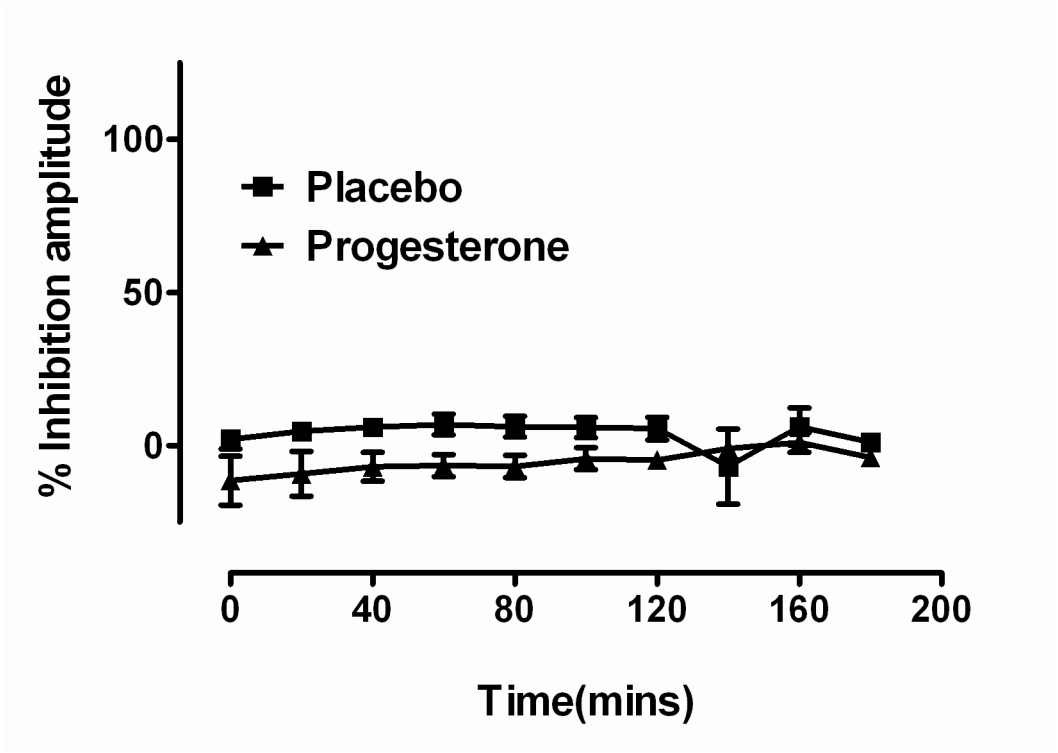

Figure 43: Graph showing the percentage inhibition of spontaneous contraction amplitude over time. There is no evident difference between the placebo and progesterone samples.

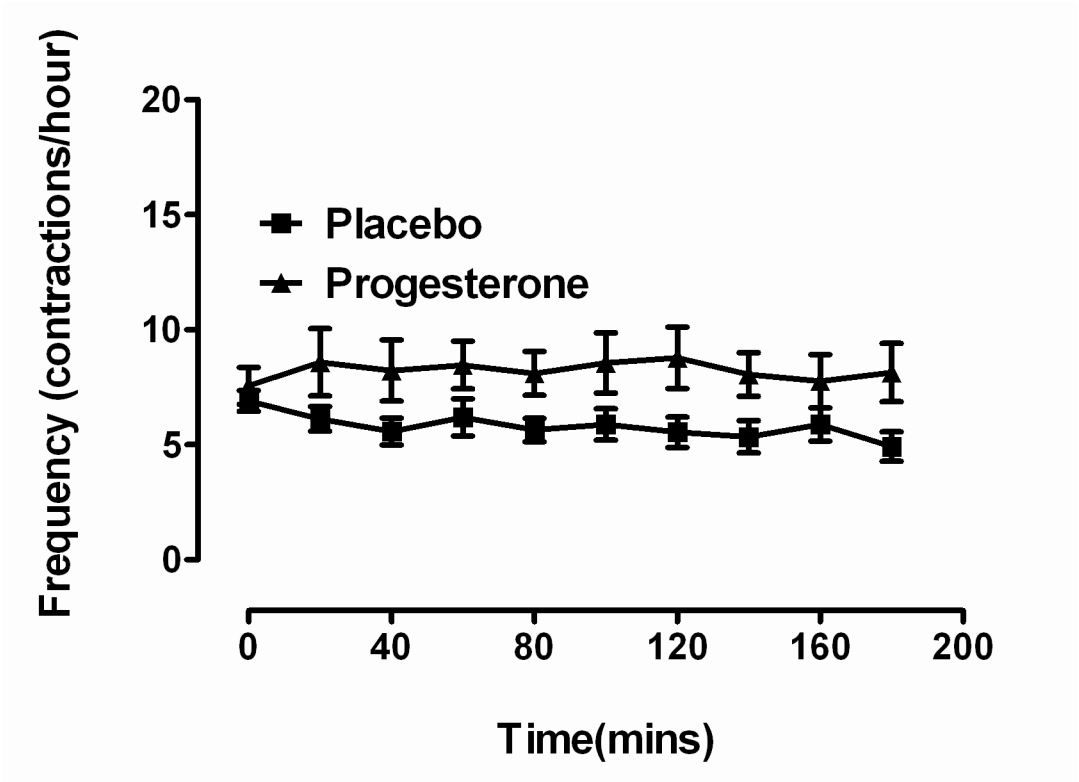

Figure 44: Graph displaying the baseline frequency data (contractions/hour) for both placebo and progesterone over time. There is no difference in frequency between placebo and progesterone.

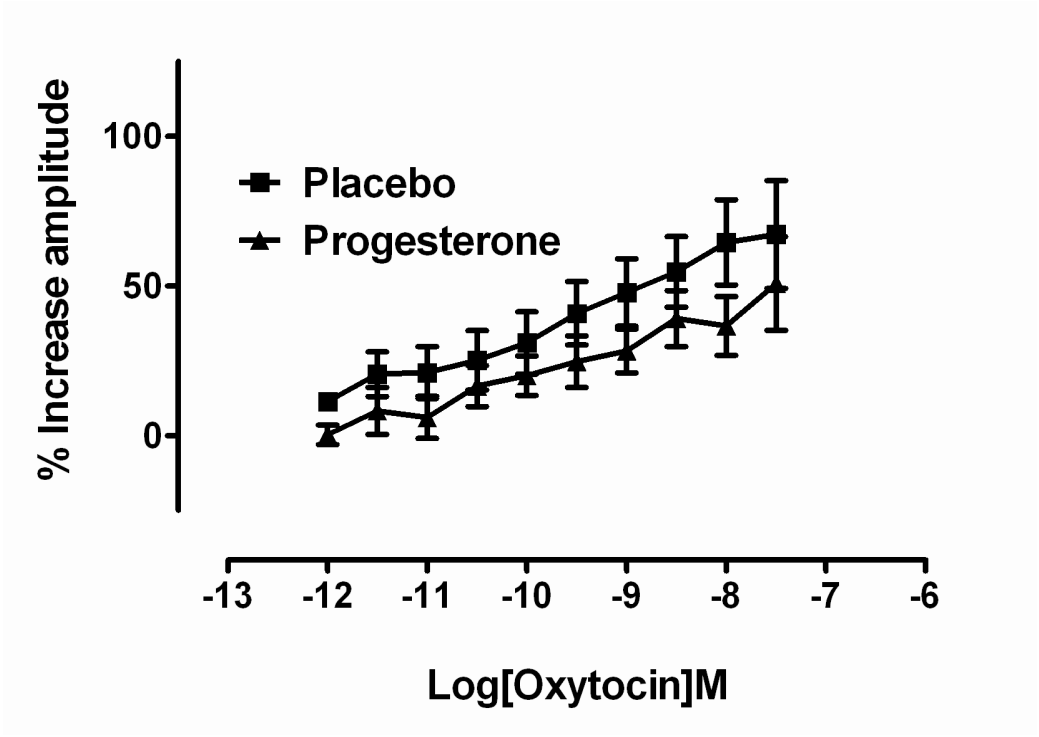

Figure 45 : Concentration-response curve showing the effects on contraction amplitude of oxytocin in placebo and progesterone groups. No right shift of the curve is seen within the progesterone group.

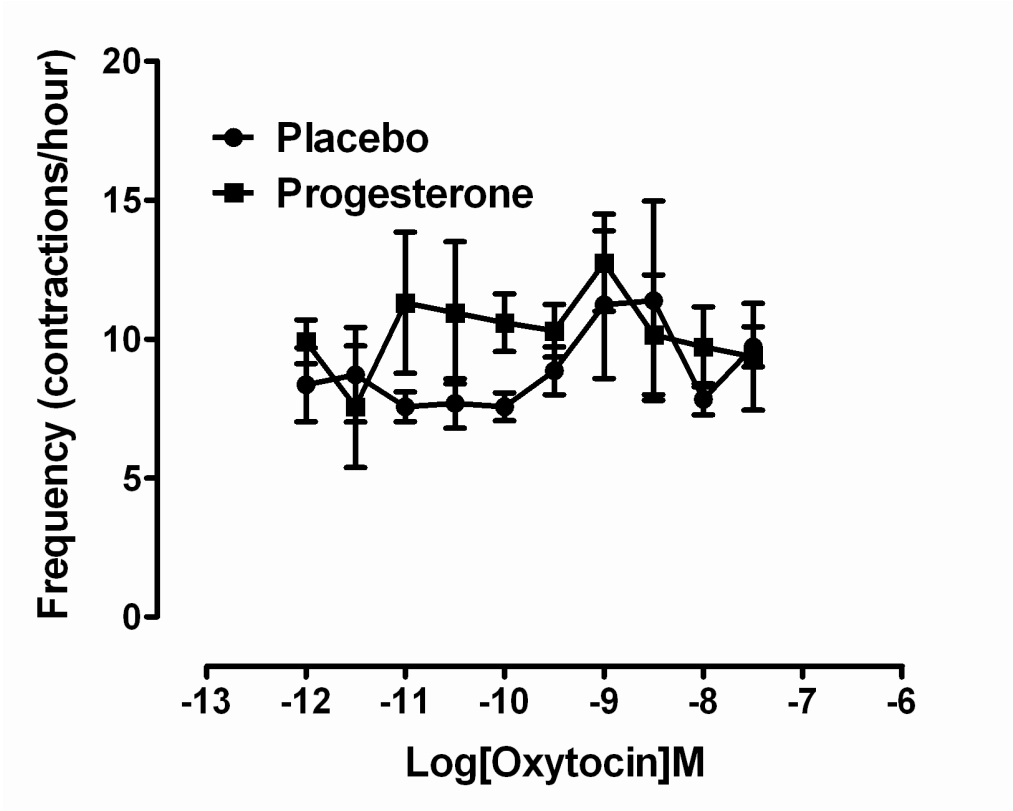

Figure 46: Concentration-response curve showing the effects on contraction frequency of oxytocin in placebo and progesterone groups. No right shift of the curve is seen within the progesterone group.

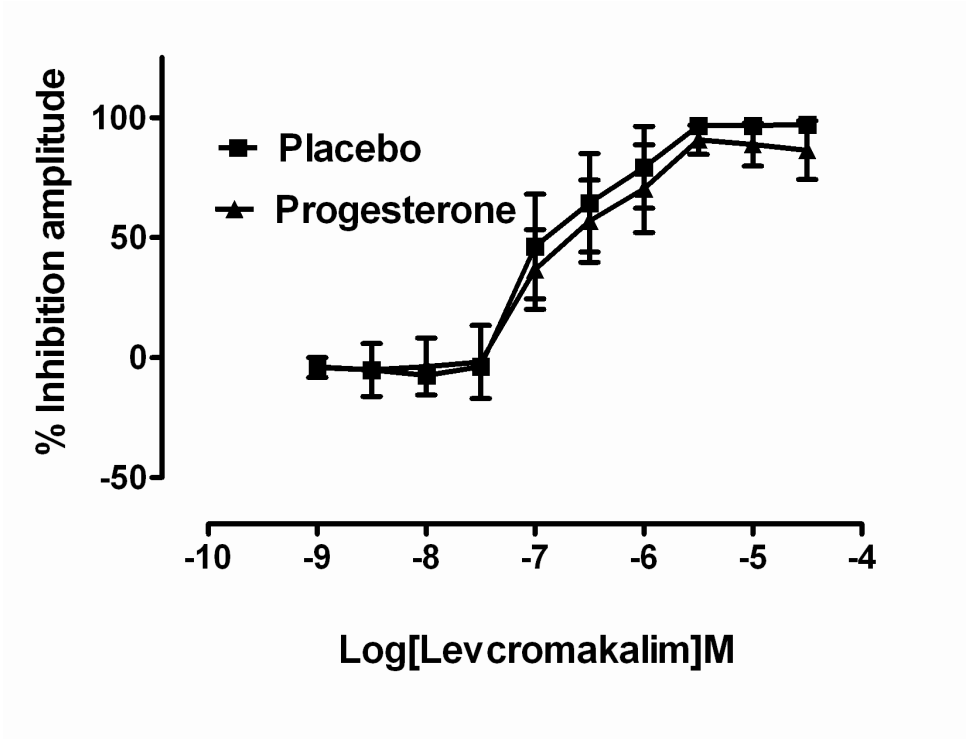

Figure 47 : Concentration-response curve showing the effects on contraction amplitude of levcromakalim in placebo and progesterone groups. No left shift of the curve is seen within the progesterone group.

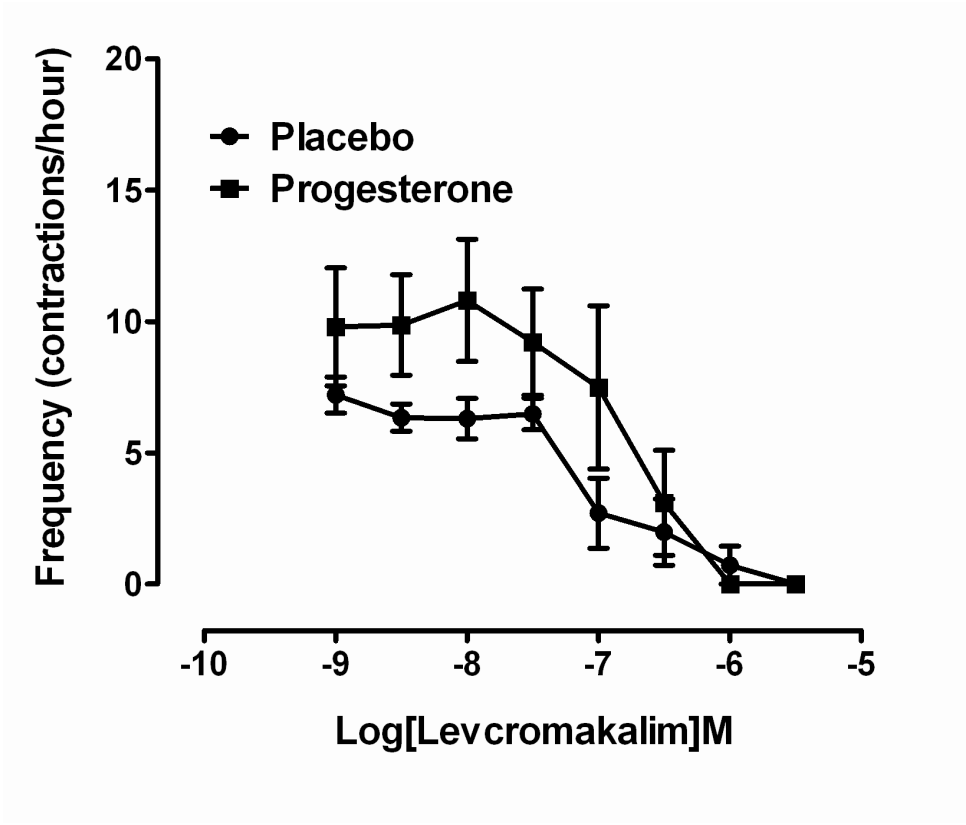

Figure 48 : Concentration-response curve showing the effects on contraction frequency of levcromakalim in placebo and progesterone groups. No left shift of the curve is seen within the progesterone group.

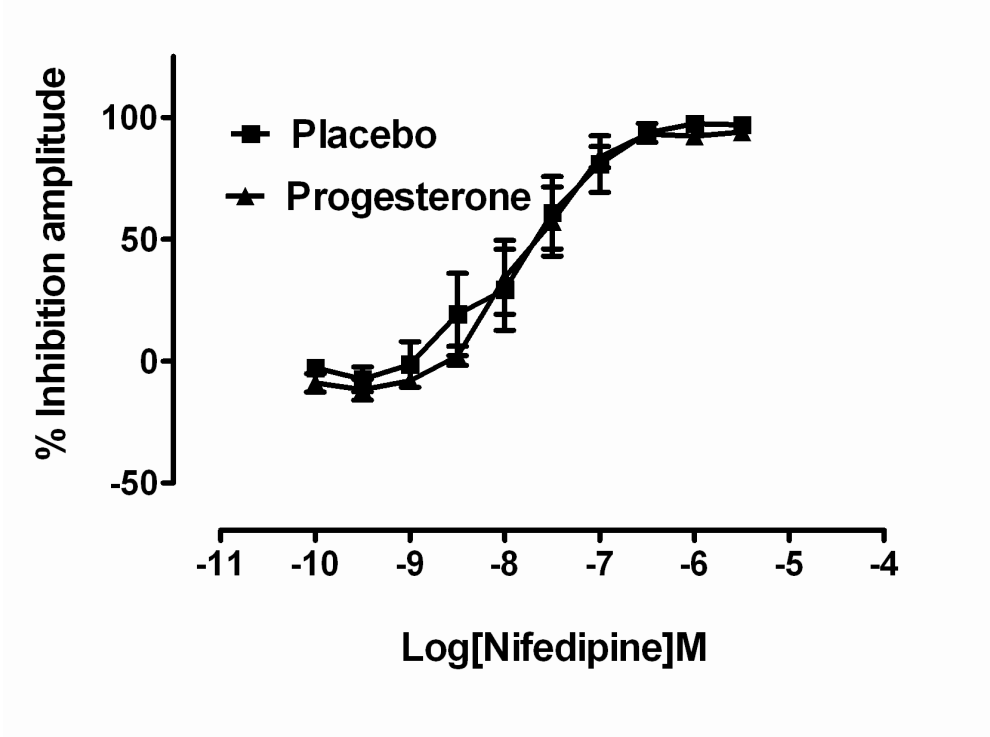

Figure 49 : Concentration-response curve showing the effects on contraction amplitude of nifedipine in placebo and progesterone groups. No left shift of the curve is seen within the progesterone group.

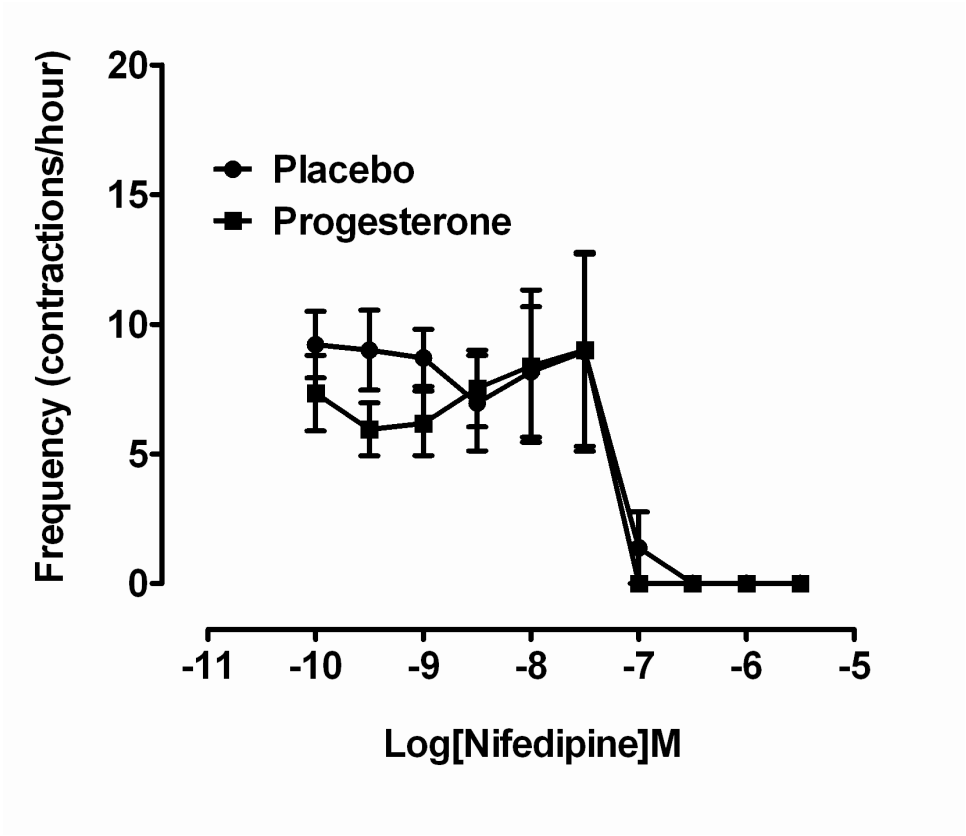

Figure 50 : Concentration-response curve showing the effects on contraction frequency of nifedipine in placebo and progesterone groups. No left shift of the curve is seen within the progesterone group.

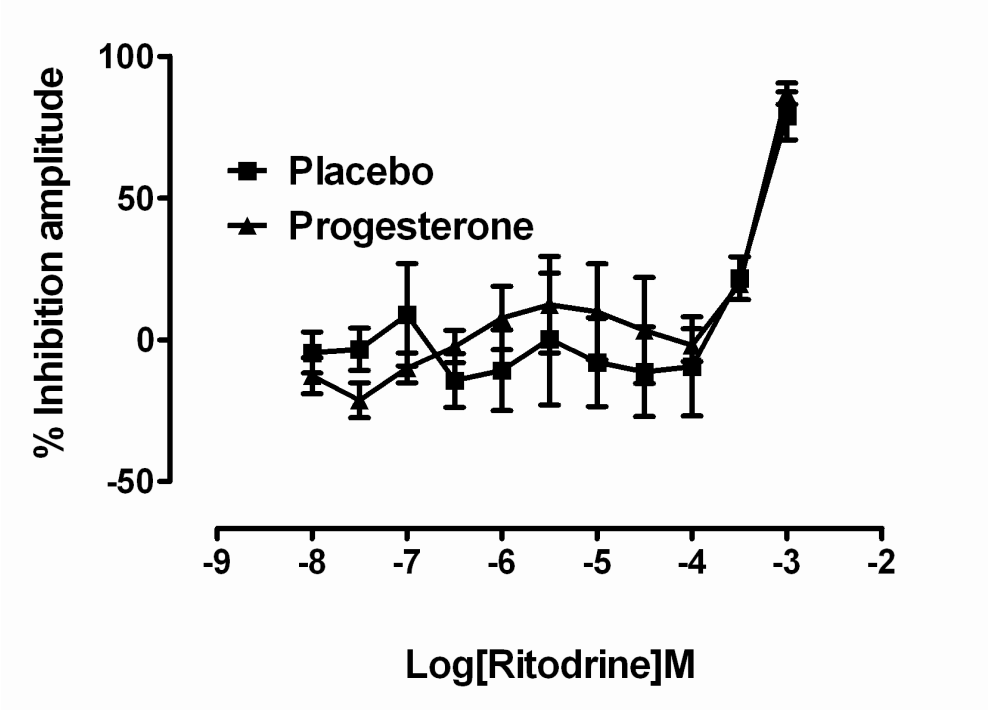

Figure 51: Concentration-response curve showing the effects on contraction amplitude of ritodrine in placebo and progesterone groups. No left shift of the curve is seen within the progesterone group.

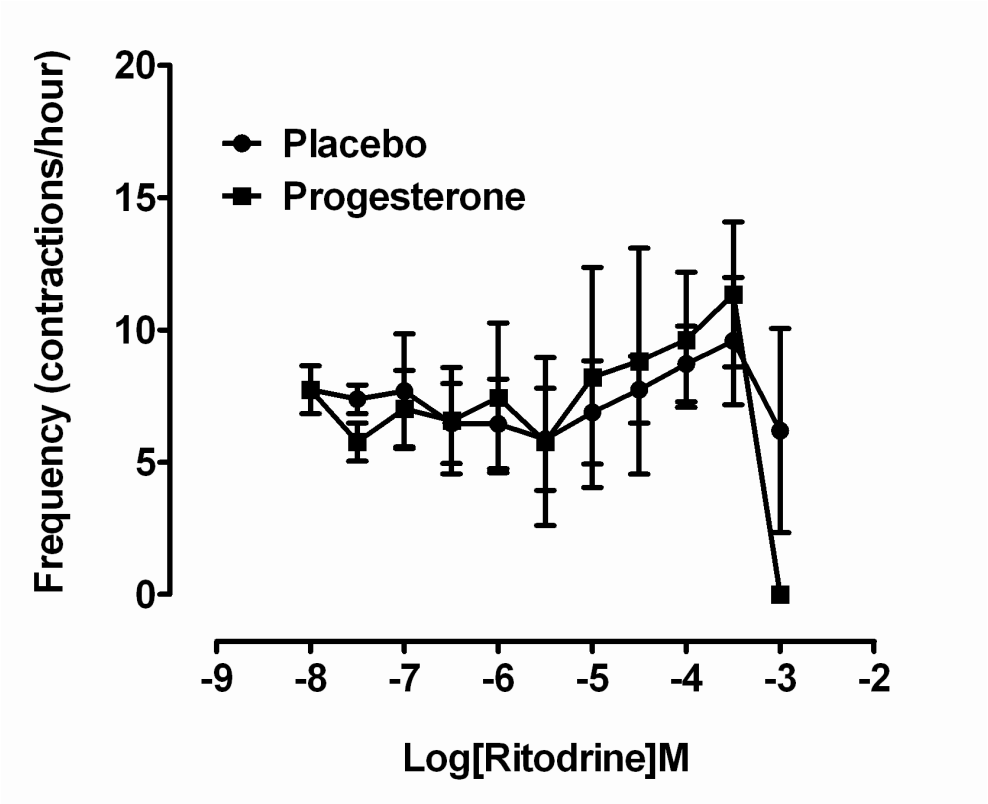

Figure 52 : Concentration-response curve showing the effects on contraction frequency of ritodrine in placebo and progesterone groups. No left shift of the curve is seen within the progesterone group.

Discussion

Contractility Results

We have examined human myometrial contractility both inherent and in response to several different drugs important to labour and contractions. This has also been done in the presence of progesterone which we hypothesised would have inhibitory effects on uterotonins and stimulatory effects on tocolytics.

In view of difficulties in obtaining upper segment myometrial biopsies from either classical caesarean section or performing a biopsy at extra risk to study patients we felt it appropriate to use lower segment for the purpose of our analyses. We have previously directly compared upper and lower segment and found qualitatively similar results (23, 25). Other groups (147) have also shown no difference between upper and lower segment biopsies in terms of myometrial contractility *in vitro* and concluded that the lower segment is appropriate for this purpose and for organ bath contractility analysis.

Oxytocin is a nonapeptide hormone synthesised within the paraventricular nuclei of the hypothalamus and secreted by the posterior pituitary. It is linked to a G protein coupled receptor and its effects are mediated by tissue-specific oxytocin receptor expression which lead directly to contraction within the myometrium (73). The contraction is achieved by sarcoplasmic reticulum Ca^{2+} release and sensitisation of the contractile mechanisms to Ca^{2+} (148). Oxytocin also modifies the contraction threefold 1) increases the frequency of contractions, 2) Increases the basal tone i.e. not complete relaxation after a contraction and 3) Increases the amplitude and duration of overall contractions (71). We observed all of these findings in our own use of oxytocin on spontaneous myometrial contractions and at concentrations of greater than 10nM we observed a decrease in overall amplitude and observed a bell shaped curve evident with agonist activity. This was due to the incomplete relaxation and failure to return to the original baseline as is noted with oxytocin at high doses.

Levcromakalim is an ATP- sensitive potassium channel opener (64) which allows an increase plasma membrane K^+ permeability causing hyperpolarisation. The

membrane potential is therefore driven away from the action potential threshold. It is a potent smooth muscle relaxant and has been shown to have an inhibitory effect on human myometrial contractions in other groups and our own (64, 92). Levcromakalim has been used previously in the treatment of angina as a vascular smooth muscle relaxant (149). The sulfonylurea, glibenclamide, is an apparent competitive inhibitor of K_{ATP} channels (144).We demonstrated a concentration-response curve on spontaneous human myometrial contractions and also one that was antagonised by glibenclamide 1.5 μM. Levcromakalim is not used clinically as a tocolytic within labour ward settings but this could represent a potential new approach for tocolysis via potassium channels as suggested by Morrison et al (64).

Calcium channel blockers are highly specific inhibitors of L-type voltage operated calcium channels and are smooth muscle relaxants that act by inhibiting the intracellular influx of calcium ions (150). Nifedipine is the most commonly used calcium channel blocker for tocolysis (151). It is still used clinically within the UK as a short term treatment for preterm labour, as recommended by the Royal college of Obstetricians and Gynaecologists (141). The use of nifedipine as a tocolytic for 48 hours may enable administration of a short course of corticosteroids. These has been shown to help mature the fetus' lungs and reduce the risk of neonatal respiratory distress syndrome (152). The use of tocolytics such as nifedipine, may also allow transfer of the fetus *in utero* to a tertiary referral unit with adequate neonatal facilities if threatened preterm labour is diagnosed. Nifedipine, however, is not licensed for tocolytic use in the UK. Our group has also shown previously that nifedipine produced a concentration-dependent inhibitory effect on both amplitude and frequency of spontaneous human myometrial contractions (92).

Ritodrine, a $ß_2$-adrenergic agonist has, in the past has been used as a tocolytic clinically (153). We found that it produced a concentration-dependent inhibition of the amplitude of contractions. The frequency of contractions showed, at doses greater than 100μM, a transient excitatory effect prior to inhibition of the contractions overall. This is thought to occur due to a transient excitement of contractile alpha-adrenoreceptors (154). There is also evidence to support a structural and functional link between the $ß_2$-adrenergic receptor and BK_{Ca} in maintaining uterine quiescence with levels of both decreasing in labour (155)

(156). Nifedipine and atosiban(an oxytocin receptor antagonist) are now recommended prior to consideration of ritodrine as a tocolytic in the prevention of preterm labour (141).

Our study has shown that in vitro progesterone, albeit at high concentrations, exerts consistent, rapid and sustained inhibition of the amplitude of spontaneous myometrial contractions. The rapidity of action of progesterone implies a non-genomic effect. Although the effects on the frequency of contractions were less consistent than that of amplitude the net effect was one of overall inhibition of contractile activity, as measured by activity integral. This unexpected effect on frequency has been noted before in vitro (101, 102).

The concentrations of progesterone required to inhibit contractions *in vitro* are unlikely to be achieved with therapeutic doses of progesterone *in vivo*. However, it may not be the absolute concentration of progesterone that is important in vivo, given that it has been suggested that patterns of change as opposed to absolute concentrations of progesterone and other steroid hormones may be the important factor in affecting myometrial contractility (157).

Unlike natural progesterone, however ,17OHPC did not exert any inhibitory effect on myometrial activity over and above that see with its vehicle (70% ethanol), thus confirming the work of another group (158). Despite this, a number of clinical trials administering intramuscular injections of 17OHPC have been successful in preventing preterm delivery in high risk women. Sexton et al suggests that this positive clinical outcome may well be mediated through long-term genomic pathways which cannot be reproduced under organ bath conditions (158); we were only able to conduct experiment for 8 hours *in vitro* before myometrial contractility began to decline. It is possible therefore that 17OHPC requires *in vivo* conditions to exert an effect. A contractility study investigating progesterone and 17OHPC carried out by Ruddock et al showed that 17OHPC levels, when incubated with myometrial tissues were reduced by between 90- 98%. Progesterone was less effected with a loss of 29-50% detected (100).They used ethanol as opposed to DMSO as a solvent and showed less vehicle effects. 17OHPC however did not appear to present within the organ baths at the end of the experiments. It also remains unclear as to whether 17OHPC is active itself or requires metabolic activation. One suggested pathway

is hydrolysis by plasma and tissue esterases, but a recent study showed that 17OHPC was not hydrolysed to by esterases *in vitro* suggesting again either *in vivo* requirements or alternative mechanisms of metabolism (159). Progesterone may also differ from 17OHPC *in vitro* as they potentially act upon different receptors. 17OHPC has recently been found to have lower affinity than progesterone for the progesterone PR receptor (160). The non-genomic pathways by which progesterone itself inhibits myometrial contractility *in vitro* may operate through the cell membrane progesterone receptors α, ß and γ, which are similar to G-protein coupled receptors (109) . Their role in the process of labour is, however, not yet understood and progesterone's main action in maintaining uterine quiescence is generally accepted to result via the PR-B receptor (48, 77). An alternative non-genomic pathway of progesterone action is interaction with gamma amino butyric acid A ($GABA_A$) receptors. These receptors appear to be important in the emerging effects that progesterone has on the brain (reference). Additionally, initial studies of rat myometrium $GABA_A$ antagonists prevented progesterone induced inhibition of contractions, suggesting that progesterone might mediate its action via the GABA receptor (107). A prior study found that relaxation produced by the above steroids was not blocked by the $GABA_A$ receptor antagonists, picrotoxin or bicuculline, but was reversed by calcium, they suggest that blockade of calcium influx appeared to be responsible this relaxation (108).

These results could not be replicated in more recent studies of human myometrium, with the authors concluding that blockade of calcium influx appears to be responsible (108).

In this study, which made use of selective inhibitors, we clearly demonstrate that progesterone does not exert its inhibition of myometrial contraction via a range of potassium channels, including $K_{ATP,}$ BK_{Ca}, IK_{Ca} and SK_{Ca}. Progesterone treated strips showed no significant difference in either contraction amplitude or frequency after addition of each potassium channel blocker. Apamin did not alter the spontaneous myometrial contractility and as a selective blocker of SK_{Ca} channels demonstrated no role for this potassium channel in regulating spontaneous contractions (161). Recent mice studies however, examining small conductance Ca^{2+}-activated potassium (SK) channel, SK3 suggest that this channel plays an important role in regulating uterine function by limiting influx

through L-type Ca^{2+} channels and disrupting the development of phasic contractile events (162). We observed normal phasic contractions prior and post addition of apamin which disrupts these channels. The lack of effect of glibenclamide which selectively blocks K_{ATP} channels (163) also suggests it has little or no role in this regulation. Iberiotoxin and charybdotoxin are both able to block BK_{Ca} channels, charybdotoxin however, is less selective and also blocks intermediate IK_{Ca} and voltage operated Kv channels (164) .These BK_{Ca} are large conductance and calcium sensitive potassium channels. Iberiotoxin had no independent effects on myometrial contractility indicating they have little or no role in regulating spontaneous contractility .Charybdotoxin had no independent effect on myometrial contractility but in combination with progesterone it appeared to increase the frequency of contractions (10nM progesterone).This effect was not maintained after the initial addition of progesterone and therefore does not give a clear indication of an intermediate IK_{Ca} or BK_{Ca} role within this mechanism of action. Other studies have shown evidence which conflicts with this. In human non -labouring myometrium others have observed increased levels of BK_{Ca} channels but decreased BK_{Ca} channel expression was seen in preterm and term labouring myometrium. Consequently, these channels may be a part of the mechanism to induce spontaneous labour contractions (156, 165) .

Other groups have studied vascular smooth muscle and progesterone, which causes a relaxation of rat mesentery They demonstrated a lack of effect of potassium channel blockers on progesterone-induced relaxation suggesting that these K channels play little or no role within vascular smooth muscle (166).Similarly ,we have been unable to demonstrate any effect of potassium channel blockers on progesterone-induced relaxation of myometrial smooth muscle.

Alternative pathways by which progesterone may exert its inhibitory effect could be via sodium or chloride channels. There is some evidence to suggest that sodium channels may be up regulated in myometrium at late gestation (167) but the distinct roles of specific sodium channels is not clearly understood. Ion channels may well have regulatory pathways related to myometrial function but whether progesterone utilises them in its mechanism to prevent preterm labour remains unclear.

The other major finding of the study is that *in vivo* progesterone therapy to prevent pre-term labour does not appear to modify contractility *ex vivo*. This may, however, be due to study design limitations. Our sample group from the progesterone-treated arm of the clinical trial discontinued their administration of progesterone at 34 weeks (238 days). The mean sample gestation within the progesterone group was 262 days, therefore providing a significant period (24 ±6, mean days ±SD) in which patients were no longer actively receiving progesterone. Whether progesterone treatment *in vivo*, would manifest changes in myometrial contractility *ex vivo* if examined during the treatment window, remains to be determined. Small sample numbers from this STOPPIT substudy may also contribute to lack of evidence of functional effect by progesterone. Due to initial study design we were unable to collect the proposed number >40 in order to have an adequately powered study therefore these results must be interpreted with caution.

The progesterone metabolite 5 ß-dihydroprogesterone is thought to reduce oxytocin binding to it's receptor in cultured cells (168).There is evidence to suggest that progesterone interacts with the rodent oxytocin receptor but not the human receptor(168) Thornton et al also demonstrated a right-shift in the activity integral of the oxytocin concentration-response curve with 100μM of the metabolite (126).With progesterone we were unable to demonstrate any change in the oxytocin concentration-response. Other groups have reported a small inhibitory response with progesterone and even less with 5 α-reduced metabolites of progesterone suggesting significant differences between progesterone and its many metabolites (169).

A previous study has reported enhanced responses to ß-sympathomimetics following both *in vivo* and *in vitro* treatment with progesterone (104). However, we did not see an enhanced effect of any tocolytic, including levcromakalim (a K_{ATP} channel opener), nifedipine (a calcium channel blocker) and ritodrine (a ß-2 agonist) in either our *in vitro* or *in vivo* progesterone groups. This is in contrast to previous work carried out by Chanrachakul et al, where they demonstrated an enhancement of the tocolytic effect of ritodrine using a lower concentration of progesterone (0.01 μM) *in vitro* (99). Their experimental design involved using oxytocin-induced contractions as opposed to spontaneous contractions in our study. It is possible that different progesterone preparations and regimes may

underlie experimental outcome differences however both clearly show a rapid non-genomic effect of progesterone alone. Additionally, they used progesterone dissolved in ethanol (against appropriate ethanol controls) in contrast to our own study where we used progesterone in cyclodextrin. The differences between the Chanrachakul study and are own are hard to explain unless progesterone's effect in enhancing the tocolytic effects of ritodrine require either ethanol or stimulation with oxytocin to become apparent. Their experimental design involved using oxytocin-induced contractions as opposed to spontaneous contractions in our study. Lastly although a potential weakness of our study may be the use of lower segment myometrial biopsies, others have previously shown no difference between upper and lower segment biopsies in terms of myometrial contractility *in vitro* (147) .

If active progesterone administration directly alters myometrial contractility *in vivo* as well as indirectly enhancing tocolytic activity, it is possible our *in vivo* progesterone samples are out with this window of "active progesterone therapy" to show clear evidence to support this theory. One previous clinical trial found that administration of a large bolus of progesterone into the amniotic fluid of women at term in labour decreased spontaneous contraction frequency and intensity within minutes, but study numbers were small (127). Alternatively, progesterone administration may not alter fundamental tissue contractility and such evidence to support progesterone's inhibitory effect on myometrial contractility may be due to high pharmacological doses in organ bath conditions as well as *in vivo*. Our group would suggest that to inhibit myometrial contractility *ex vivo* that active progesterone treatment is required, as this is likely to act via short term non genomic pathways.

In conclusion we have demonstrated that progesterone can inhibit myometrial contractility *in vitro* however its mechanism of action both *in vitro* and *in vivo* warrants further investigation.

CHAPTER 4

The effects of progesterone and 17 α hydroxyprogesterone caproate on cytokine release, uterine relaxants and contraction associated proteins within myometrium *in vitro*

Introduction

A wide variety of mediators have been implicated in the control of labour and the initiation of preterm labour including pro-inflammatory cytokines (170) and contraction associated proteins (171). We hypothesised that progesterone may have an impact on these mediators and that this might contribute to the mechanism of action of progesterone in preventing preterm birth. We looked at a several groups of labour and inflammatory associated genes as well as known smooth muscle relaxants and promoters of uterine quiescence.

As well as progesterone itself, 17OHPC has been demonstrated to prevent preterm labour (111) particularly in high risk groups. Again, by looking at various different genes as above we compared 17OHPC with progesterone.

Aim of Chapter

The aims of the following experiments were to determine cytokine release into its surrounding cell culture medium from myometrial tissue *in vitro* in the presence of various concentrations of progesterone over a 24 hour period. Myometrial tissue samples were incubated with nil (control), progesterone (water soluble) or 17OHPC at 10^{-6}M, 10^{-5}M and 10^{-4} M concentrations. DMSO controls were carried out for 17OHPC. By weighing three small myometrial explants from each patient's biopsy we were able to standardise the amount of tissue being used per gram wet weight .Each set of experiments was performed in duplicate and with myometrial biopsies obtained from 7 separate patients for every cytokine measured. We then measured the final mRNA density levels within the myometrial explants of various uterine relaxants, stimulants and contraction associated proteins at the final 24 hour time-point. We used the Bio-Plex® suspension array technology and were able to quantitate seven pro-inflammatory cytokines from the supernatants of the myometrial tissues. Our assay was customised by the manufacturer to measure cytokines: IL1ß, IL6, IL8, IL10, IFN γ, MCP and TNF α. The suspension array is composed of colour-coded bead sets, each of which may be conjugated with a unique reactant specific to a given target, listed above, creating an immunoassay. We performed an operational-validation kit with the system in order to validate the performance of the system. The kit included a series of standards to generate a standard

curve for each antibody. This technique can be likened to ELISA (enzyme linked immunoassay) in which we detected the presence of an antibody or antigen within a sample that is linked to a detected enzyme such as peroxidise. Although not an identical system the background theory is comparable and this flow-cytometric technology assay uses monoclonal antibodies linked to fluorescent beads. We also elected not to stimulate cytokine release as a marker of inflammation or labour rather we measured the basal levels over time produced from myometrium within culture medium only. An example of this is by using lipopolysacharide (LPS) to stimulate an inflammatory response (172). Inflammation is certainly one cause of preterm labour however in twin pregnancy (as recruited in STOPPIT) stretch and other mechanisms are implicated as underlying mechanisms relating to the higher rates of preterm labour. We felt it therefore more appropriate to make any comparison with our STOPPIT data that the myometrium should not be stimulated by an inflammatory mediator.

We also carried out an assessment of lactate dehydrogenase (LDH) release as a marker of cell death. LDH is released from cells as cell membrane integrity is lost. By measuring LDH release prior to the commencement of a cell culture and again measuring LDH release over time up to and beyond our selected 24 hour time-points we were able to rule out loss of tissue viability within our myometrial explants and during the allocated time-points selected for our cell culture.

We then measured expression of mRNA for a number of pro labour substances, contraction associated proteins, endogenous uterine stimulants and endogenous inhibitors of uterine contractility using RT-PCR. We were only able to carry this out at the final 24 hour time-point, at the end of the cell culture experiment. We selected primer probe sets for IL1ß, IL6, IL8, COX-2, INOS, bNOS, eNOS, cGRP, cGRPR, ep2, Cx-26, Cx-43 and Progesterone receptor A+B. A table of these genes is listed within materials and methods (Table 3, Table 4) .DMSO affected mRNA expression and cytokine release on its own therefore in view of the impact that the vehicle control DMSO had on both BioPlex results and RT-PCR the results for 17OHPC are difficult to interpret, we planned to use a water soluble version of 17OHP but were unable to source one.

Statistical testing and graph display as carried out using GraphPad Prism, this calculates a p value of greater or less than 0.05 as shown. This indicates significance but does not give an exact p value number.

Results

Release of Cytokines from myometrial explants in the presence of progesterone and 17 OHPC

IL1β

This pro-inflammatory cytokine was released into the supernatant in increasing amounts over time but was produced in low levels relative to other cytokines. Using time and the progesterone treatment as independent variables, time had a significant effect on the levels (all expressed as $pgml^{-1}/g$ wet weight, mean ± SEM) detected with 24 hours showing the highest levels n=8, $p<0.001$ compared with baseline. Cytokine production (without additional progesterone) measured 0.8 ± 0.3 $pgml^{-1}/g$ wet weight after 1 hour and reached 12.6 ± 1.8 at 24 hours . Treatment with 17 OHPC did not alter IL1 ß levels at low concentration, $10^{-6}M$. However, at higher concentrations of 10^{-4} M there was a significant vehicle effect compared to control, $p<0.05$, therefore at high levels 17OHPC had no effect different from that of its vehicle dimethyl sulfoxide (DMSO) .Treatment with progesterone 10-6M, 10-5M and 10-4M did not alter the amount of IL1ß released into the supernatant when compared with control, (two-way ANOVA, GraphPad Prism, Figure 53, Figure 54).

IL6

IL6 was more readily released into the supernatants than IL1ß without any inflammatory stimulation. Again, using time and progesterone treatment as the sources of variation we found that time had a significant effect on the release of IL6 $p<0.001$ (e.g.1 hour control levels measured 177 ± 55, 24 hours levels 3003 ± 475) but progesterone concentrations $10^{-6}M$, $10^{-5}M$, $10^{-4}M$ had no impact on IL-6 levels (all expressed as $pgml^{-1}/g$ wet weight, mean ± SEM) (Figure 55).Treatment with 17OHPC had no effect on IL6 different from that of its vehicle control (Figure 56).

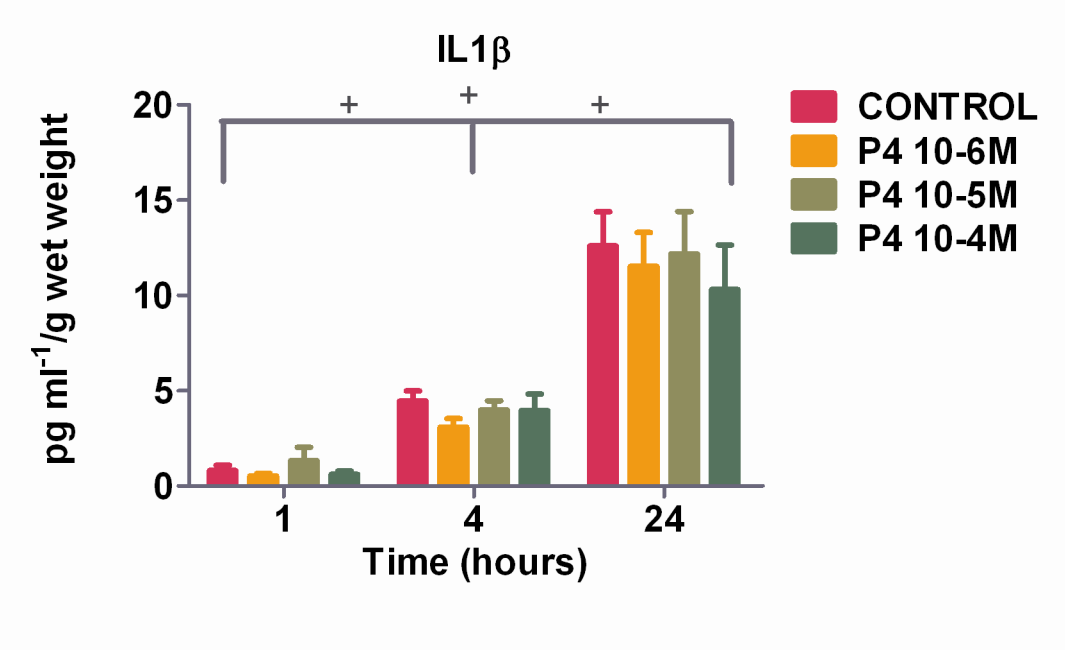

Figure 53 : Levels of IL1β detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Over time there was a significant increase in levels of IL1β + p<0.001 (ANOVA) but progesterone did not have any effect when compared with control.

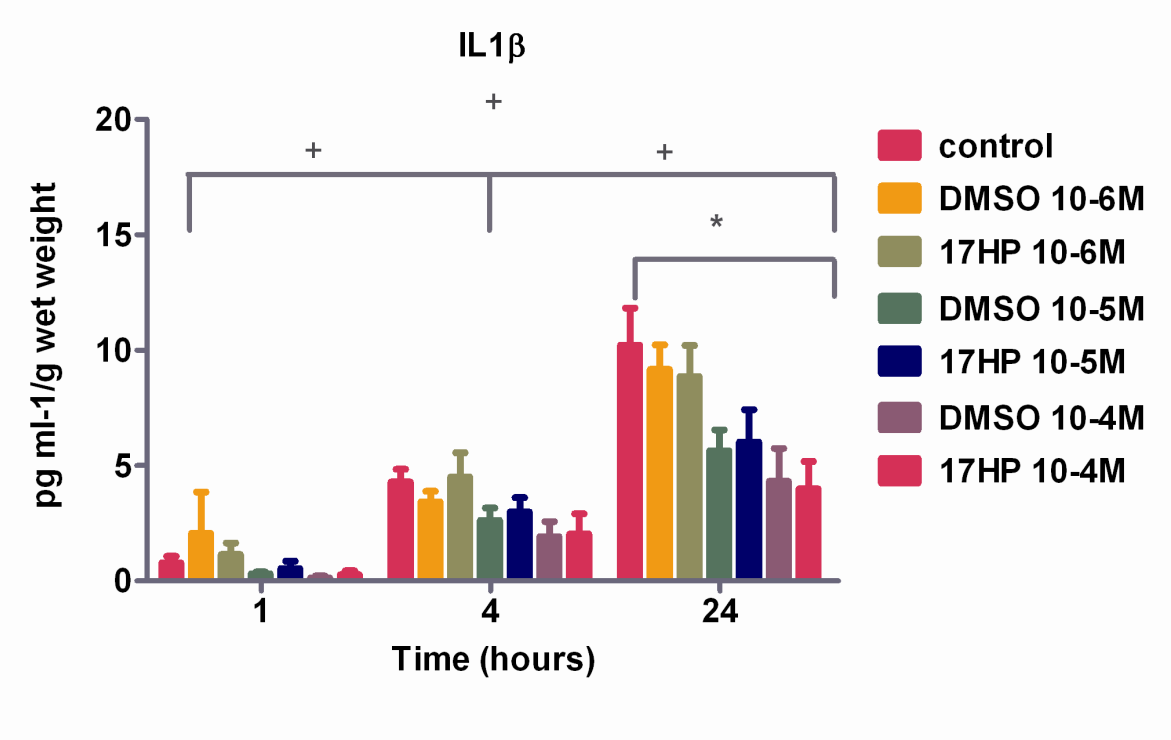

Figure 54 : Levels of IL1β detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of IL1β + p<0.001 (ANOVA). The DMSO vehicle significantly reduced levels of IL1β at 100 µM * p<0.05. The 17OHPC results do not differ from its vehicle at this concentration.

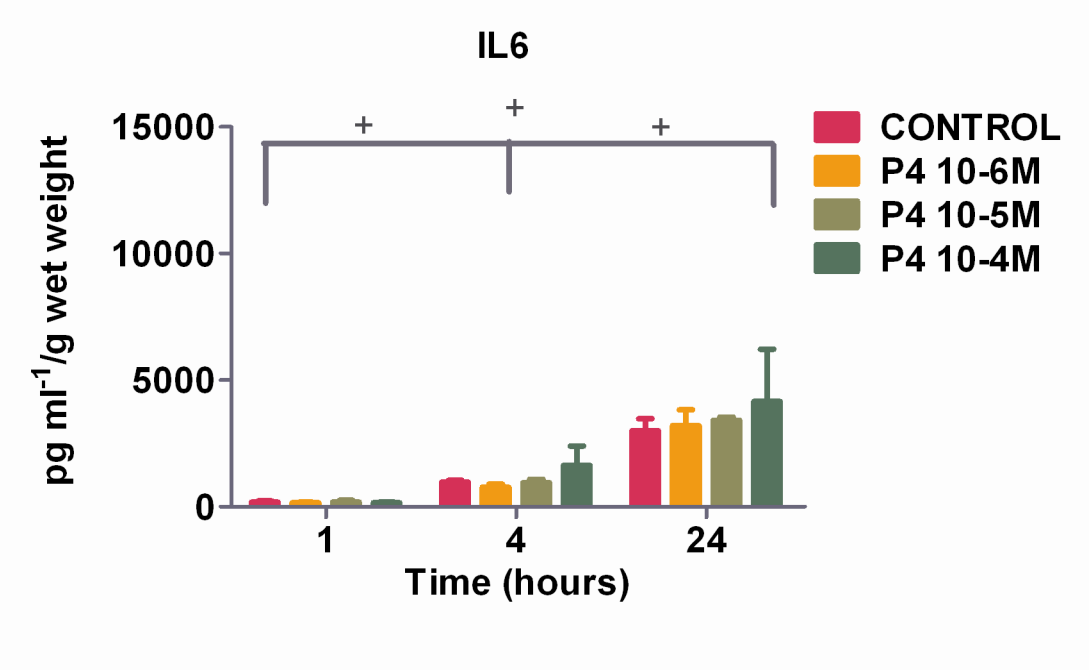

Figure 55 : Levels of IL6 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Over time there was a significant increase in levels of IL6 +p<0.001 (ANOVA) but progesterone did not have any effect when compared with control.

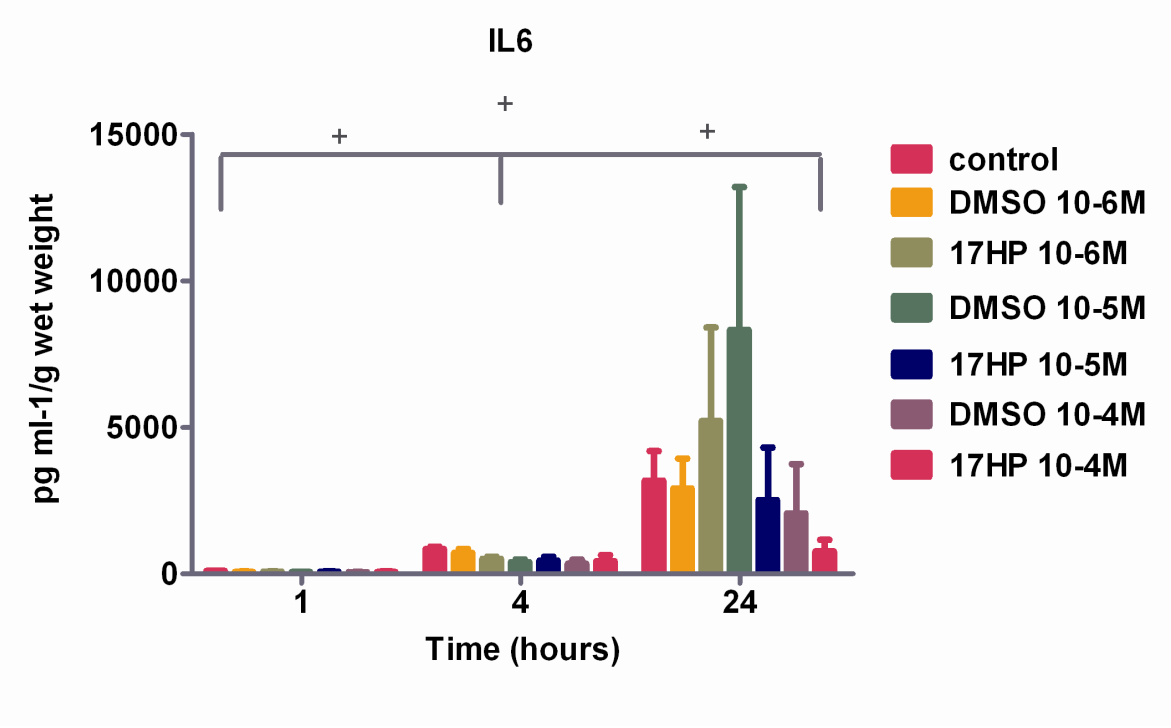

Figure 56 : Levels of IL6 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of IL6 +p<0.001 (ANOVA). 17OHPC had no effect on IL6.

IL8

This pro- inflammatory cytokine similarly to IL6 was readily released into the supernatants from non-stimulated myometrial explants. Low levels were detectable at 1 hour compared with 4 hours and 24 hours $p<0.001$. Progesterone had no effect at any of the concentrations on the overall levels of IL8 detected $p>0.05$ and time again was the only source of variation (two-way ANOVA, GraphPad Prism) (Figure 57). Levels of the control at time 1 hour measured 42 ± 23 (all expressed as pgml-1/g wet weight, mean ± SEM). Treatment with 17 OHPC did not alter IL8 levels at low concentration, 10-6M. However, at higher concentrations of 10-4 M there was a significant vehicle effect compared to control, $p<0.05$, therefore at high levels 17OHPC had no effect different from that of its vehicle (DMSO) (Figure 58).

IL10

This cytokine is an anti-inflammatory cytokine, our hypothesis that progesterone may increase levels of IL10 rather than decrease (as with pro-inflammatory cytokines) in order to maintain uterine quiescence. Low levels of IL10 were detected using this Bio-Plex® array. Time again, was the significant variation factor with an increase in release over the time-points, $p<0.001$(Figure 59) (ANOVA). Progesterone at 10-6M, 10-5 and 10-4M showed no change in the amount of IL10 released compared with control $p>0.05$ (two-way ANOVA, GraphPad Prism). Levels of the control at time 1 hour measured 9.8 ± 4.4 (all expressed as pgml-1/g wet weight, mean ± SEM).Treatment with 17OHPC had no effect on IL10 different from that of its vehicle control at 10-4 M there was a significant vehicle effect compared to control, $p<0.05$ (Figure 60).Progesterone at these concentrations does not appear to increase the amount of IL10 released compared with a control.

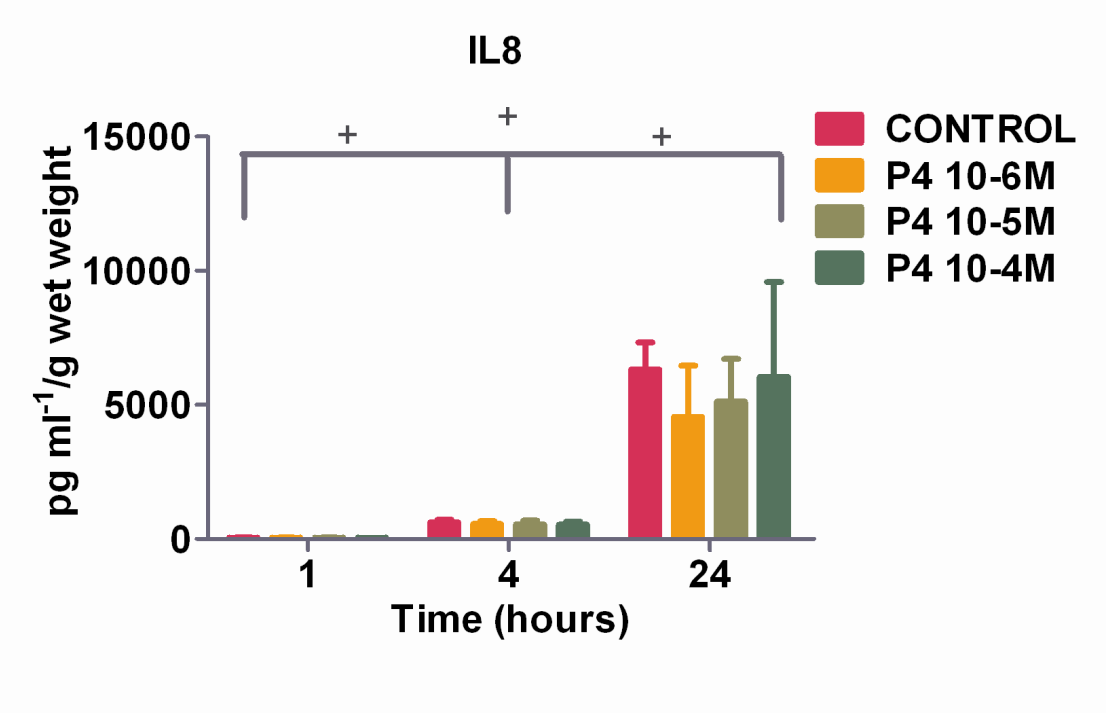

Figure 57 : Levels of IL8 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Very low levels were seen at 1 hour in comparison with 24 hours. Over time there was a significant increase in levels of IL8 + $p<0.001$ (ANOVA) but progesterone did not have any effect when compared with control.

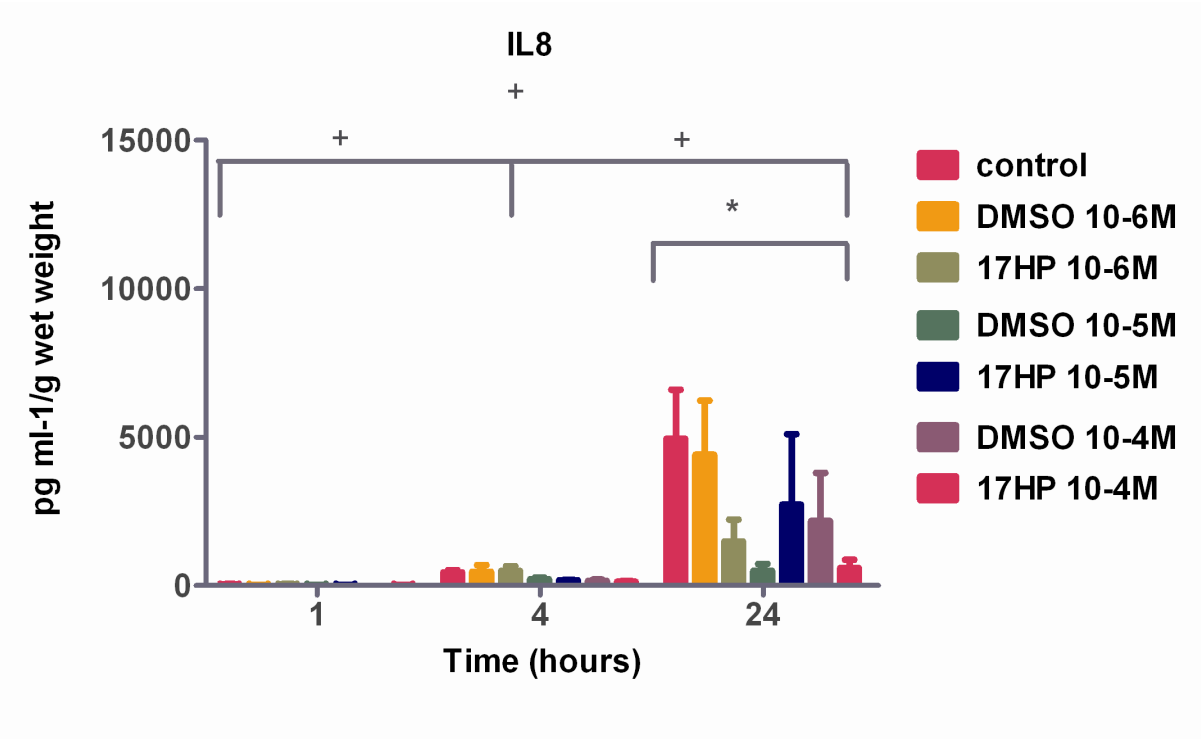

Figure 58 : Levels of IL8 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of IL8 + $p<0.001$ (ANOVA). The DMSO vehicle significantly reduced levels of IL8 at 10 µM and 100 µM * $p<0.05$. The 17OHPC results do not differ from its vehicle at this concentration.

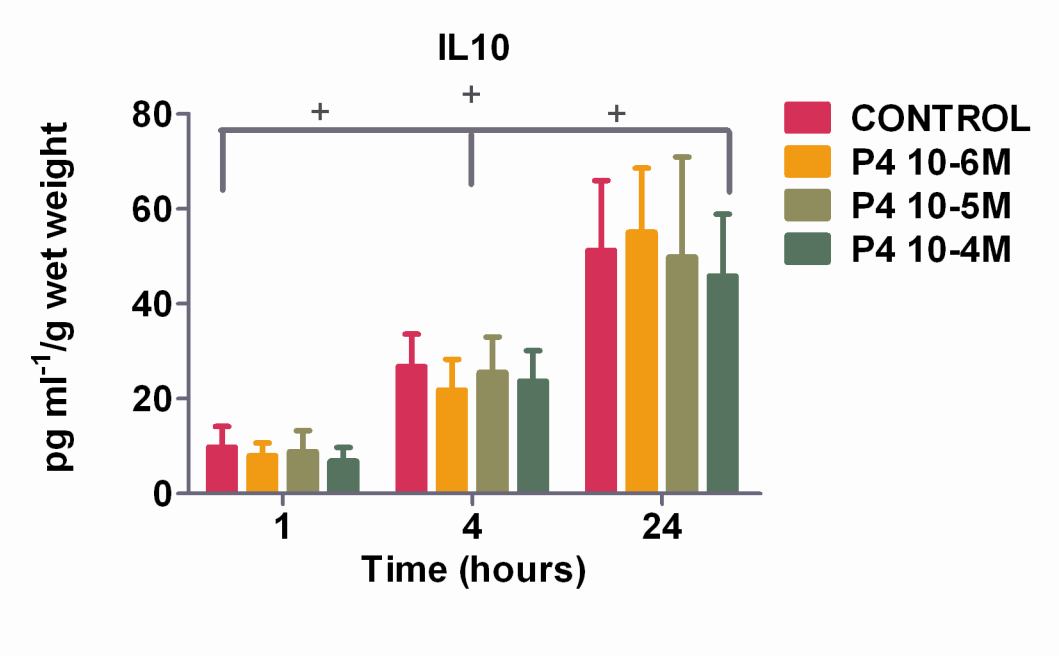

Figure 59 : Levels of IL10 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Very low levels were seen at 1 hour in comparison with 24 hours. Over time there was a significant increase in levels of IL10 + $p<0.001$ (ANOVA) but progesterone did not have any effect when compared with control.

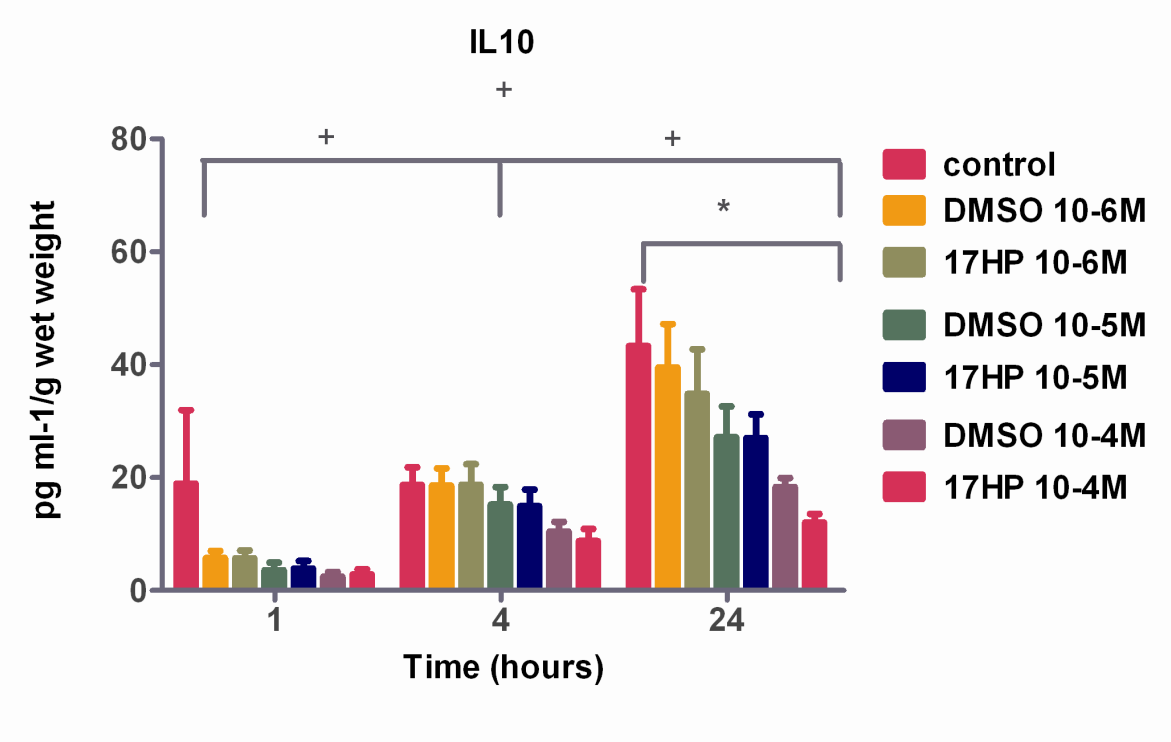

Figure 60 : Levels of IL10 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of IL10 + $p<0.001$ (ANOVA). The DMSO vehicle significantly reduced levels of IL10 at 100 µM * $p<0.05$. The 17OHPC results do not differ from its vehicle at this concentration.

IFNγ

Moderate levels of IFNγ were detected with this assay from cell culture. Progesterone had no effect at any of the concentrations on the overall levels of IFNγ detected $p>0.05$ and time again was the only source of variation $p<0.001$ (two-way ANOVA, GraphPad Prism). Levels of the control well at 1 hour measured 34 ± 18 (all expressed as $pgml^{-1}$/g wet weight, mean ± SEM) (Figure 61). Treatment with 17 OHPC did not alter IL8 levels at low concentration, 10^{-6}M. However, at higher concentrations of 10-5 M and 10-4 M there was a significant vehicle effect compared to control, $p<0.05$, therefore at high levels 17OHPC had no effect different from that of its vehicle (DMSO) (Figure 62). We also detected significantly lower levels overall of IFNγ released into the supernatants with 17OHPC. Progesterone at these concentrations does not appear to reduce the amount of IFNγ released into the supernatants compared with control *in vitro*.

MCP-1

Moderate levels of MCP-1 again, were detected with this assay from cell culture. Progesterone had no inhibitory effect at any concentration on the overall levels of MCP-1 detected $p>0.05$.Time again was the only source of variation (two-way ANOVA, GraphPad Prism) $p<0.001$. Levels of the control well at 1 hour measured 114 ± 42 (all expressed as pgml-1/g wet weight, mean ± SEM) (Figure 63). Treatment with 17 OHPC did not alter MCP-1 levels at low concentration, 10-6M. However, at higher concentrations of 10-4 M there was a significant vehicle effect compared to control, $p<0.05$, therefore at high levels 17OHPC had no effect different from that of its vehicle (DMSO) (Figure 64).Progesterone at these concentrations does not appear to reduce the amount of MCP-1 released into the supernatants compared with control in vitro.

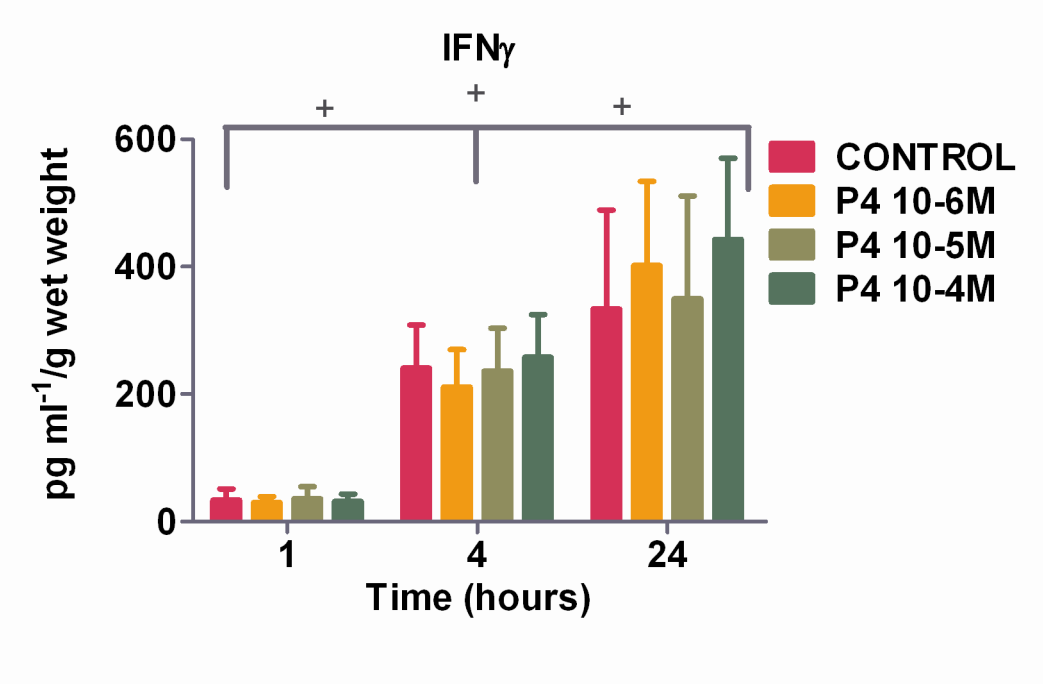

Figure 61 : Levels of IFNγ detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Over time there was a significant increase in levels of IFNγ + p<0.001 (ANOVA) but progesterone did not have any effect when compared with control.

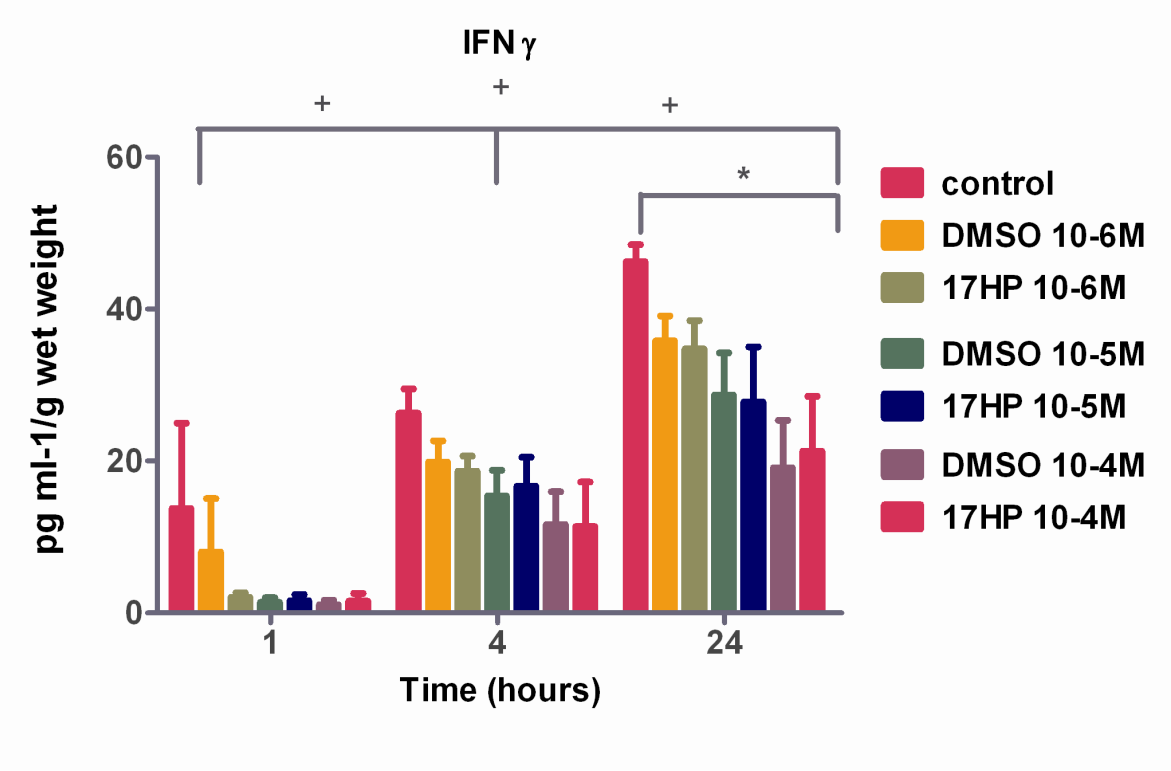

Figure 62 : Levels of IFNγ detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of IFNγ + p<0.001 (ANOVA). The DMSO vehicle significantly reduced levels of IFNγ at 100 µM * p<0.05. The 17OHPC results do not differ from its vehicle at this concentration.

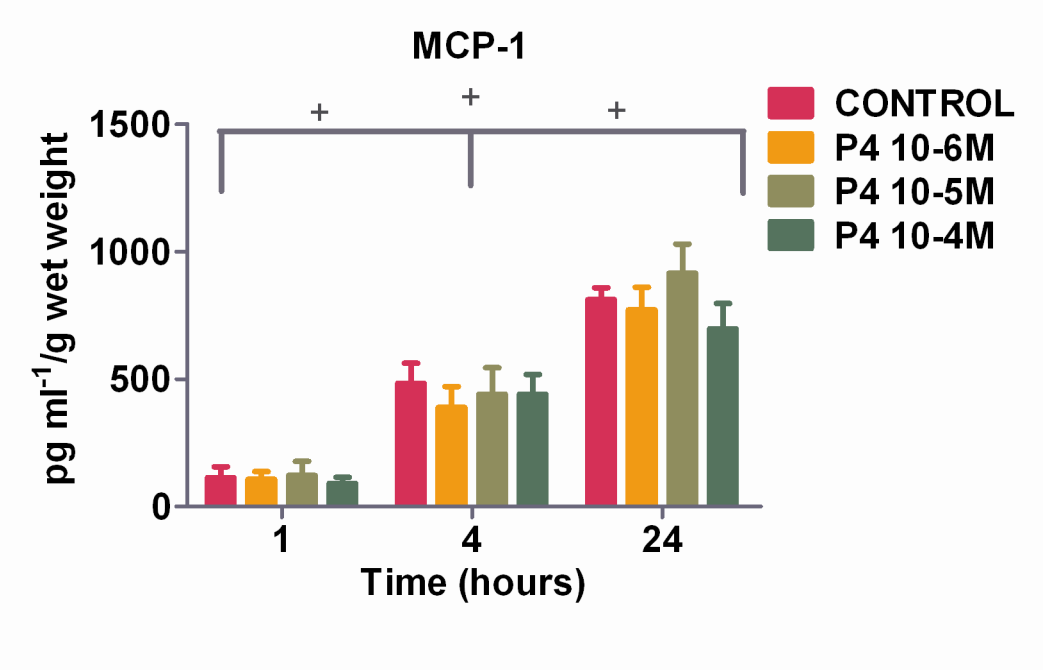

Figure 63 : Levels of MCP-1 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Over time there was a significant increase in levels of MCP-1, + p<0.001 (ANOVA) but progesterone did not have any effect when compared with control.

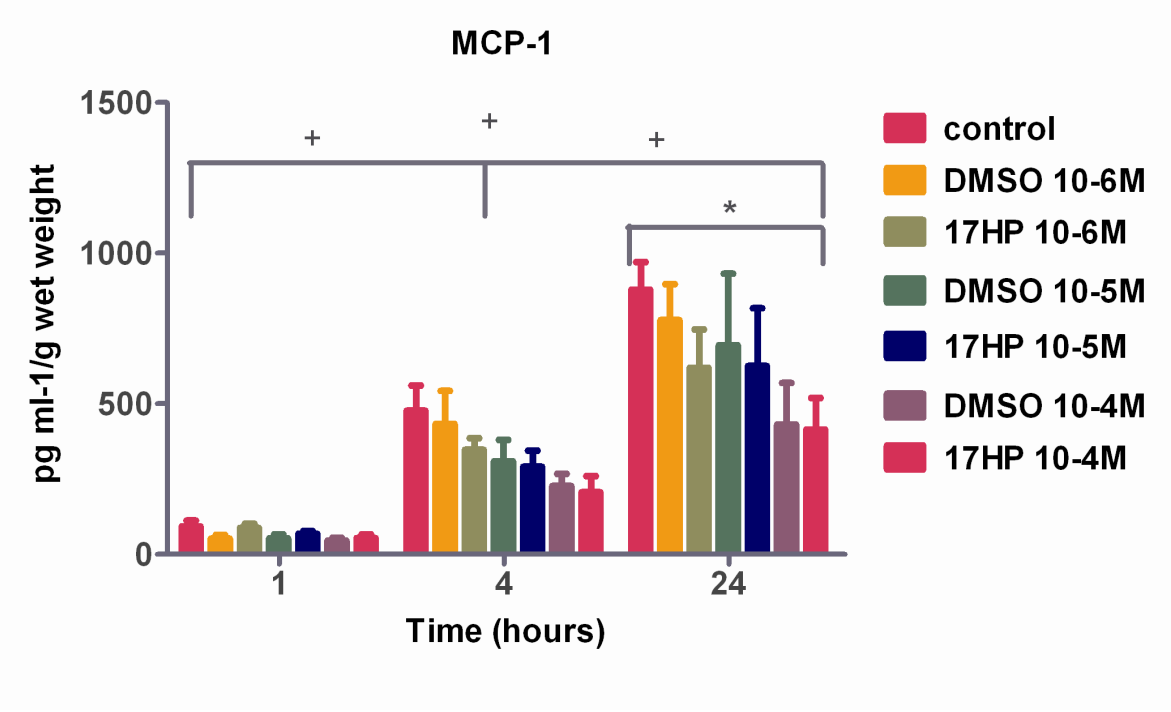

Figure 64 : Levels of MCP-1 detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of MCP-1 + p<0.001 (ANOVA). The DMSO vehicle significantly reduced levels of MCP-1 at 100 µM, * p<0.05. The 17OHPC results do not differ from its vehicle at this concentration.

TNF-α

Low levels of TNF-α were detected with this assay from cell culture. Progesterone had no inhibitory effect at any concentration on the overall levels of TNF-α detected, $p>0.05$.Time again was the only source of variation (two-way ANOVA, GraphPad Prism) $p<0.001$. Levels of the control well at 1 hour measured 5.2 ± 1.5 (all expressed as $pgml^{-1}/g$ wet weight, mean ± SEM) (Figure 65). Treatment with 17OHPC had no effect on MCP different from that of its vehicle control (Figure 66). Progesterone at these concentrations does not appear to reduce the amount of TNF-α released into the supernatants compared with a control *in vitro*.

Tissue Viability

We evaluated our own LDH assay kit by performing a standard curve in order to ascertain that it was detecting correctly. Measurements were detected spectrophotometrically using optical density (OD) at 490-690 nanometer (nm) wavelength .We then carried out an assessment of LDH release from a control well at 24 hour intervals up to 96 hours (Figure 67); however this did not give us an accurate time zero assay of LDH release. Because the tissue is removed from the patient using dissection and placed in medium we required an assessment of the basal LDH levels due to initial tissue trauma from dissection, removal from patient and transport to the laboratory. We therefore compared a control tissue specimen with an identical specimen treated with a lysis buffer. The lysis treated sample was used as an indicator of maximum superficial cell death for the purposes of this experiment. The results showed that there was little change over the 24 hour period and therefore the overall percentage cytotoxicity was within an acceptable range (Figure 68). Although there is a degree of LDH release within our experiment, as an overall estimate of tissue viability there appears to be some initial cell damage at time zero likely caused by initial removal from the patient and sampling. The overall estimate of tissue viability calculated as a percentage of the maximum LDH released is approximately 80% at 1 hour (estimate of % viability= 100-% of overall maximum LDH release). This estimate of tissue viability falls to 60% at the 24 hour time point (Figure 69).

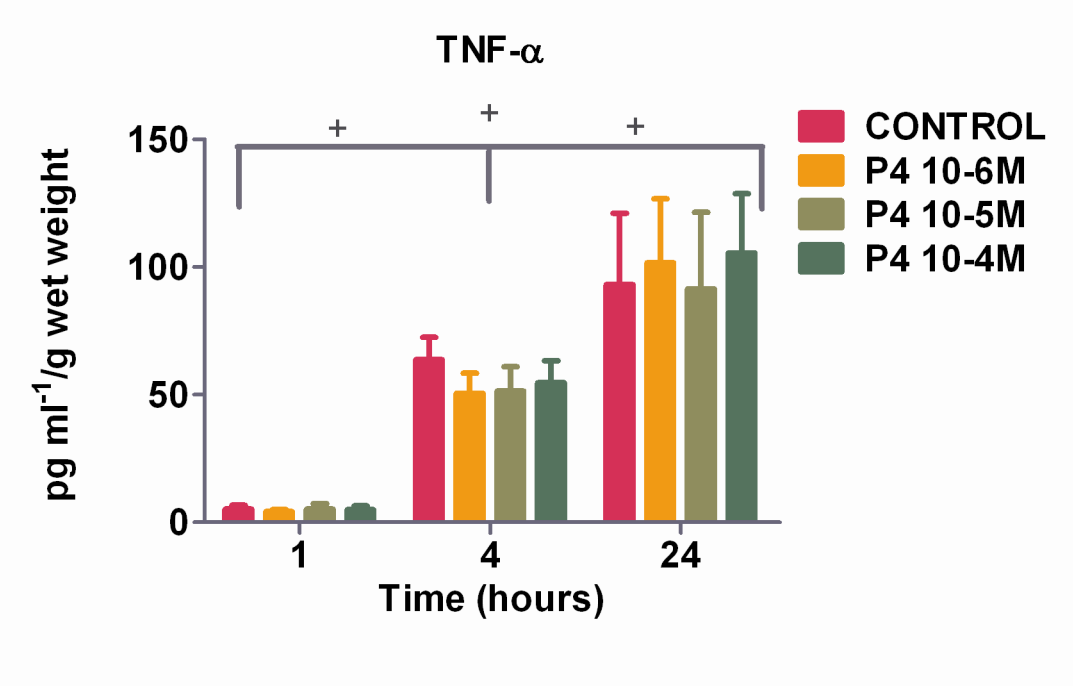

Figure 65 : Levels of TNF-α detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with progesterone 1µM, 10 µM and 100 µM. Over time there was a significant increase in levels of TNF-α, + p<0.001 (ANOVA) but progesterone did not have any effect when compared with control.

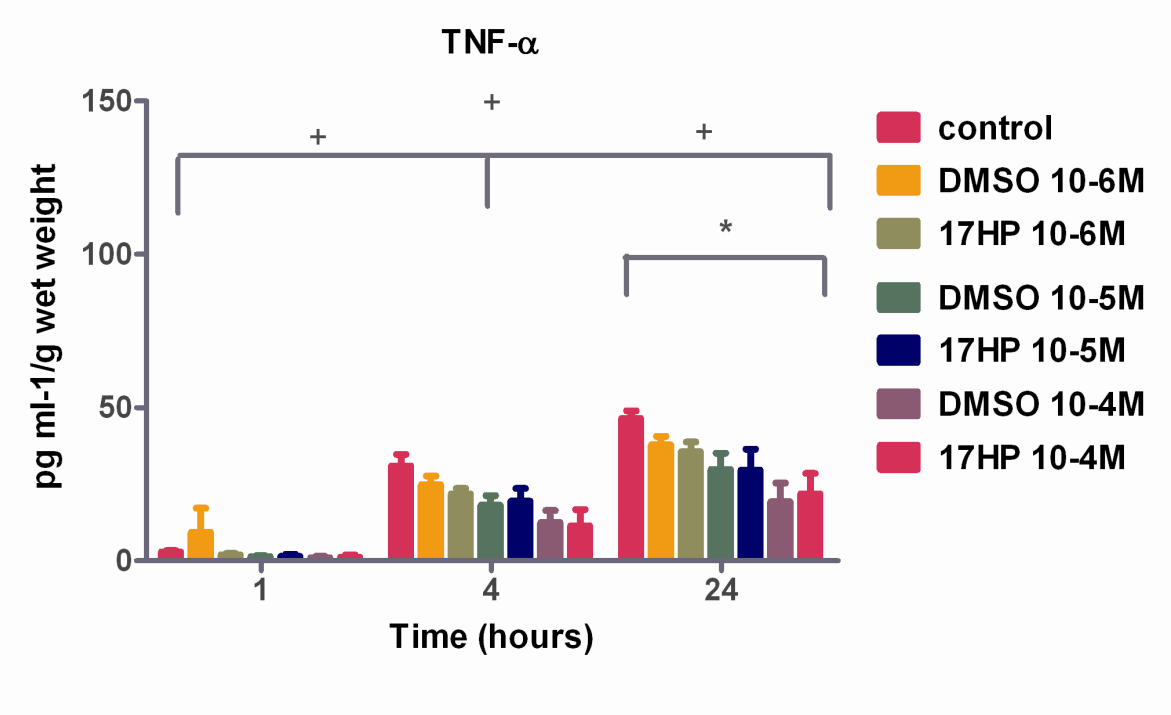

Figure 66 : Levels of TNF-α detected n=7 (pgml-1/g wet weight, mean ± SEM) at 1, 4 and 24 hour time-points within the supernatant after treatment with 17OHPC 1µM, 10 µM and 100 µM. and the equivalent DMSO control. Over time there was a significant increase in levels of TNF-α + p<0.001 (ANOVA). The DMSO vehicle significantly reduced levels of TNF- α at 100 µM, * p<0.05. The 17OHPC results do not differ from its vehicle at this concentration.

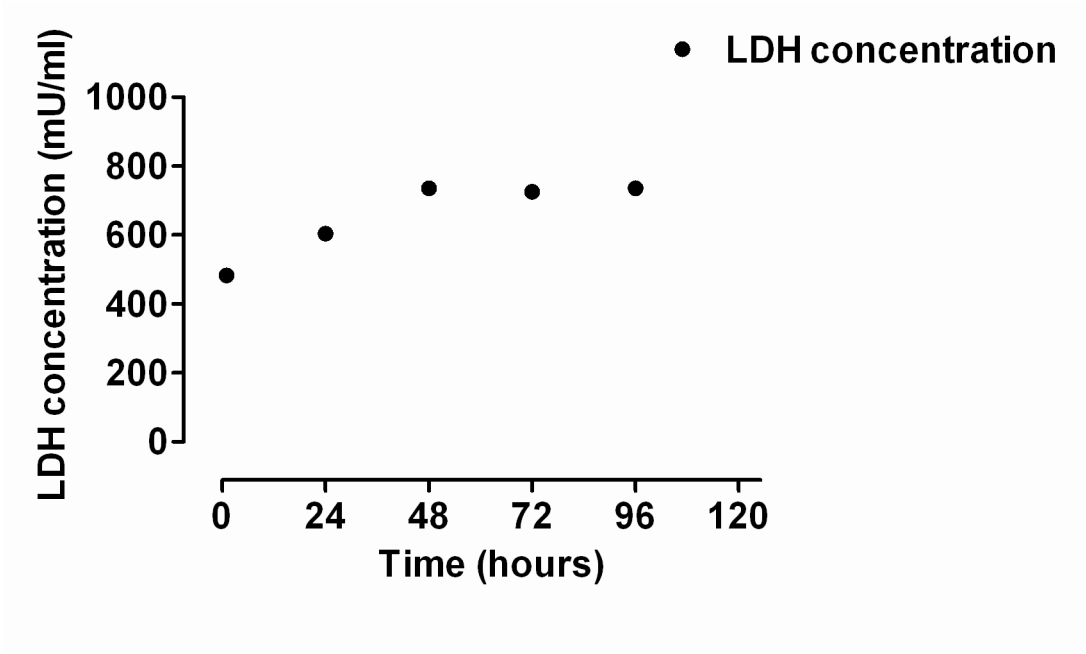

Figure 67 : LDH levels from the supernatants gathered over a 5 day period at 1, 4, 24, 48, 72, 96 and 120 hours. We used 24 hours as our final time-point. This gives an indicator of cell membrane integrity over time.

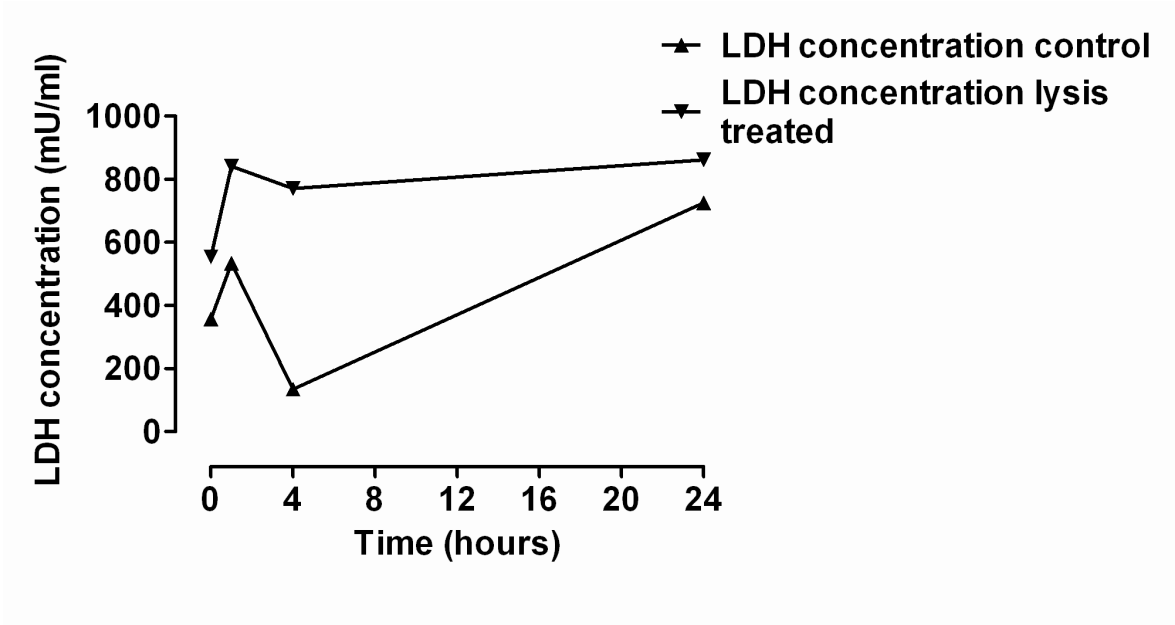

Figure 68 : LDH levels at our selected time-points for cell culture including a zero time-point. There were also samples treated with a lysis buffer to induce cell death as a comparison. Relative levels at time zero indicate an acceptable amount of cell death within the accepted 24 hour time period.

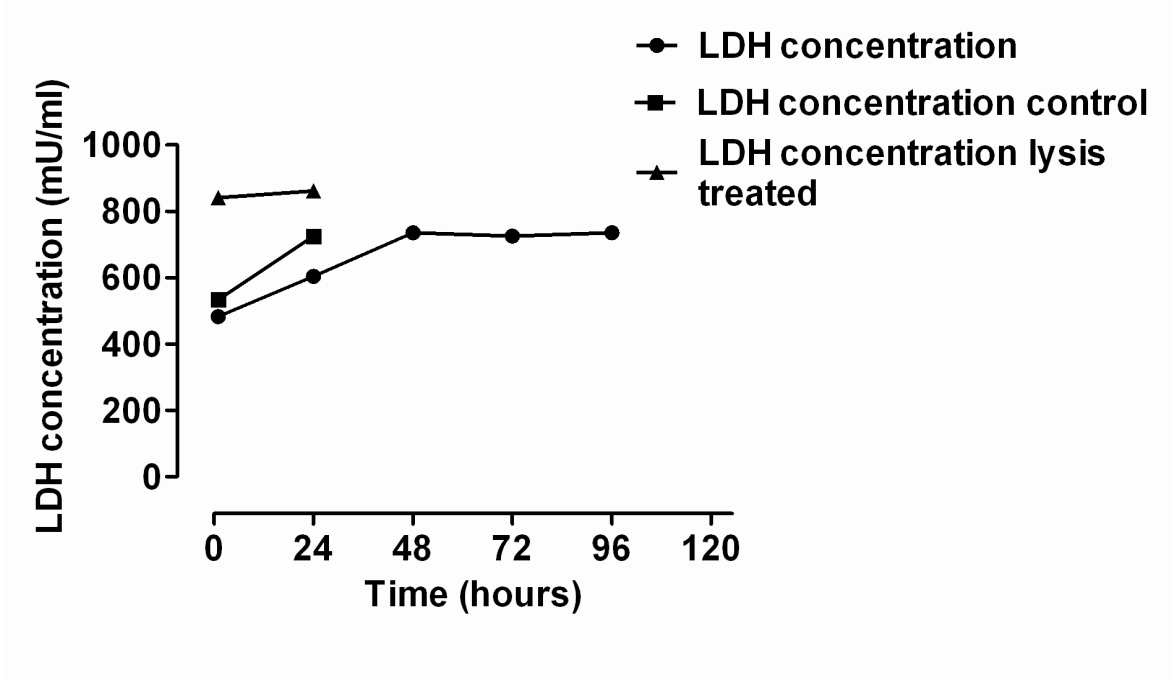

Figure 69 : This figure shows calculated mU/ml LDH released from the control preparation as well as LDH released from tissue incubated with a lysis buffer. The tissue with lysis buffer is a marker of maximum superficial cell death. Although there is a degree of LDH release, as an overall estimate of tissue viability there appears to be some initial cell damage at time zero. The overall estimate of tissue viability is 80% at 1 hour and falls to 60% at the 24 hour time point.

Results RT-PCR

After assessing the cytokine release into the surrounding supernatants we measured the relative tissue mRNA expression of various labour and uterine related genes relative to a control gene within myometrial tissue. Real time RT-PCR (Abi, Taqman) was used to quantitate endogenous inhibitors of uterine contractility (cGRPt, ep2), promoters of uterine relaxation (NOS, pgdh) cytokines (IL6, IL8, IL1ß), prostaglandin production enzyme (COX-2) and gap junction components (connexin 26 and connexin 43). We also measured progesterone receptor A+B in combination. All results were expressed relative to 18s and ß-actin. For continuity we have expressed our results in this chapter relative to housekeeping gene 18S as our later analysis of STOPPIT samples using RT-PCR this housekeeping gene was also used. This housekeeping gene has been widely used within myometrial and parturition studies as a suitable control (173). The synthetic progestin, 17 OHPC had significant vehicle effects (DMSO) therefore we analysed only the tissues from our control versus progesterone groups at each concentration.

Nitric-oxide synthase

We looked at three separate isoforms of NOS, iNOS (inducible), eNOS (endothelial) and bNOS (neuronal).They produce NO which activates guanylate cyclise and promotes smooth muscle relaxation. The 24 hour progesterone incubation at 10^{-6}M, 10^{-5} and 10^{-4}M did not modify any iNOS, eNOS or bNOS levels detectable within the myometrium when compared to the control sample (ANOVA with Bonferoni comparison test). Although it did not reach statistical significance inos does show a trend with a reduction with each concentration of progesterone. As the uterus is non-neuronal it expresses very little bNOS (Figure 70, Figure 71, Figure 72).

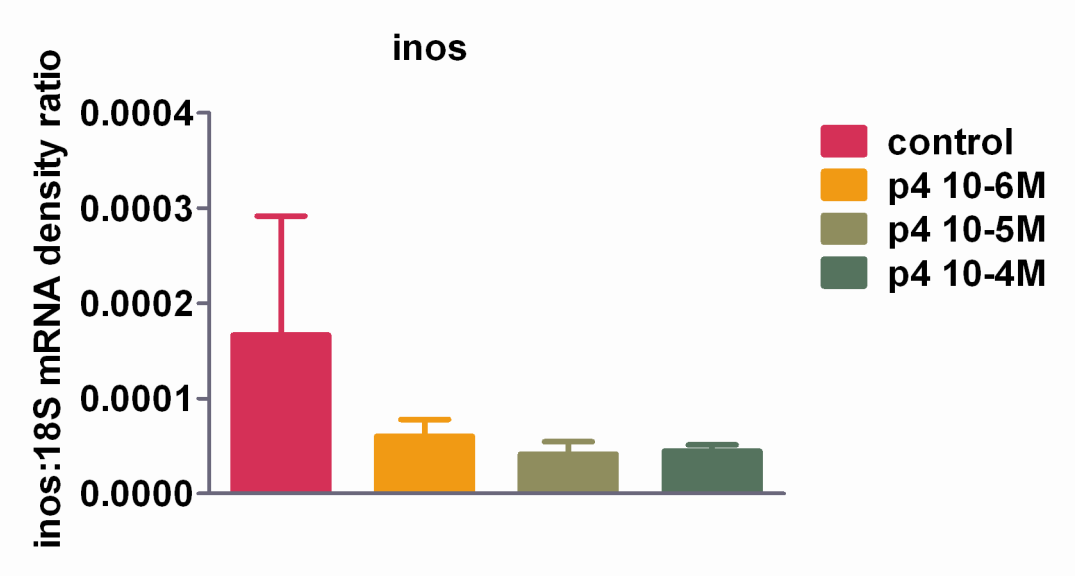

Figure 70 : This graph shows inos gene mRNA expression levels, relative to 18S.The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). Low levels were of inos were detected overall.

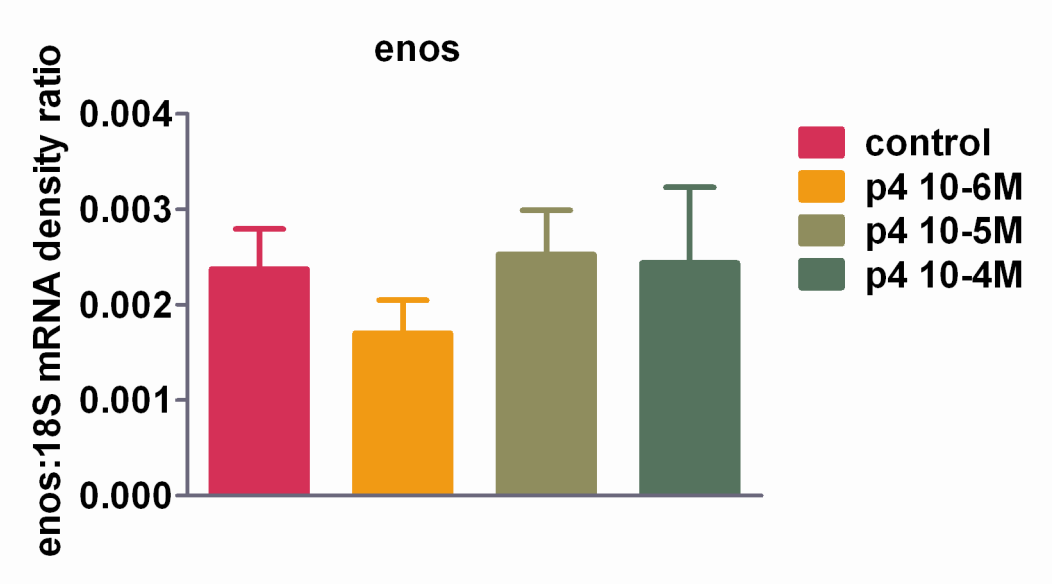

Figure 71 : This graph shows enos gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). Higher levels of enos were detected compared with inos and bnos.

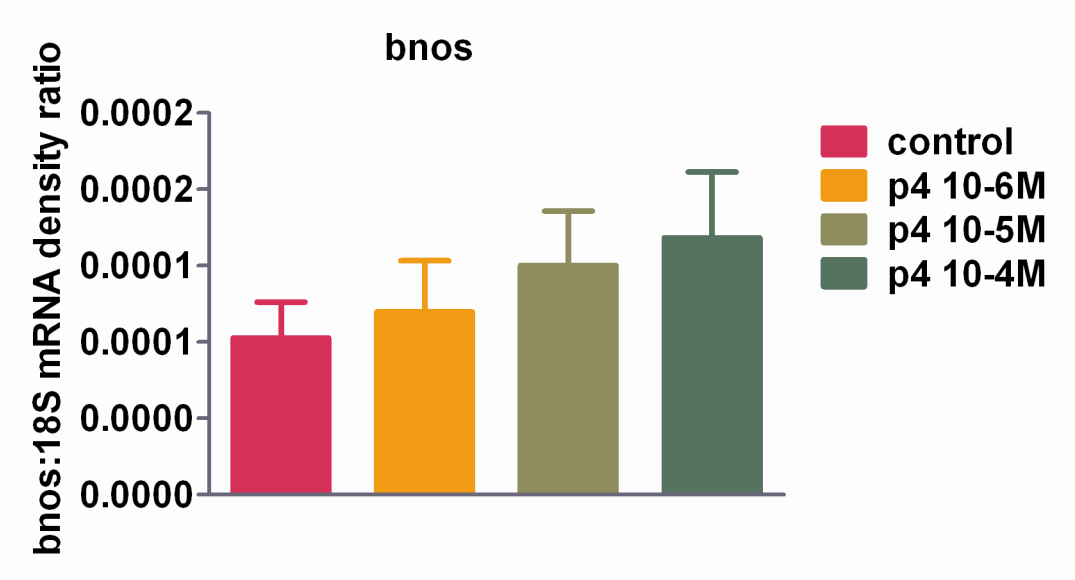

Figure 72 : This graph shows bnos gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). Low levels were of bnos were detected overall.

Calcitonin gene related peptide and receptor

CGRP inhibits myometrial contractile activity. We measured mRNA for both the peptide and its receptor. There was no detectable difference of CGRP or CGRPR after 24 hour incubation with 10^{-6}M, 10^{-5} and 10^{-4}M progesterone when compared with control (ANOVA with Bonferoni comparison test). Expression of the receptor relative to control gene was higher than the peptide expression itself in human non labour myometrium at term (Figure 73, Figure 74).

Endothelial phosphate receptor EP2

This is a subgroup of receptor for prostaglandin E_2 and is G protein coupled. Its activation leads to a rise in cAMP and myometrial relaxation. There was no detectable difference in mRNA of EP2 relative to 18S in the progesterone treated groups with any concentration of progesterone compared with control (ANOVA with Bonferoni comparison test) (Figure 75).

15-hydroxyprostaglandin dehydrogenase PGDH

This enzyme is associated with the catabolism of prostaglandins, which are strongly implicated in the onset and maintenance of parturition. Again, 24 hour incubation with 10^{-6}M, 10^{-5} and 10^{-4}M progesterone did not modify levels of PGDH detectable against the control (ANOVA with Bonferoni comparison test) (Figure 76).Although graphically it may appear that progesterone reduced expression at concentrations of 10^{-5} and 10^{-4}M this does not reach statistical significance.

Pro-inflammatory cytokines

We measured IL1ß, IL6 and IL8 all of which are generated in the uterus and cervix and implicated in labour and preterm labour. In a similar pattern to the BioPlex supernatant results we demonstrated no difference in the levels of IL1ß, IL6 and IL8 following 24 hour incubation with 10^{-6}M, 10^{-5} and 10^{-4}M progesterone compared to control (ANOVA with Bonferoni comparison test) (Figure 77, Figure 78 and Figure 79). Low levels of IL1ß compared to IL6 and Il8 were detected with this analysis which is in agreement with the Bio-Plex assay results.

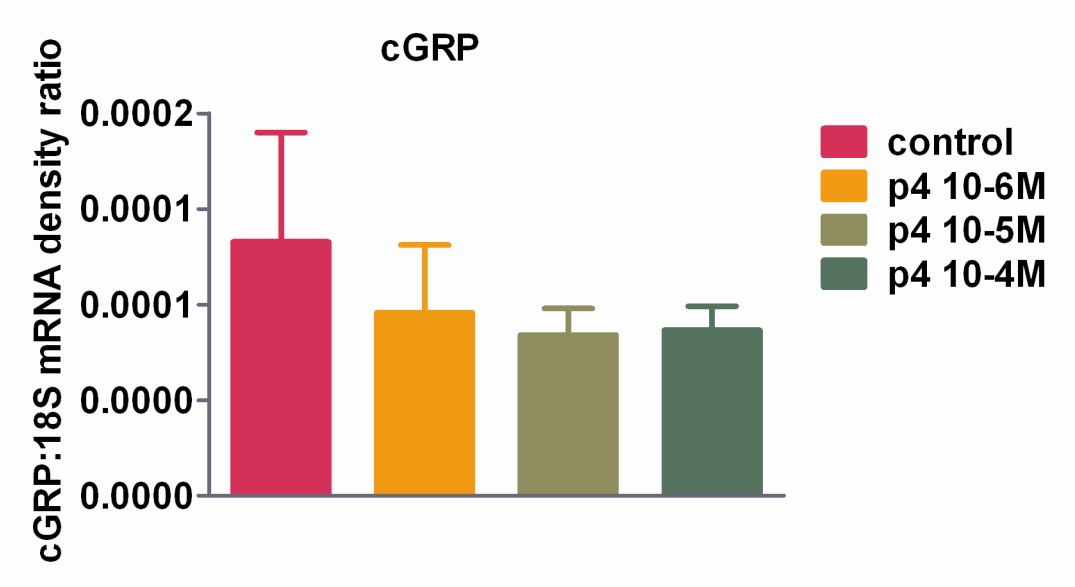

Figure 73 : This graph shows cGRP gene mRNA expression levels; relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). Low levels were of cGRP were detected overall.

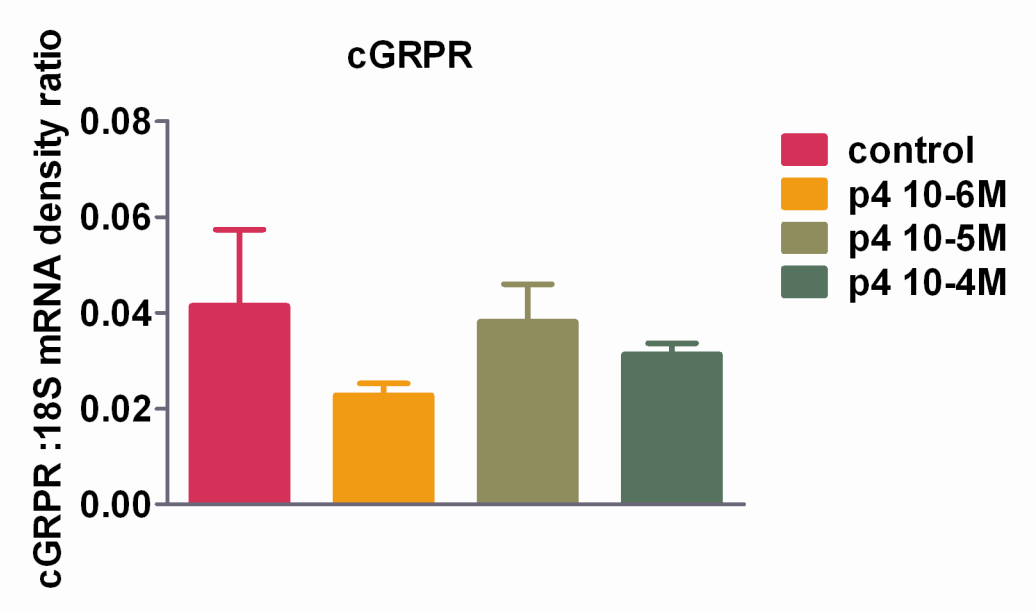

Figure 74 : This graph shows cGRP receptor gene mRNA expression levels; relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). Higher levels were of cGRP receptor were detected relative to its peptide.

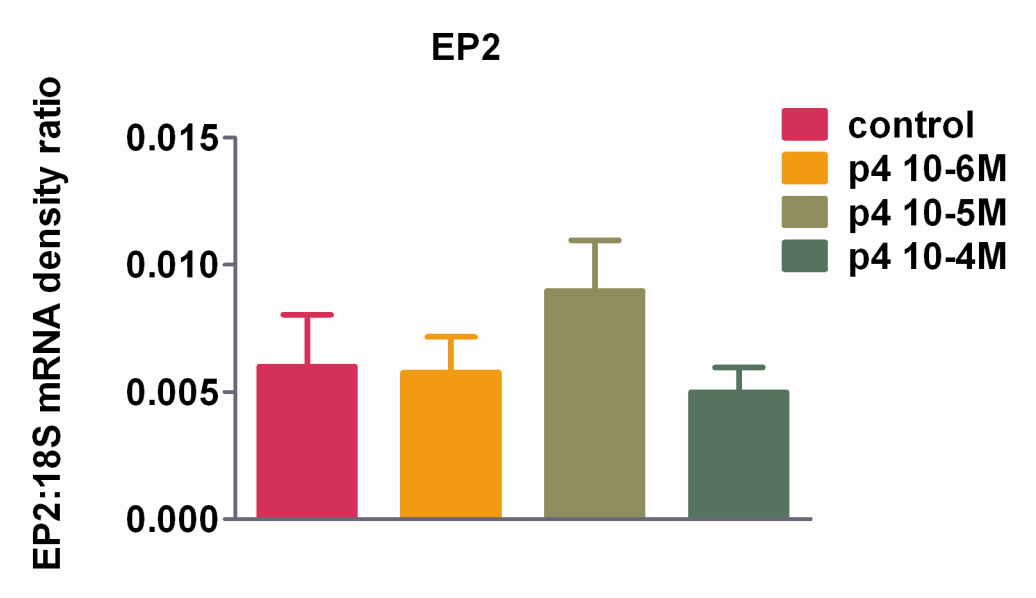

Figure 75 : This graph shows EP2 gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA).

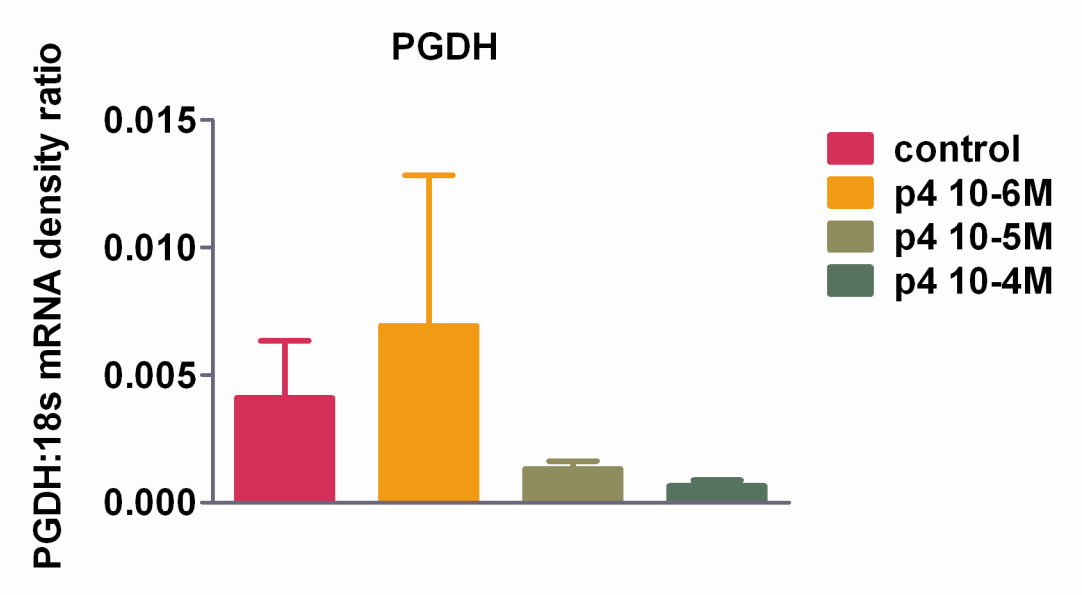

Figure 76 : This graph shows PGDH gene mRNA expression levels; relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA).

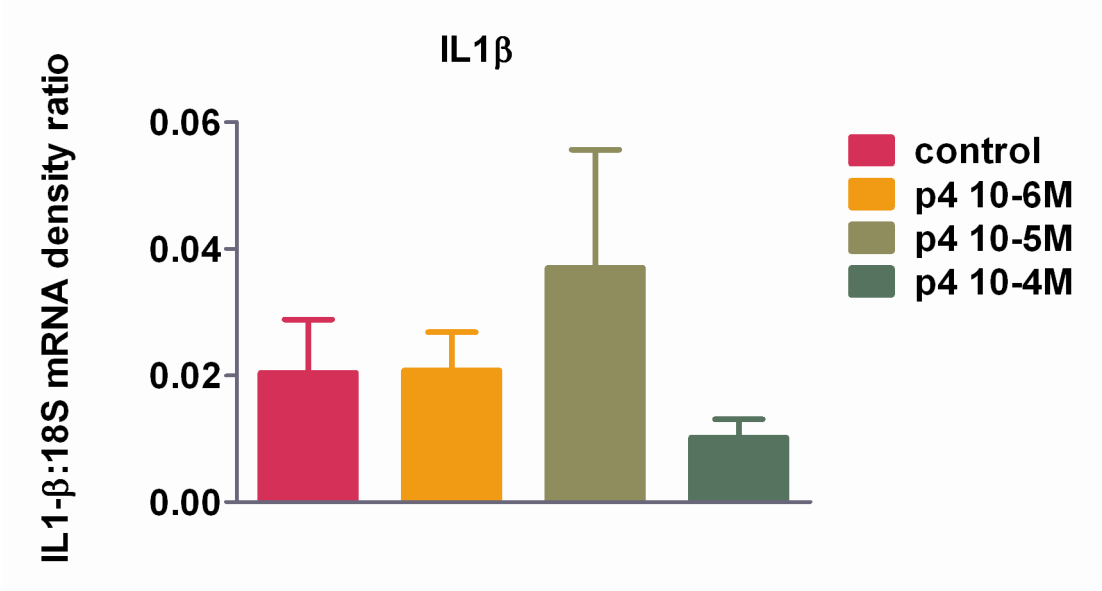

Figure 77 : This graph shows IL1β gene mRNA expression levels; relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). Low levels of this pro-inflammatory cytokine were detected overall.

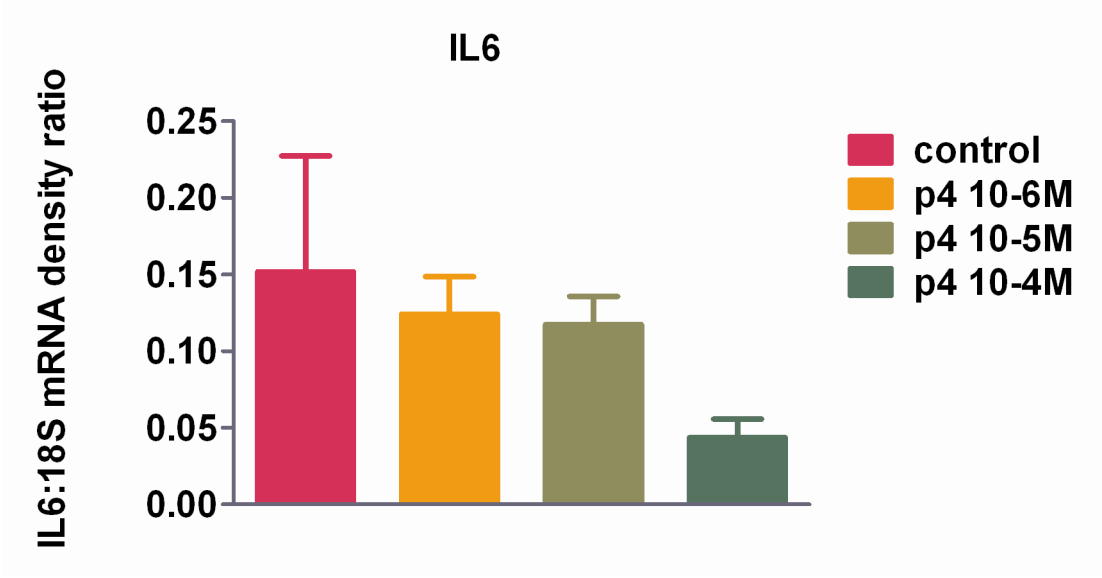

Figure 78 : This graph shows IL6 gene mRNA expression levels; relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA).

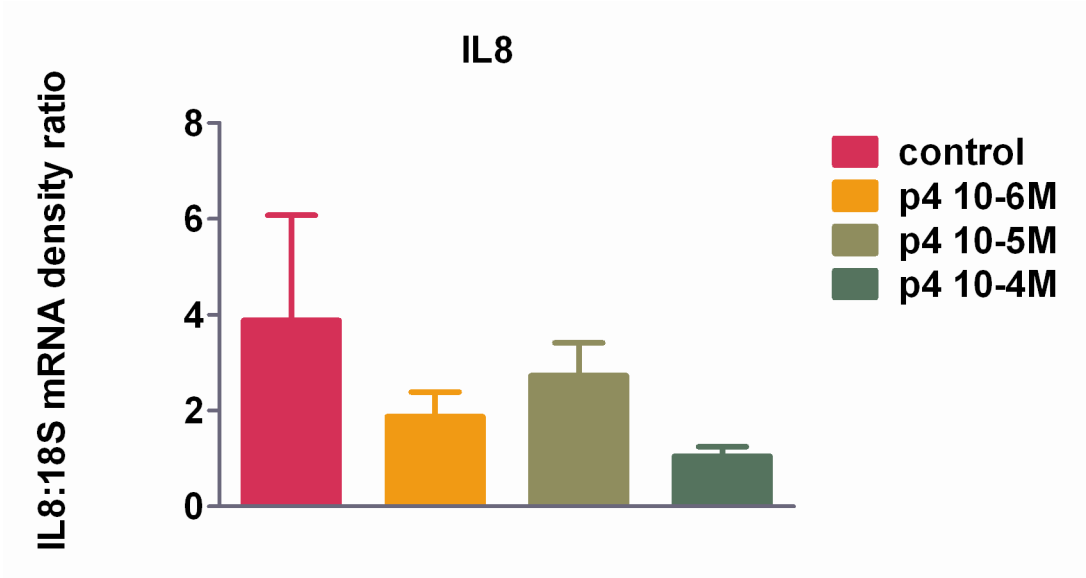

Figure 79 : This graph shows IL8 gene mRNA expression levels; relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA).

COX-2

COX-2 is a contractile agent which promotes prostaglandin synthesis. Again, 24 hour incubation with 10^{-6}M, 10^{-5} and 10^{-4}M progesterone did not modify levels of COX-2 detectable against the control (ANOVA with Bonferoni comparison test, GraphPad prism).

Gap junction proteins

We measured contraction associated proteins connexin 26 and 43. These form components of gap junction transmembrane proteins which aid to formulate synchronous contractile activity. Again, 24 hour incubation with 10^{-6}M, 10^{-5} and 10^{-4}M progesterone did not modify levels of connexin 26 detectable against the control. In contrast we did observe an inhibition in connexin 43 levels in the progesterone 10^{-6}M and 10^{-4}M incubations with progesterone incubation compared to control $p<0.05$ (ANOVA with Bonferoni comparison test, GraphPad prism).

Progesterone receptor A+B

We measured the progesterone receptor A+B together. PRB is the full length gene and also encodes for PRA (truncated form by 164 N-terminal amino acids). The incubation with 10^{-6}M, 10^{-5} and 10^{-4}M progesterone did not modify levels of PRA+B together, detectable against the control.

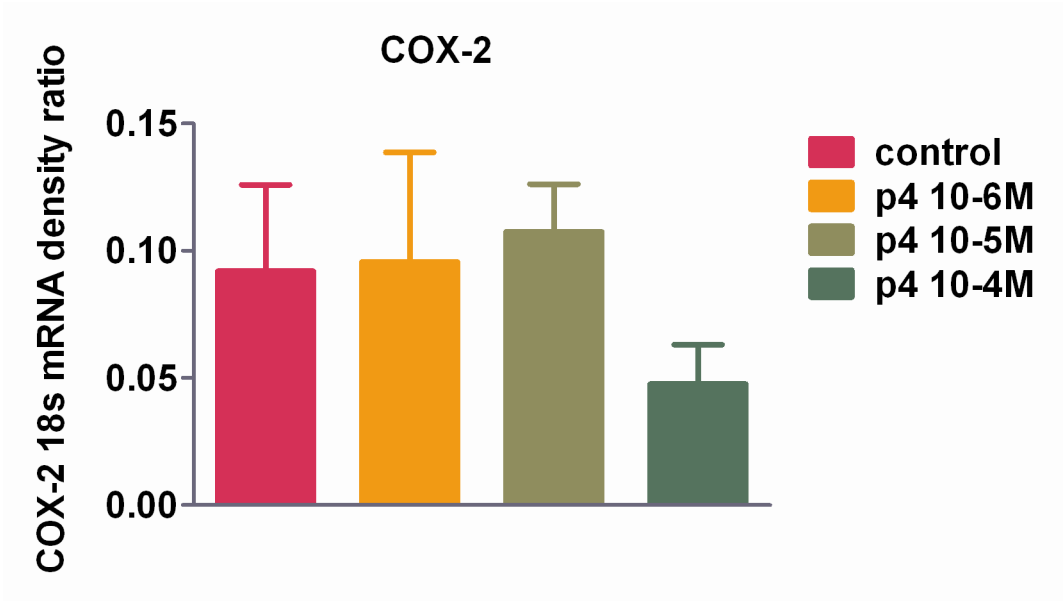

Figure 80 : This graph shows COX-2 gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA).

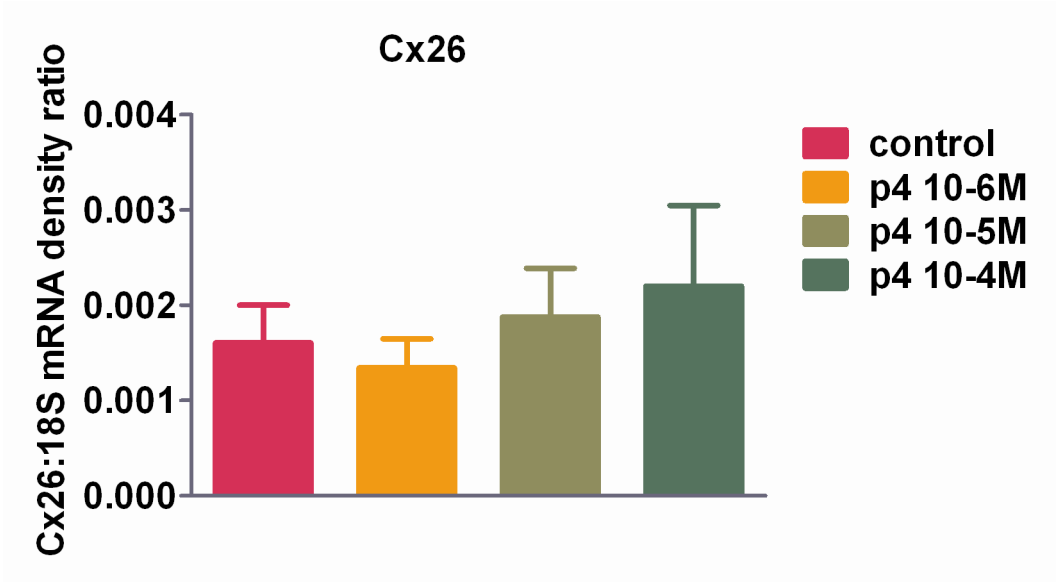

Figure 81 : This graph shows Cx26 gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA).

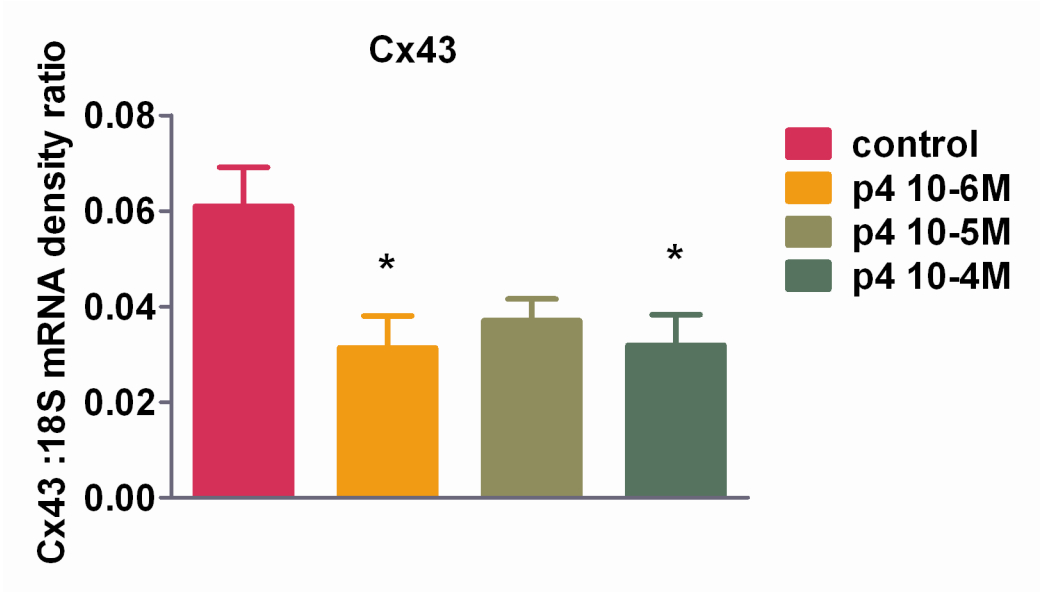

Figure 82 : This graph shows Cx43 gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was a significant change in expression after the 24 hour incubation with progesterone 1µM and 100 µM compared with control myometrium (ANOVA) $p<0.05$.

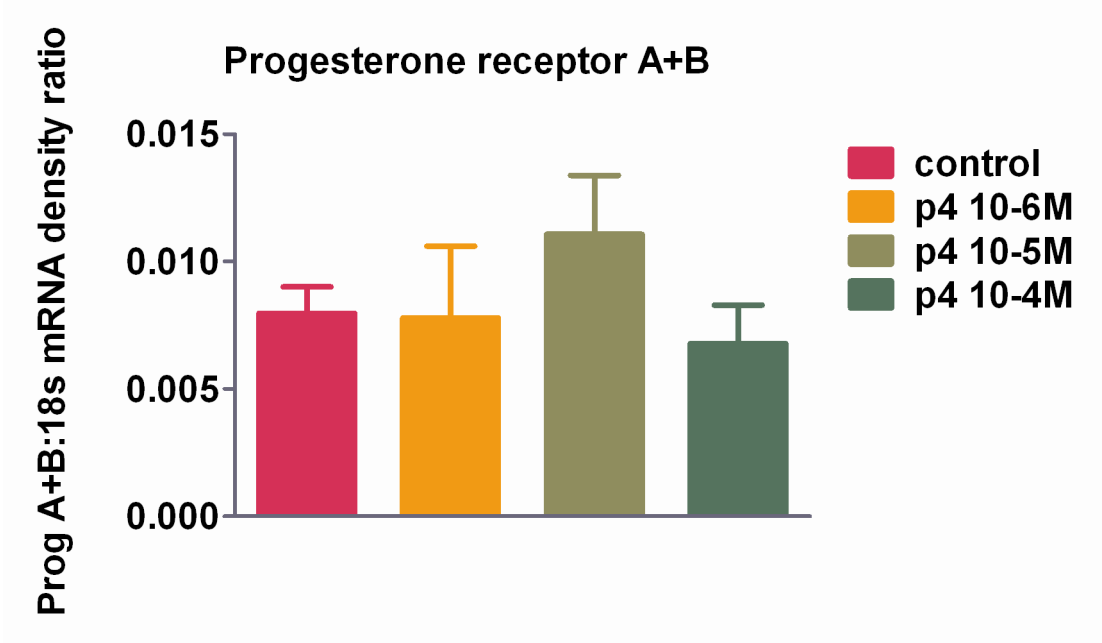

Figure 83 : This graph shows progesterone receptor A and B gene mRNA expression levels, relative to 18S. The density ratio for mRNA n=6 expressed as mean ± SEM.There was no significant change in expression after the 24 hour incubation with progesterone 1µM, 10 µM and 100 µM compared with control myometrium (ANOVA). We measured the progesterone receptor A and B together.

Discussion

The effects of progesterone and 17 α hydroxyprogesterone caproate on cytokine release, uterine relaxants and contraction associated proteins within myometrium *in vitro*

In this chapter we aimed to look specifically at progestogenic effects *in vitro*. A wide variety of mediators have been implicated in the control of labour and preterm labour, including pro-inflammatory cytokines and contraction associated proteins. The aims of the following experiments were to determine cytokine release into the surrounding cell culture medium from myometrial tissue *in vitro* with three concentrations of progesterone over a 24 hour period. We aimed to determine the myometrial effects of progesterone and 17-α hydroxyprogesterone caproate (17OHPC) after tissue culture over a 24 hour period.

We again used lower uterine segment myometrium sampled at prelabour elective caesarean section which we have previously discussed in chapter 3.

As we were investigating the effects of progesterone on several markers we wanted to make a comparison between our *in vitro* and *in vivo* (chapter 5) experimental models. Within STOPPIT, patients received vaginal progesterone, 90mg daily. Progesterone can be administered orally, vaginally or intramuscularly. Oral preparations show a wide variation in levels due to individual's gastric filling and enterohepatic circulation. The vaginal route provides higher uterine circulating levels but this is not consistently reflected in serum levels (174). There is also evidence to support the uterine first pass effect with vaginal progesterone preparations giving targeted higher uterine concentrations of the vaginally delivered drug (175). The half-life of vaginal progesterone 100mg is approximately 13 hours (176) and within STOPPIT (123) trial this vaginal tablet was replenished at the same time every 24 hours. Therefore for the *in vitro* comparison we selected shorter times 1 and 4 hours in addition to 24 hours. The shorter time-points were selected after the contractility studies carried out had shown maximal effects of progesterone at 1 hour on myometrial contractility which was maintained for over 4 hours. We also selected three concentrations of progesterone and 17OHPC. In view of difficulty

determining exact myometrial progesterone concentrations we extracted information from our previous contractility data that had shown significant inhibitory effects at high doses of 100μM.

17OHPC does not appear to modify any of the cytokines released, however we did not continue our analysis with this progestin as there were significant vehicle effects detected. As mentioned in chapter 3, a contractility study investigating progesterone and 17OHPC and the effects on human myometrial contractions carried out by Ruddock et al showed that 17OHPC levels, when incubated with myometrial tissues were reduced by between 90- 98%. Progesterone was less effected with a loss of 29-50% detected (100).They used ethanol as opposed to DMSO as a solvent and showed less vehicle effects. 17OHPC however did not appear to present within the organ baths at the end of the experiments. As it is strongly lipophilic and difficult to dissolve, preparations that have been used in clinical trials are dissolved in castor oil (119). Its half life is unclear in women and its maternal metabolic pathway unclear as well as its target organ site. With significant vehicle effects our results are difficult to interpret and indeed show no discernible difference in any inhibition towards cytokines without similar vehicle effects. It is also possible as in the paper published by Rudduck et al that the 17OHPC is not reaching the myometrial tissue and is therefore not able to actively modify it *in vitro*.Our further analysis of myometrial mRNA expression of various contraction associated proteins and inflammatory markers therefore only takes into account the progesterone treated and control samples.

The pathophysiology of labour both term and preterm is poorly understood. It is likely a complex culmination in a series of events and therefore to halt this process at one single step such as with tocolysis to stop contractions has not been successful in either prolonging the pregnancy or improving the neonatal outcome. Progesterone and synthetic progestin, 17OHPC, have been shown to reduce the incidence of preterm delivery in high risk groups (114). We examined both natural progesterone, which was used in the STOPPIT (123) study and we also looked at this synthetic progestin in view of ongoing clinical trials investigating progestins for the prevention of preterm labour. Smith et al recently investigated the plasma levels of progesterone amongst other hormones. They suggest that it is the patterns of change as opposed to absolute concentrations of progesterone, oestriadiol, oestriol and corticotrophin releasing

hormone that are common to most pregnancies. They noted declining progesterone to oestriol ratio that occurs later in gestation as labour approaches and this theory would support the basis of supplementary progesterone to prevent preterm delivery.

If these pro-inflammatory cytokines are implicated in labour and preterm labour this offers one possible target site for treatment or prevention of preterm labour. Our focus in particular, relates to steroid control with progesterone and was one potential mechanism of action for progesterone in the prevention of preterm labour.

We detected levels of several pro and one anti inflammatory cytokine from our assays, measured from the surrounding cell culture fluid (supernatants) and released into the medium by the myometrial tissue. The cytokine IL8 followed by IL6 was most abundant within our assays and IL1ß was least detected. The review from Keelen et al in 2002 examines cytokines, prostaglandins in the context of term and preterm delivery. They looked at amnion and choriodecidual membranes at term not in labour and in spontaneous labour as well as preterm labour with and without intrauterine infection present. They were able to demonstrate relative expression of cytokines and chemokines cDNA analysis within the four sub-groups. They showed dramatically increased levels in a number of chemokines within membranes of preterm deliveries complicated by chorioamnionitis. Many cytokines and chemokines were also evident in tissues prior to labour but with a modest expression increase in normal term labour. For example IL8 levels within amniotic fluid increased in pregnancy towards term with further elevation after the onset of labour (177). Levels of IL8 were also increased within myometrium from lower uterine segment with the onset of labour (178). With our *in vitro* model we are able to show that each cytokine/chemokine measured was released from myometrium which had not been stimulated to simulate labour by any inflammatory agent. An inflammatory stimulus, such as LPS (lipopolysacharide) has been shown to stimulate production of cytokines in particular with IL1ß and IL6 expressed in preterm and term myometrium prior to onset of labour. Other groups have shown IL8 and TNF α were only expressed in myometrium in active labour (172). Although our *in vitro* model does not replicate their method we found a similar pattern in cytokine

levels with lower segment myometrium that had not been stimulated with the inflammatory agent LPS, and was left in cell culture for 24 hours.

Gustafsson et al found no difference in the secretion of IFN-γ, IL-4, IL-10, TGF-ß or TNF α when comparing decidual tissues before and after the onset of labour (179) but there are limited data on cytokine levels from human myometrial tissues. TNF α has a stimulatory role with prostaglandin synthesis and therefore a rise may have been expected with labouring samples. Young et al identified small amounts of TNF α in myometrium of labouring women (25) and other studies have also only detected low levels (172). We demonstrated low levels of TNF-α within the Bio-Plex ® assay but this increased at each time-point with the most abundant levels being measured from the supernatant after 24 hours in cell culture.

MCP-1,monocyte chemoattractant protein ,also known as C-C chemokine motif ligand 2, CCL-2 is up-regulated in both term and preterm labour (180).Its main role is to recruit monocytes and leucocytes to sources of inflammation and can be up-regulated by other inflammatory mediators such as cytokines IL1ß and interferon γ.We observed no difference in expression of MCP-1 following the progesterone incubation.

Our progesterone incubations at 1, 10 and 100μM had no direct inhibitory effect on cytokine release into the surrounding cell culture medium under *in vitro* conditions tested. We hypothesised that the mechanism of action of progesterone in preventing preterm delivery may occur via suppression of endogenous myometrial prostaglandin and cytokine production. This hypothesis was supported by non pregnant human studies with up-regulation of IL8 and COX-2 in response to progesterone withdrawal (181). This may indicate that our study is underpowered to detect a significant difference within the cytokines. It is also possible that our in vitro model fails to adequately replicate the physiology. We cannot be certain that progesterone was penetrating further than the outer cell layers within our cell culture model and therefore it is possible that the concentrations affecting the myometrium were indeed much lower than in the cell culture well.

There is good evidence to show that following a traumatic brain injury the excessive release of IL1ß and TNF-α is a major cause of cerebral oedema and

progesterone has been shown to attenuate the production of these pro-inflammatory cytokines (182).This may however not be replicated within myometrial systems. Our data shows no attenuation in the production of pro-inflammatory cytokines with progesterone. In his review of the preterm parturition syndrome, Romero suggests that in infection-related preterm birth, the increased concentration of IL1ß in gestational tissue could stimulate nuclear factor-kappa B (NF-κB) .This activation of NF-κB can increase COX-2 (mRNA and protein expression) and prostaglandin production and also could repress progesterone activity resulting in a functional progesterone withdrawal and, thus, preterm parturition (12). It may therefore indicate that once the inflammatory cascade has been activated that supplementary progesterone will have little anti-inflammatory effect in myometrium.

Although we did not see an effect with our *in vitro* model there is evidence to support this theory from the functional progesterone withdrawal which we have discussed previously. It remains unclear how supplementary progesterone would have any effect during pregnancy when endogenous levels are already very high and progesterone as a prevention for preterm labour has only been shown to be effective in a small subgroup of high risk women with a history of preterm birth and not in multiple pregnancy (114). If levels of progesterone are already high then the progesterone receptor ratio change towards term or labour and development of the contractile phenotype may be central to the parturition pathway. This may also be linked into the inflammatory cascade which is being linked very closely with the process of normal and abnormal labour.

Inflammatory cytokines, in particular prostaglandins (PGE_2 and $PGF_2\alpha$) may be involved in both term and infection-associated birth. In many animal species prostaglandins are able to trigger parturition by inducing the progesterone withdrawal (77, 183). Humans differ with no detectable fall in progesterone to trigger parturition. It is now thought to be a functional withdrawal in response to progesterone receptor ratios that is responsible for the loss of progesterone quiescence. Progesterone's main action in maintaining uterine quiescence is generally accepted to result via the PR-B receptor (48, 77). Prostaglandins are also used for the induction of labour in women and can trigger parturition at all stages of pregnancy. For induction of labour however, this can take over 12 hours suggesting that a latency period is required for contractile phenotype to

take over. Madsen et al demonstrated that prostaglandin $PGF_2\alpha$ increased the PRA/PRB ratio by increasing PRA but not PRB in a human myometrial cell line suggesting that $PGF_2\alpha$, either by local production or presence within the myometrium could initiate labour by modulating the nPR expression to induce functional progesterone withdrawal (184). The NF-κB transcription factor is a key mediator in inflammatory response (27) and has also been shown to stimulate expression of PRA, PRB and PRC in myometrial cells indicating an up-regulation of the inhibitory PR isoforms and a mechanism for the loss of uterine quiescence (79).

We may therefore have looked too far along the pathway within the inflammatory cascade to show a change in pro-inflammatory cytokines. It may be a response to the withdrawal of the progesterone levels and their PR isoforms receptors that causes a reduction *in vivo* and our model has over simplified what may actually be occurring *in vivo*. We also did not measure samples from amnion or choriodecidua but focused on myometrium itself and release of cytokines into the surrounding cell culture medium. Within our subsequent analysis of myometrial tissue using RT-PCR we selected to look again at IL1β, IL6 and IL8 to determine the expression of cytokines within the myometrium. There was no significant reduction following the progesterone incubations in the expression of these cytokines, supporting the data obtained from the Bio-Plex array.

Our analysis of the mRNA relative expression in myometrial explants at the 24 hour time shows no change in the nitric oxide synthase isoforms (NOS), calcitonin gene related peptide and receptor (CGRP), endothelial phosphate receptor EP2, 15-hydroxyprostaglandin dehydrogenase (PGDH), 26 (Cx-26), COX-2 and progesterone receptor A+B (PR A+B) or interleukins (IL1β, IL6 and IL8) . There was however a significant reduction in expression of the contraction associated protein, connexin 43 with progesterone compared to control.

We did not see an up-regulation in any isoforms of mRNA NOS although there was an inhibitory trend with iNOS which did not reach statistical significance following progesterone incubation. It is possible however, that our study is underpowered to detect a difference as trends were apparent within some graphical data that may indicate a progestogenic effect. Norman et al showed using Western blotting that pregnancy was associated with an up-regulation of

both endothelial and nitric oxide synthase expression and neuronal nitric oxide synthase expression within human myometrium (185).The isoforms of nitric oxide synthase have been identified within human myometrium but do not appear to be modified with the onset of labour (186, 187).Other groups were able to demonstrate all three isoforms within human myometrium but iNOS and nNOS were only detectable in small amounts as seen with our results (188). They also showed that eNOS was not correlated with gestational age suggesting it was unlikely to have a role in modulating myometrial tone throughout pregnancy. NOS have been implicated in cervical remodelling and as a smooth muscle and potential uterine relaxant. Within a rat model, Bulbul et al demonstrated increased iNOS expression and nNOS expression with 17-β estradiol and progesterone in combination. They were unable to demonstrate a change in eNOS expression with steroid hormones (189). Our hypothesis that progesterone may upregulate these endogenous inhibitors of uterine contractility like potential tocolytics as with the nitric oxide donors, glyceryl trinitrate and sodium nitroprusside which can inhibit myometrial contractions (92) was not reflected in the results from the *in vitro* cell culture.

In rat uterine myometrial smooth muscle cell lines, progesterone has been shown to up regulate calcitonin-gene related peptide receptor components, calcitonin-gene related peptide being a potent smooth muscle relaxant (134). Dong et al report that CGRP induced a concentration-dependent relaxation in spontaneously contracting myometrium from pregnant women. This relaxatory effect however, was reduced in myometrium obtained from patients during labour and in the non-pregnant state (91) .CGRP-receptors are abundant in the myometrial cells of pregnant women who are not in labour, and are minimal in uterine specimens from women in labour and in the non-pregnant state. Another endogenous inhibitor of uterine contractility, the relaxant prostaglandin E receptor subtype two (EP2) is regulated by steroid hormones within the rat uterus and progesterone lead to an increase in expression (133). Evidence showing that treatment with progesterone enhanced myometrial calcitonin gene-related peptide B receptor protein expression in cultured pregnant myometrial explants were elevated further when combined incubation with 17β-estradiol plus progesterone was carried out (190) .We were unable to replicate this effect within human myometrial explants with similar dose regimes and time courses for progesterone alone. This may be related to our original experimental

design as monolayer of cells may allow a greater degree of penetration of a drug than explants of myometrium and a longer genomic effect may have been seen beyond 24 hours. There are several EP receptor isoforms within human myometrium and some are expressed higher in the lower than upper segment, such as EP2, they also demonstrated that within the upper segment, EP2 was higher in term labour samples and lower in preterm labour samples compared to term non-labour. This expression profile is difficult to explain, as it does not correspond with the current theory supporting a contractile upper segment during labour (191).

Prostaglandins are inactivated by an NAD +(nicotinamide-adenine dinucleotide) dependent 15-hydroxyprostaglandin dehydrogenase (PGDH), which catalyzes the initial conversion of PGE2 and $PGF_2\alpha$ to their biologically inactive 15-keto derivatives and although the amnion and chorion are major sites of prostaglandin synthesis and metabolism (192), this enzyme has been located to myocytes within myometrium (131). They also showed a decrease in PGDH activity in preterm and term labour, hypothesising that this would lead to a decreased metabolism of prostaglandins within the myometrium and subsequently an overall increase in prostaglandin levels. Greenland et al examined the PGDH promoter gene and suggested that progesterone was an important stimulus for PGDH expression in the utero-placental unit. This may be a mechanism for maintenance of uterine quiescence during pregnancy. In myometrial smooth muscle cells, both isoforms of the progesterone receptor, PR-B and PR-A, caused a ligand-dependent activation of PGDH-2368/luc3 but suggested that there were multiple and complex crosstalk pathways for regulation of PGDH (193). Our *in vitro* model, again, showed no up-regulation with PGDH following progesterone incubation. Other groups have looked directly at prostaglandins and their ability to stimulate labour in women by inducing the functional progesterone withdrawal (change in PR-A: PR-B ratio). Madsen et al concluded that prostaglandins acting via the protein kinase-C pathway facilitate functional progesterone withdrawal by increasing the myometrial PR-A:PR-B expression ratio (184).

Prostaglandins are formed from the precursor arachidonic acid which itself is a substrate for at least three enzyme groups. The cyclo-oxygenase (COX) or prostaglandin endoperoxide synthase pathway produces prostaglandin

endoperoxides, which are then synthesized to prostaglandins by specific synthase enzymes.COX-2 mRNA and protein expression, has been shown to increase at term with significant up-regulation occurring prior to the onset of labour (130). Slater et al suggest that this up-regulation mediates an increase in prostaglandin synthesis in human myometrium at term, and may well have a role in the parturition process rather than as a consequence of labour. We postulated that within non labouring myometrium that progesterone could inhibit COX-2 expression. We showed no difference, however in COX-2 expression following the progesterone incubation. Others have tested a similar hypothesis that progesterone inhibits uterine contractility by blocking nuclear factor kappaB (NF-kappaB) activation and induction of COX-2.Immortalized human fundal myometrial cells were treated with IL1ß with and without progesterone. IL1ß alone caused a marked up-regulation of COX-2 mRNA, but treatment with progesterone suppressed this induction (194). The fundal myometrial cell line differs from lower segment myometrial explants and they stimulated an inflammatory response within the cells using IL1ß, these methodological differences could allow for the inherent differences seen in COX-2 mRNA expression with progesterone in our results.

In labour, synchronous contractions of the uterus depend on electrical coupling of myometrial smooth muscle cells by gap junctions (87). Normal labour is hypothesised to occur after the functional progesterone withdrawal allowing a contractile phenotype to dominate via activation of contraction associated proteins. Some of the important contraction associated proteins are the oxytocin receptor, PGDH and gap-junction protein connexin 43 (48). In the human myometrium, gap junctions are scarce in the non-pregnant uterus, but become abundant at term in preparation for labour. Connexin 26 and 43 are major components of these gap junctions and allow the uterus to function as a syncitium. Both have been identified within rat myometrium(195). Increased levels of connexin 43 mRNA have been detected in later gestation of pregnancy with further elevation of levels in labouring women(171). In human myometrial cell cultures expression of connexin 43 is up-regulated with oestriol and inhibited by progestin (4, 88). This suggested that progesterone could decrease the contraction capacity of myometrium by reducing connexin 43 expression and therefore gap junction formation. Although we demonstrated no difference with connexin 26 mRNA expression, there was a significant reduction in connexin 43

expression with both the lowest and highest progesterone incubation concentrations. This supports other data regarding these contraction associated proteins and is one potential mechanism of action that progesterone may prevent uterine contractions.

We finally looked at the progesterone receptor A and B but due to commercial availability we were unable to look at the receptor expressions separately. There was no change in expression of the receptors following progesterone incubation however this may be explained by the PRA: PRB ratio. The functional progesterone withdrawal suggests that genomic actions of progesterone are mediated by their nuclear receptors. The full length PRB and truncated PRA are controlled by separate promoters but studies suggest that PRB is the principal mediator. PRA is also thought to have an inhibitory effect on PRB and the extent of this will depend on its amount relative to PRB. It is therefore thought that the ratio or PRA:PRB is inversely related to the progesterone response(48, 77, 78). Measuring both in combination with progesterone may well show no change in expression as it is the overall ratio that can determine an effect.

In summary we were able to show cytokine release from myometrial explants that was not inhibited by progesterone over a 24 hour time period. We demonstrated a reduction in gap junction component connexin 43 with progesterone in agreement with other literature. We speculate that this may well be an important mechanism of action of progesterone for the prevention of preterm birth and warrants further investigation.

CHAPTER 5

The impact of prolonged maternal progesterone on human myometrium: The myometrial effects of prolonged *in vivo* treatment with progesterone

Introduction

We elected again to look at the group of mediators that have been implicated in the control of labour and preterm labour: such as the pro-inflammatory cytokines and contraction associated proteins mentioned in chapter 4. We hypothesised that progesterone would alter expression of these agents in *vivo*. We anticipated this would enable us to compare the effects of progesterone on myometrium both *in vivo* and *in vitro*.

Aim of Chapter

Using RT-PCR we quantified pro-labour genes, contraction associated proteins, endogenous uterine stimulants and endogenous inhibitors of uterine contractility. We selected probes for IL1β, IL6, IL8, iNOS, bNOS, eNOS, cGRP, cGRPR, ep2, Cx-26, Cx-43 as we have previously looked at the *in vitro* effects of progesterone on the expression of these genes in myometrium in chapter 4.

We localised expression of inflammatory mediators IL1β, IL6, IL8 and TNFα to examine using immunohistochemistry techniques established and published from our laboratory. We also used the leucocyte cell marker CD 45 to calculate the density of inflammatory cells and compare the placebo to progesterone groups as had been done before with labouring versus non labouring myometrium. Young et al showed that parturition was associated with a significant increase in IL1β, IL6 and IL8 expression in leucocytes in labouring cervix and myometrium and we have also previously shown that leucocytes (predominantly neutrophils and macrophages) infiltrated the cervix coinciding with the onset of labour (25). We aimed to observe if there were any differences in these events in the placebo and progesterone groups.

Our final aim within this chapter was to determine if changes in gene expression were accompanied by changes in protein expression. Hence we investigated the latter using Western blotting on proteins extracted from STOPPIT samples. We investigated the contraction associated protein, connexin 43.

STOPPIT samples

The molecular effects of progesterone *in vivo* on human myometrium are largely unknown and with our unique access to this cohort of women participating we were able, with appropriate consent and ethics to carry out this substudy. Although we have previously mentioned the STOPPIT trial and samples collected from this cohort in chapter 2 and 3, the focus in this previous chapter was on contractility studies. As previously explained the 10 x10 x 30 mm lower uterine segment sample was divided to allow separate analyses. The myometrium used for contractility studies had to be freshly transported in Krebs-Henseleit solution and experiments carried out within 12 hours as discussed in chapter 3. The other samples were stored in either mRNA later for subsequent molecular studies or in formalin for subsequent paraffin embedding and immunohistochemistry studies. It is these molecular and immunohistochemistry studies that we will discuss in this chapter.

In total, we collected 18 samples over the 17 month period. Samples collected at elective lower uterine segment caesarean section were nine from the placebo group and only three from the progesterone group. Patients were consented within the antenatal clinic and therefore prior to labour or their planned caesarean delivery. The total number of samples including some from labouring patients reached twelve in the placebo group and six within the progesterone group. After consenting, only a single specimen was not obtained; this patient required an emergency caesarean for the second twin, and it was therefore not deemed appropriate to collect the myometrium at this time. Three other consented STOPPIT patients went on to have a successful vaginal delivery of their twins. We were therefore confident that we had obtained as many samples as was practically possible within the limits of obstetric care for multiple pregnancies. The demographic data is shown again in this chapter (also in chapter 3) showing there was no detectable significant difference in either groups for age, BMI, gestation or baby weight for twin one and two (unpaired t-test).

The majority of specimens were collected at pre –labour elective caesarean section however three patients from placebo and three from progesterone were sampled at emergency caesarean section (Table 8). The reasons for emergency caesarean section varied and were failure to progress in the 1st stage of labour,

spontaneous rupture of membranes with twin one breech presentation and failed induction of labour with prostaglandins.

The time between sampling and completion of the placebo or progesterone also varied and we were only able to collect a total of three biopsies that were on treatment or within 2 weeks of completion of treatment. All three of these were within the placebo group. The mean gestation of delivery in the STOPPIT group was 36+4 weeks, the median gestation was 37+1 weeks.

The clinical trial finished recruiting in 2007 therefore there were no additional samples after summer 2008 once all patients within the trial had delivered. Over 500 patients were recruited in this multicentre trial. The results of the STOPPIT study were published in June 2009: there were no differences in the primary outcome (preterm delivery) between the progesterone and the placebo group.

	STOPPIT Placebo	STOPPIT Progesterone
Number women	12	6
Age (Years)	29.3(1.2)	31.8(1.1)
BMI (kg/m2)	25.9(1.5)	25.0(3.1)
Gestation (days)	252(4.6)	262(2.6)
Baby weight (Kg)		
Twin 1	2.3(0.1)	2.6(0.1)
Twin 2	2.3(0.2)	2.6(0.1)
Previous LUSS	0	3
ELLUCS	9	3
Parity		
0	4	3
1	6	3

Table 8 : The STOPPIT sample patient demographic data (mean ± SEM) showing there was no detectable significant difference in either groups for age, BMI, gestation or baby weight for twin one and two (unpaired t-test). The majority of specimens were collected at prelabour elective caesarean section however three patients from placebo and three from progesterone were sampled at emergency caesarean section.

Results RT-PCR

We examined the inherent mRNA expression of various labour and uterine related genes within the myometrial tissue. Real time RT-PCR (Abi,Taqman) was used to quantitate endogenous inhibitors of uterine contractility (cGRP, cGRPR, EP2), promoters of uterine relaxation (NOS,PGDH) cytokines (IL1ß, IL6, IL8) and gap junction components (connexin 26 and 43). For continuity we have expressed our results in this chapter relative to housekeeping gene 18S as in our previous analysis of *in vitro* progesterone samples this housekeeping gene was also used.

For analysis purposes we looked at the total group of STOPPIT patients, placebo versus progesterone. The initial proposal was only to collect samples from uncomplicated patients undergoing routine caesarean deliver not in labour "uncomplicated not in labour participants". However, in view of the limited numbers all suitable STOPPIT patients, including labouring ones that required caesarean delivery were included within our sample cohort. Some additional samples were therefore collected from women who did not fulfil our initial entry criteria. These women were either in labour or had required some form of medical intervention either due to labour, induction of labour or rupture of membranes prior to caesarean section. In view therefore that these were not a "clean" cohort of not in labour samples we carried out two separate analyses. The first included all STOPPIT samples, the "total STOPPIT cohort" group and the second included only uncomplicated not in labour samples, the "standardised STOPPIT cohort". The latter consists of patients within the STOPPIT trial, not in labour with no complications. As we know from previous data, many of the inflammatory markers can be up regulated within human myometrium in labour. We therefore felt it necessary to look at both the "Total" cohort and "standardised" cohort prior to analysis of the data.

We analysed our data by log transforming our results and confirmed a normal distribution with Kolmogorov-Smirnov[a] test. We then performed unpaired t-test and equal variances were not assumed. Data within the graphs was presented in an untransformed format and levels were expressed as mean ± SEM for box plot graphs and individual levels obtained for each sample in the scatter plot graphs.

We plotted all of our data on a scatter plot to assess overall distribution. Although women at the extremes of low gestation were in the placebo group, there were no significant differences in mean gestation in the progesterone and placebo group. We have shown one example of a scatter plot graph.

On plotting individual values against gestation, the question of an effect with some of our measured genes arose. Although there was no statistically significant difference in gestation between the groups we wanted to make a full comparison of our data. We have therefore performed scatter plot graphs for all individual values against gestation. This shows that there were no samples from within the progesterone group taken under 250 days gestation. The other scatter plot graphs are presented within the appendix of this thesis.

Nitric-oxide synthase

We looked at three separate isoforms of NOS, iNOS (inducible), eNOS (endothelial) and bNOS (neuronal).

Inos

Levels of inos detected were relatively low with similar expression as seen with our *in vitro* myometrial model. There was no difference in expression between placebo and progesterone groups for both the total STOPPIT cohort and the standardised STOPPIT cohort (unpaired t-test, log transformed data, Figure 84, Figure 85). In view of small numbers, we also plotted the individual values obtained for both groups against the pregnancy gestation (Figure 86, Figure 87). We were able to see therefore that the majority of progesterone treated samples were within the latter gestations obtained and that any early ones had been within the placebo group.

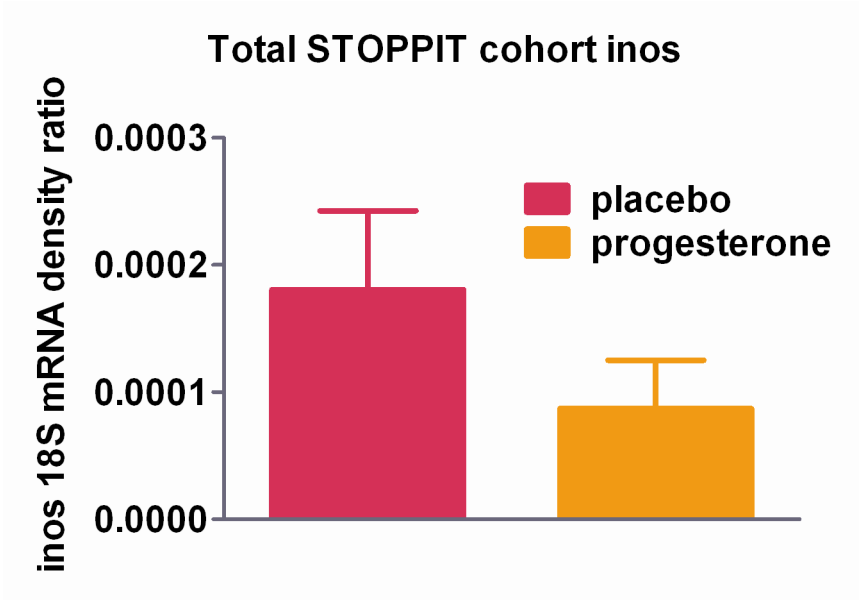

Figure 84: This graph shows inos gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

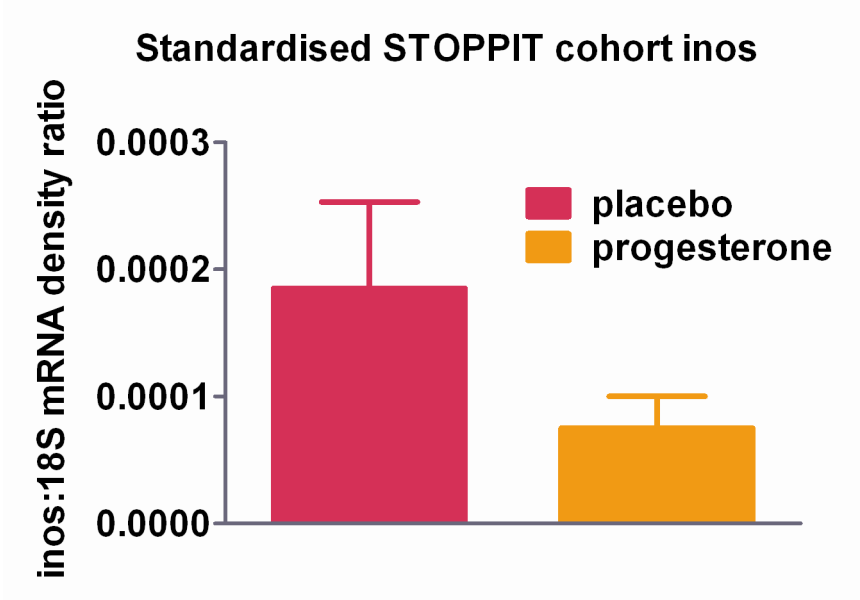

Figure 85 : This graph shows inos gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups within the standardised STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

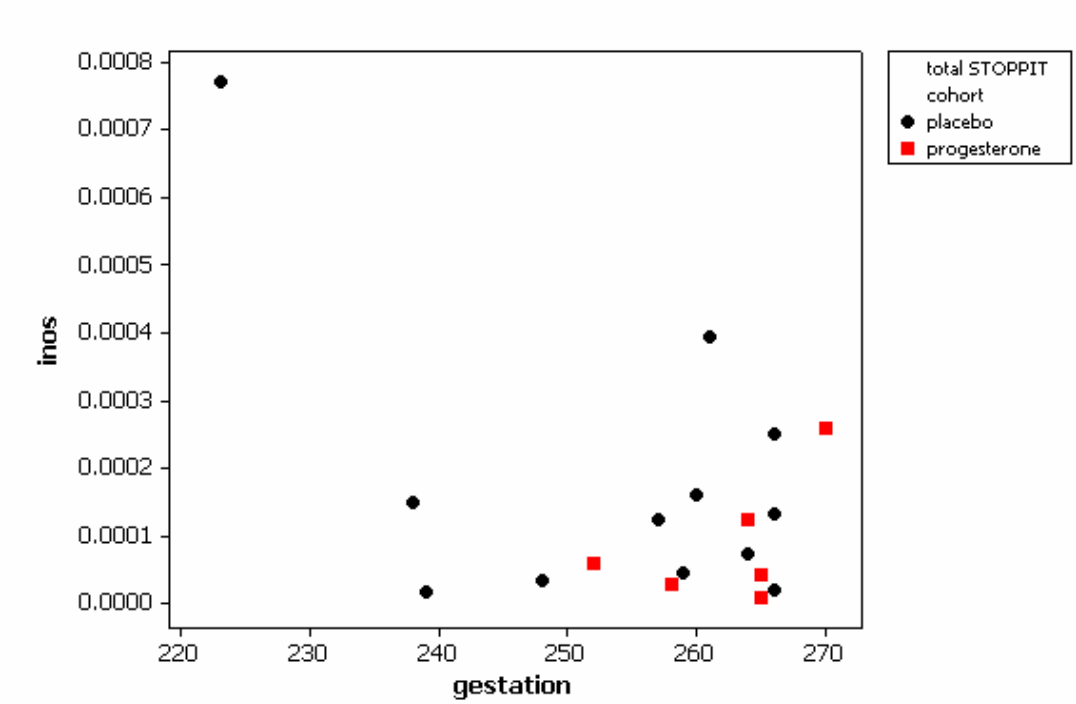

Figure 86 : Scatter plot graph showing inos gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

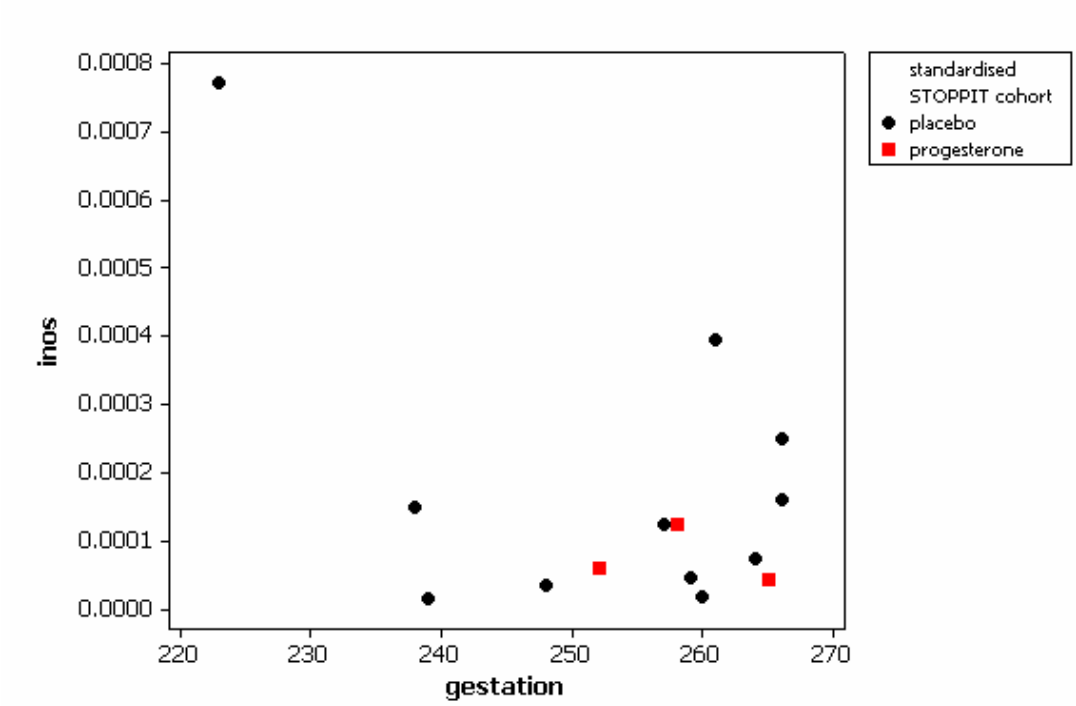

Figure 87 : Scatter plot graph showing inos gene mRNA expression levels, relative to 18S. Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

Enos

Enos expression again, showed similar values as seen in with our *in vitro* myometrial model. Enos showed higher levels of expression than both inos and bnos. There was no difference in expression between placebo and progesterone groups for both the total STOPPIT cohort, $p>0.05$ (Figure 88). There was a significant difference however when analysis was restricted to the standardised STOPPIT cohort, $p <0.05$ (unpaired t-test log transformed data, Figure 89). We also plotted the individual values against gestation (appendix). All samples within the progesterone group were obtained at a gestation of greater than 250 (35+5) days. All samples from earlier gestations were within the placebo group.

Bnos

Levels of bnos detected showed low expression in human myometrium, similar to our *in vitro* myometrial model. There was no difference in expression between placebo and progesterone groups for the total STOPPIT cohort and standardised STOPPIT cohort, $p>0.05$ (unpaired t-test, log transformed data, Figure 90, Figure 91). We also plotted the individual values obtained for both groups against the pregnancy gestation (appendix).

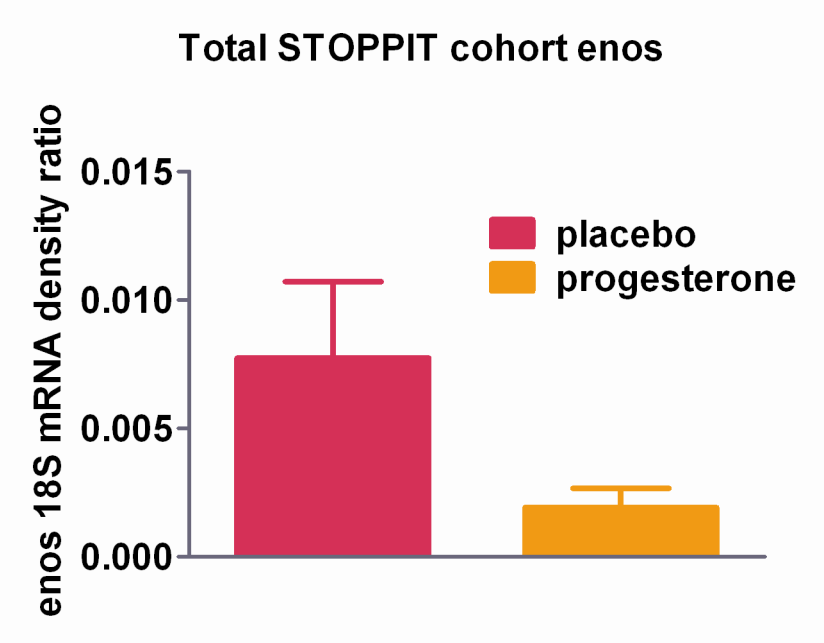

Figure 88 : This graph shows enos gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, $p>0.05$ (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

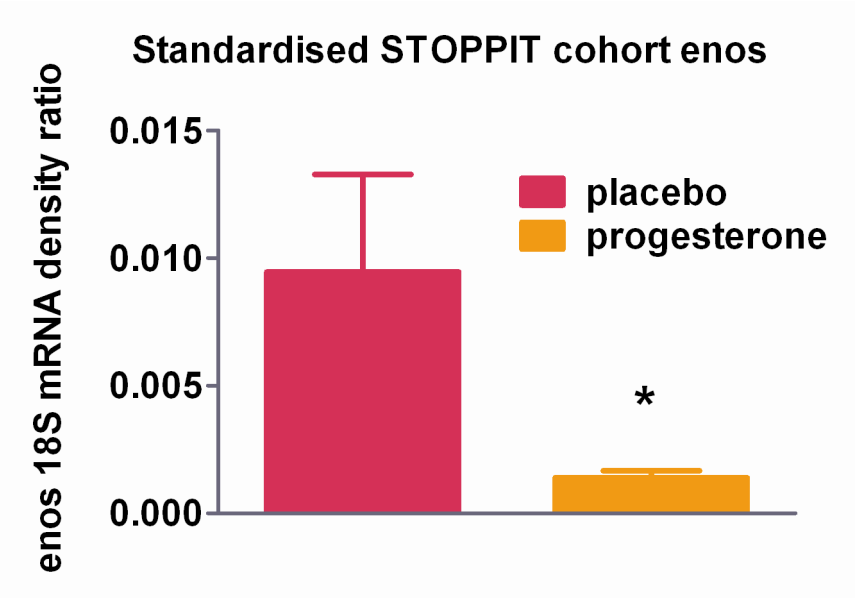

Figure 89 : This graph shows enos gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was a significant difference between placebo and progesterone groups when analysis was restricted to the standardised STOPPIT cohort of patients, $p<0.05$ (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

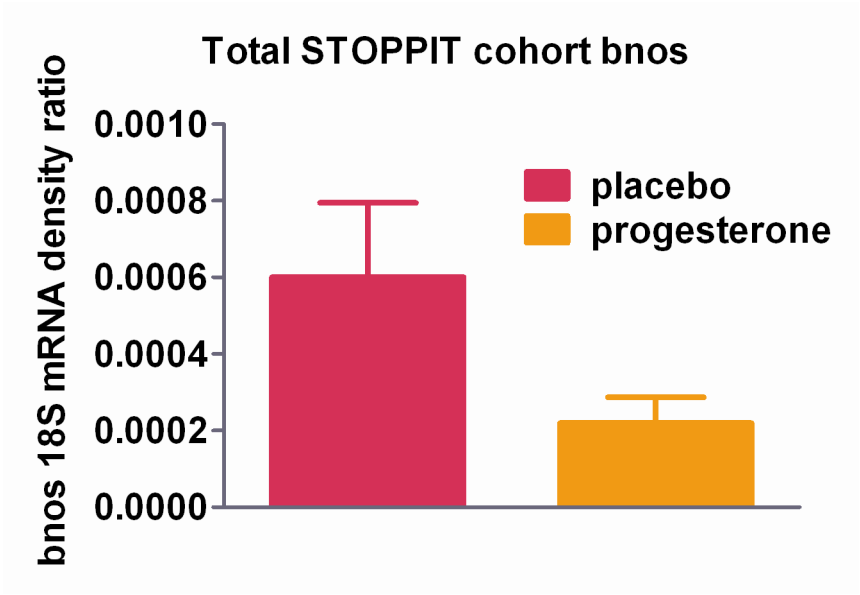

Figure 90 : This graph shows bnos gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was no detectable significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

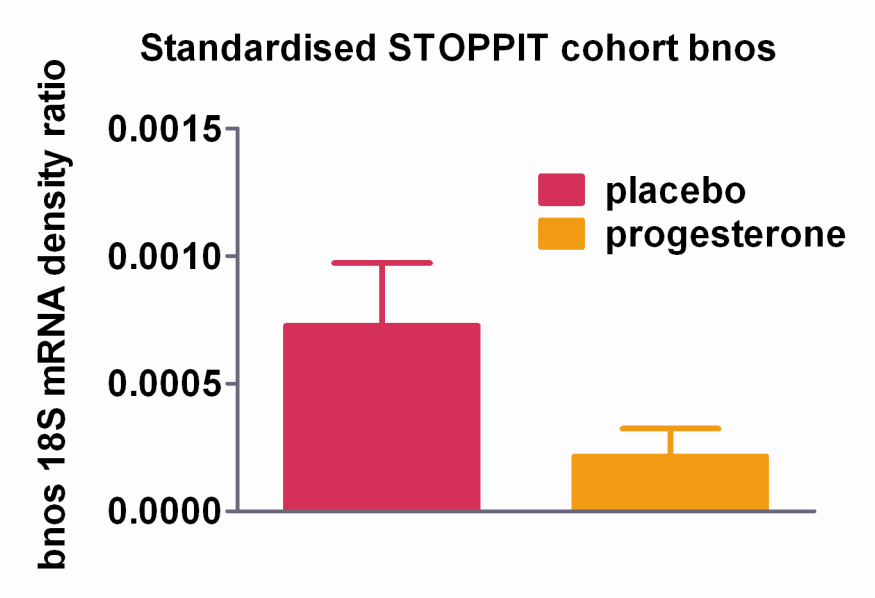

Figure 91 : This graph shows bnos gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was no detectable significant difference between placebo and progesterone groups within the standardised STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

cGRP, cGRPR

CGRP inhibits myometrial contractile activity. We measured both the peptide and its receptor. Levels again, were comparable to our in vitro model of expression of both cGRP and its receptor within human myometrium. Levels of the receptor were higher than the peptide expression itself in human myometrium. There was no difference in cGRP or its receptor expression between placebo and progesterone groups for both the total STOPPIT cohort and standardised STOPPIT cohort only , $p>0.05$ (unpaired t-test, log transformed data Figure 92, Figure 93, Figure 94, Figure 95).We also plotted the individual values obtained for both groups against the pregnancy gestation (appendix) .

EP2

This is a subgroup of receptor for prostaglandin E_2 and is G protein coupled. . Its activation leads to a rise in cAMP and myometrial relaxation. Levels of expression were again comparable to levels within our *in vitro* model looking at EP2 within human myometrium. There was no statistically significant difference between the placebo and progesterone groups in the total STOPPIT cohort , $p > 0.05$, however when analysis was restricted to standardised STOPPIT cohort there was a lower expression of EP2 in the placebo group, $p <0.05$ (unpaired t-test log transformed data, Figure 96, Figure 97). We also plotted the individual values obtained for both groups against the pregnancy gestation (appendix).

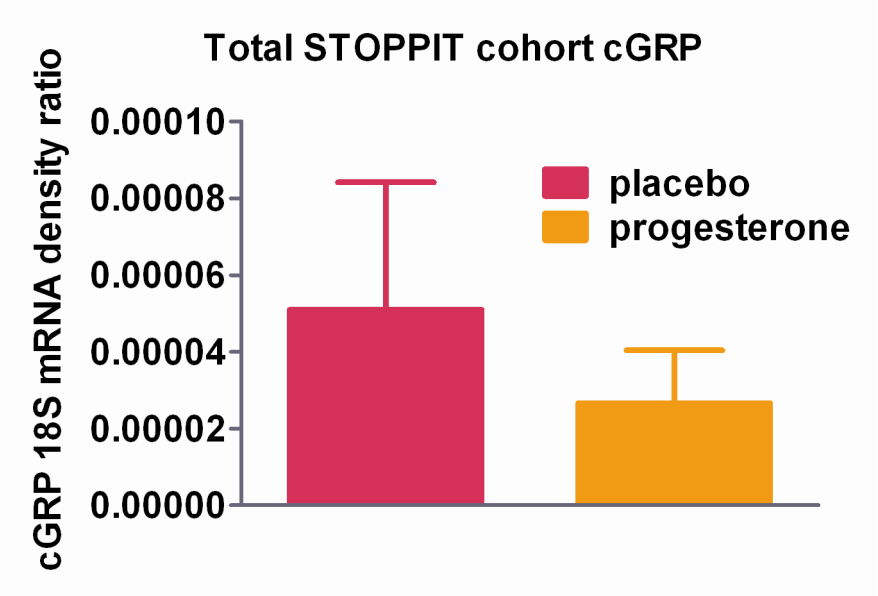

Figure 92 : This graph shows cGRP gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

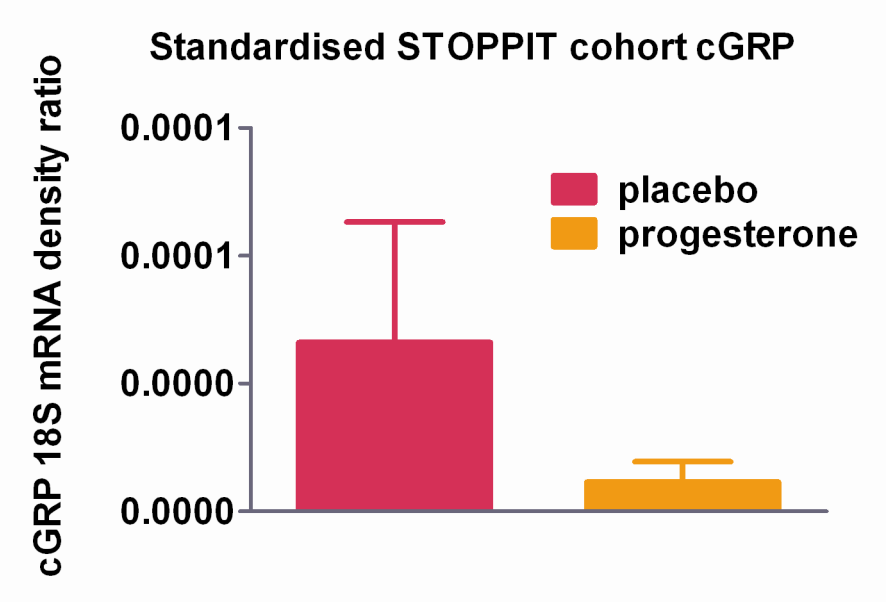

Figure 93 : This graph shows cGRP gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups within the standardised STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

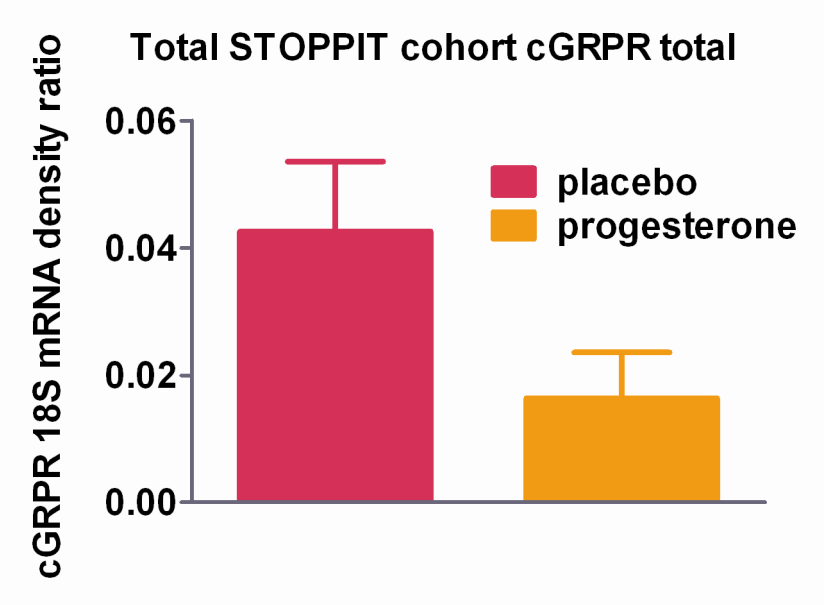

Figure 94 : This graph shows cGRPR gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

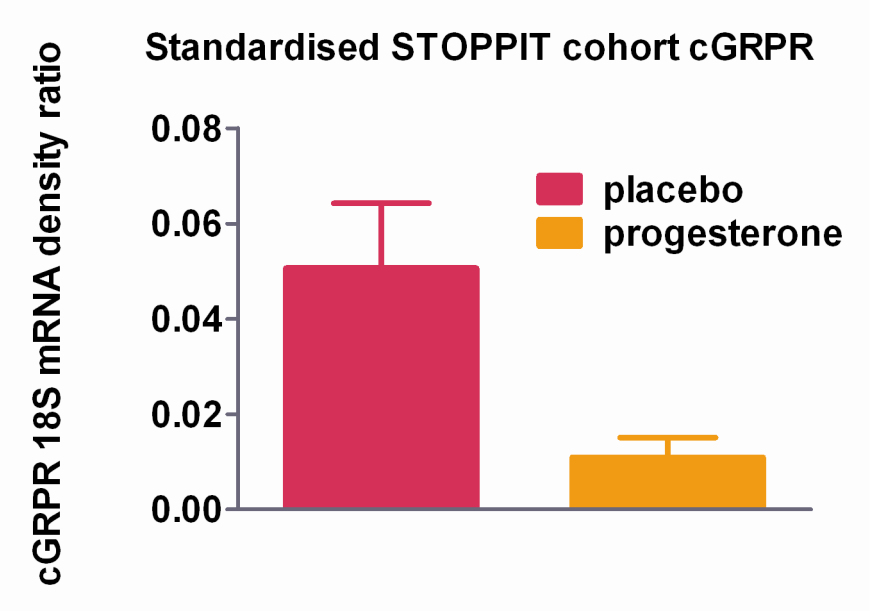

Figure 95 : This graph shows cGRPR gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no detectable significant difference between placebo and progesterone groups with the ELLUCS STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

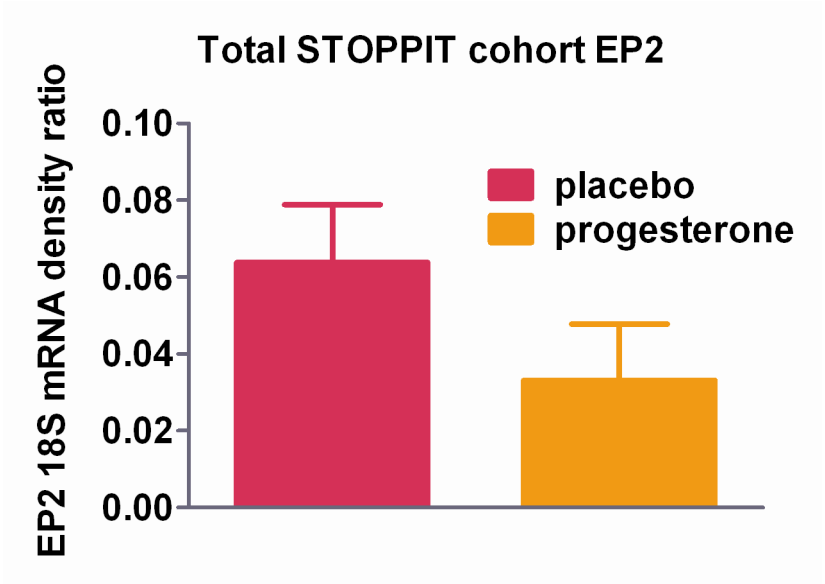

Figure 96 : This graph shows EP2 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

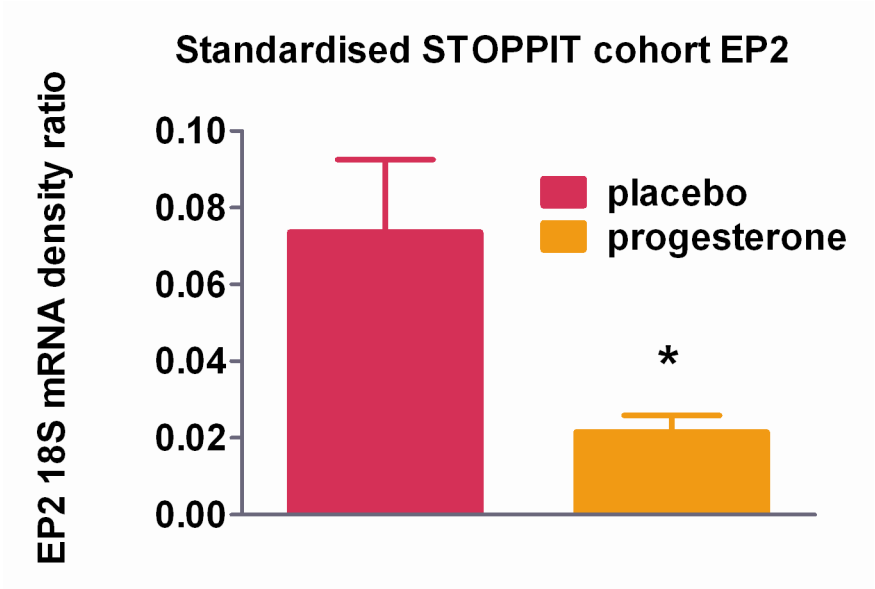

Figure 97 : This graph shows EP2 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was a significant difference between placebo and progesterone groups when analysis was restricted to the ELLUCS STOPPIT cohort of patients, p<0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

Pro-inflammatory cytokines

We selected IL1ß, IL6 and IL8 all of which are generated in the uterus and cervix and implicated in labour and preterm labour. There was no difference in expression between placebo and progesterone groups for both the total STOPPIT cohort and standardised STOPPIT cohort with all three pro-inflammatory cytokines, IL1ß (Figure 98, Figure 99), IL6 (Figure 100,

Figure 101,) and IL8 (Figure 102, Figure 103), $p>0.05$ (unpaired t-test log transformed data).

Contraction associated proteins connexin 26 and 43

Within our group we observed a lower expression of the contraction associated protein connexin 26 within the standardised STOPPIT cohort and the total STOPPIT cohort (Figure 104, Figure 105) , $p>0.05$ (unpaired t-test log transformed data). We observed no statistical difference in connexin 43 between placebo and progesterone within the total STOPPIT cohort however; when analysis was restricted to the standardised STOPPIT cohort there was a lower expression within the progesterone group (Figure 106, Figure 107).

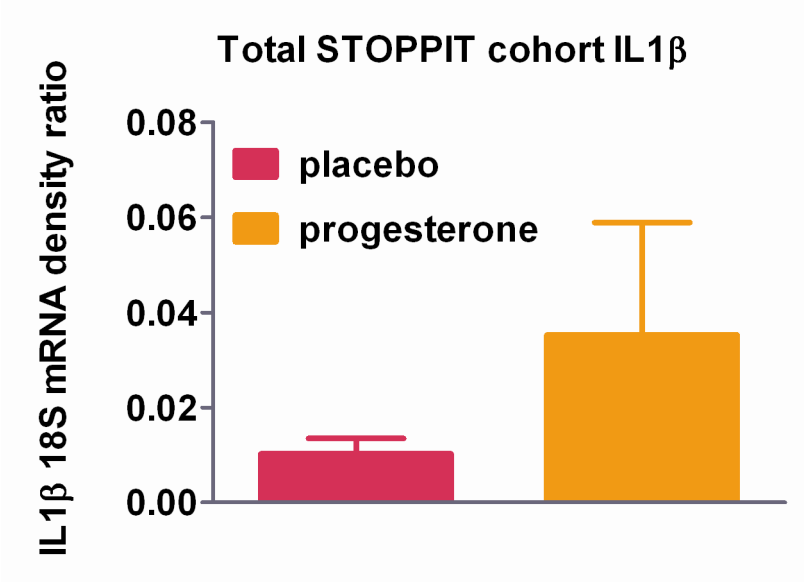

Figure 98 : This graph shows IL1β gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

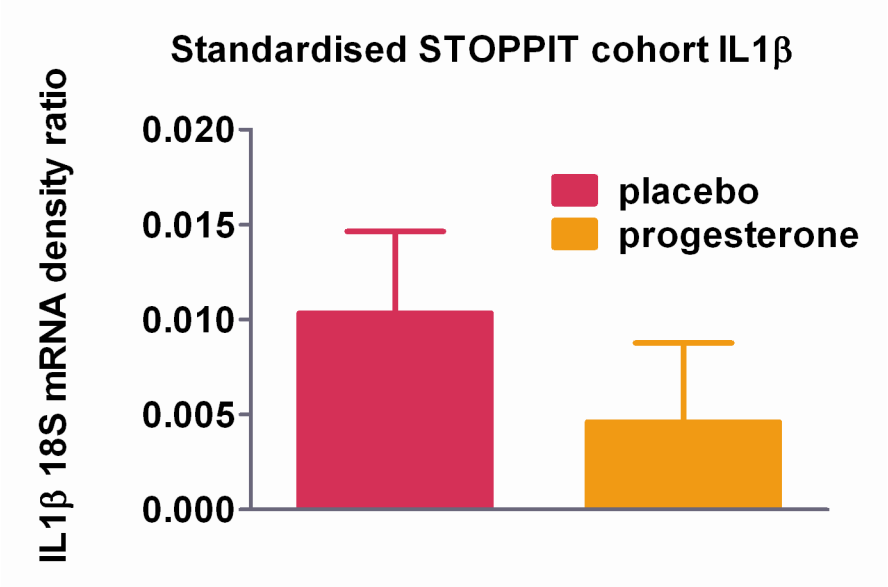

Figure 99 : This graph shows IL1β gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups within the standardised STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

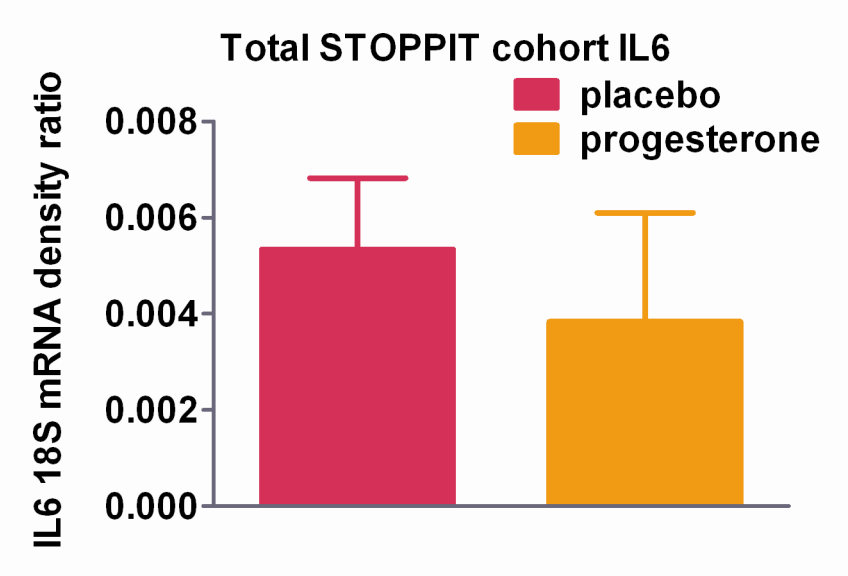

Figure 100 : This graph shows IL6 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

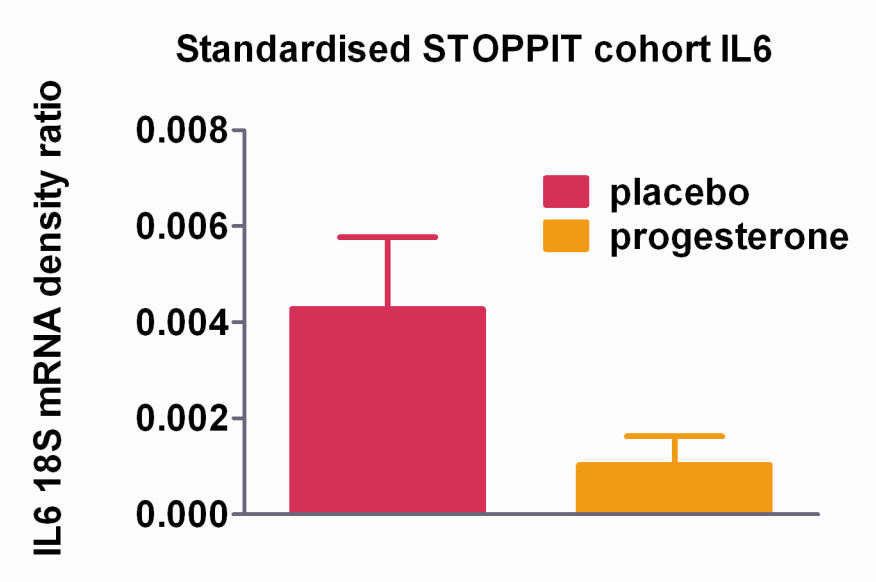

Figure 101 : This graph shows IL6 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups within the standardised STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

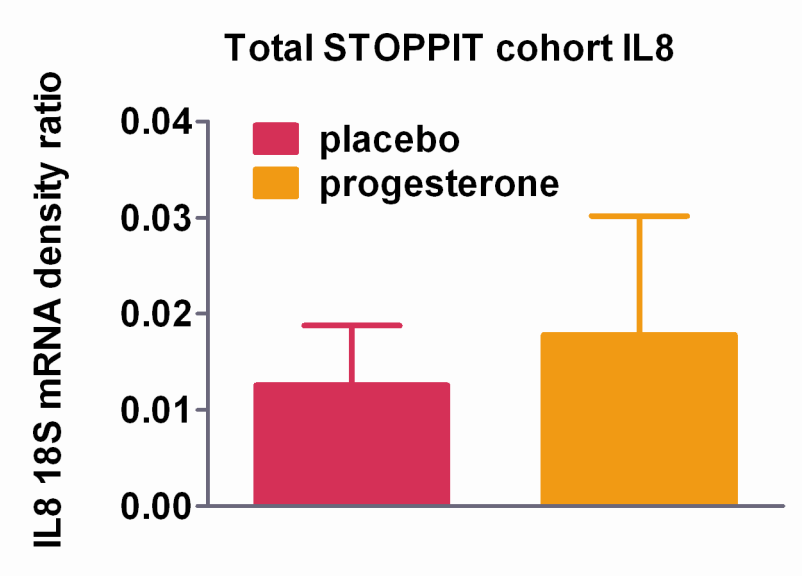

Figure 102 : This graph shows IL8 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

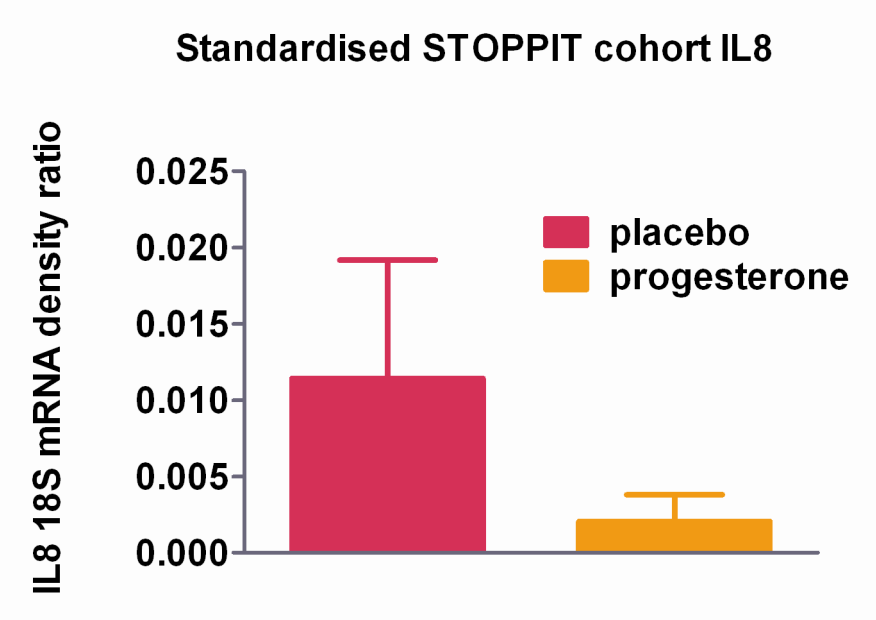

Figure 103 : This graph shows IL8 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was no significant difference between placebo and progesterone groups within the standardised STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

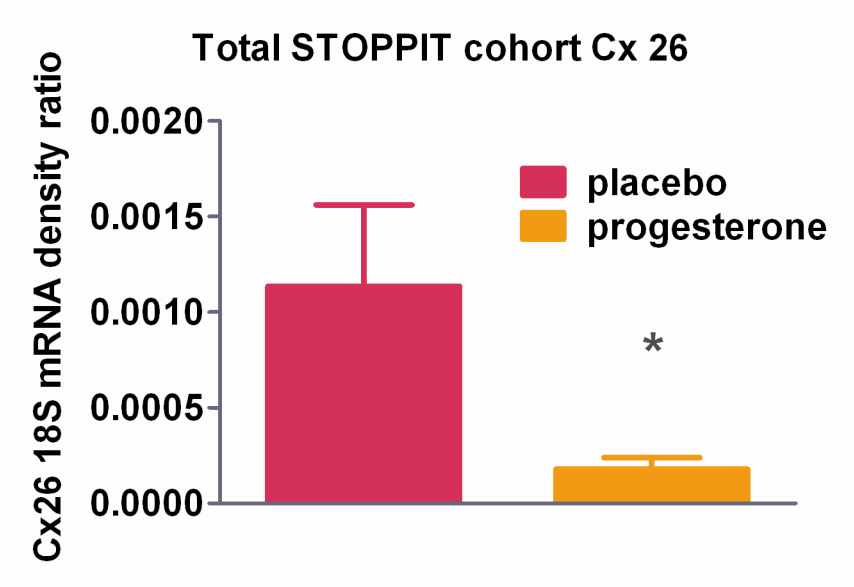

Figure 104 : This graph shows Cx 26 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was a relative reduction between placebo and progesterone groups with the total STOPPIT cohort of patients, $p<0.05$ (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

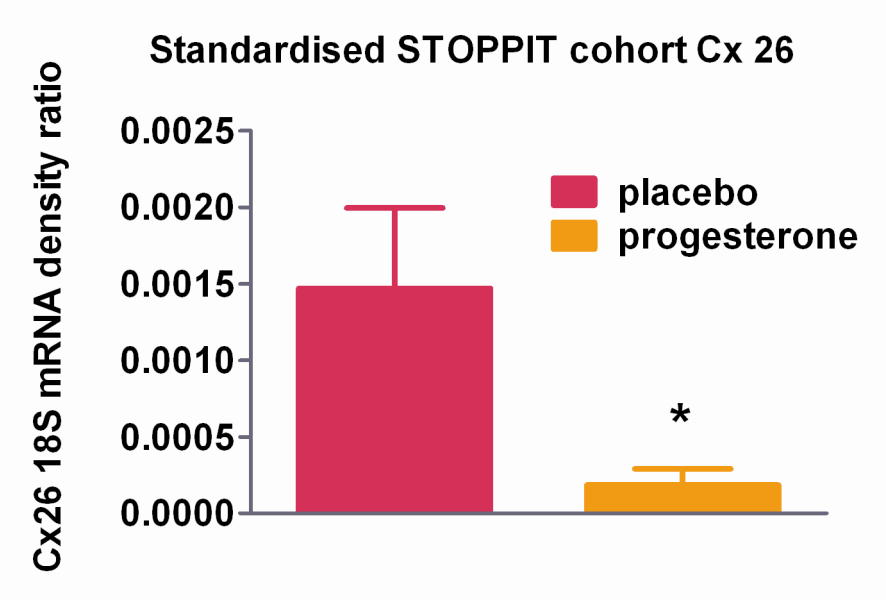

Figure 105 : This graph shows Cx 26 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM).There was a relative reduction between placebo and progesterone groups within the standardised STOPPIT cohort of patients, $p<0.05$ (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

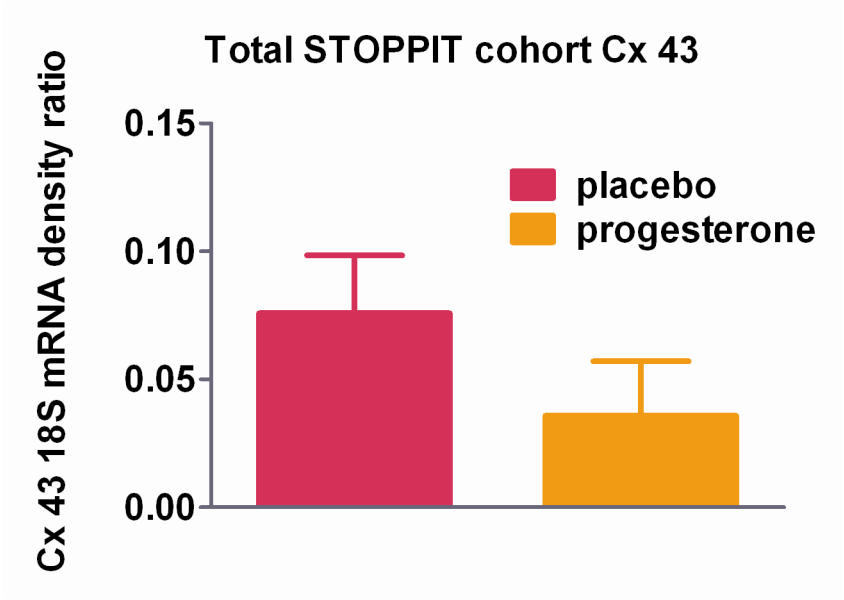

Figure 106 : This graph shows Cx 43 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was no significant difference between placebo and progesterone groups with the total STOPPIT cohort of patients, p>0.05 (unpaired t-test log transformed data). Patients within placebo group n=12, progesterone n=6.

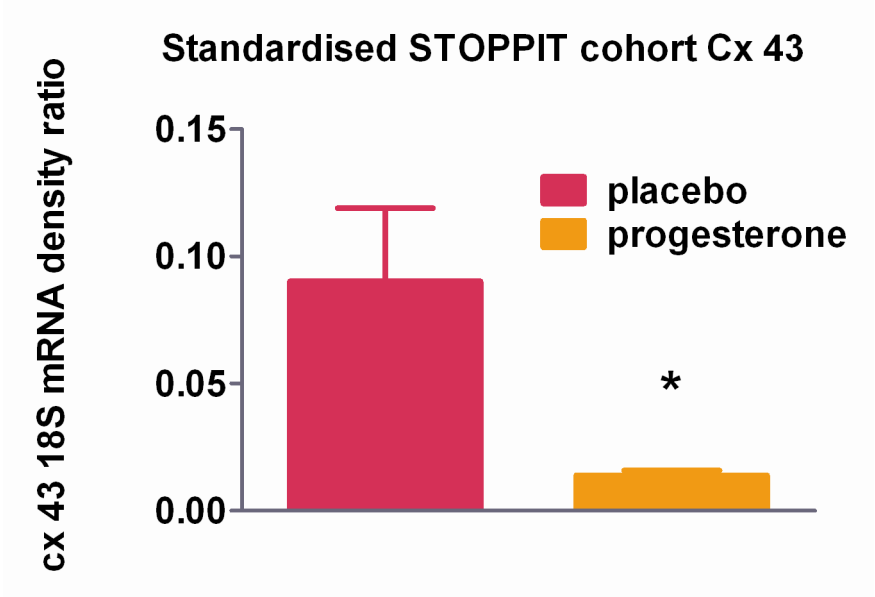

Figure 107 : This graph shows Cx 43 gene mRNA expression levels, relative to 18S. Data display is of untransformed data, expressed as mean ± (SEM). There was a significant difference between placebo and progesterone groups when analysis was restricted to the standardised STOPPIT cohort of patients, p<0.05 (unpaired t-test log transformed data). Patients within placebo group n=9, progesterone n=3.

Immunohistochemistry Results

We examined all 18 samples stained for each pro-inflammatory cytokine and the CD 45 leucocyte marker. At the time of analysis we remained blinded to the STOPPIT group and made our comparison with the evidence gathered at this stage. We stained the tissue for cytokine localisations however we did not attempt to analyse staining intensity semi-quantitatively. The leucocytes were identified using histological analysis in ten randomly selected high powered fields (x 400 objective magnification) and were counted by two independent observers who were blinded to the specimen details. We were then able to comment if any difference existed between the placebo and progesterone groups for CD45 only. We looked at the total STOPPIT cohort as a group. We did not have adequate numbers of samples to look at in labour versus not in labour where previous studies have shown differences. All magnifications are shown at x20 unless stated.

IL1β

The positive control using tonsil showed clear staining and the negative control showed blue staining only. There was strong staining evident within the myocytes for IL1β (Figure 108). A gradient of staining was sometimes evident however, myocytes generally stained brown with their darker nuclei evident within the cell. The surrounding stroma and showed little evidence of staining. There was strong staining within the endothelial lining of blood vessels. There was also darker staining in areas within blood vessels or adjacent to the blood vessel.

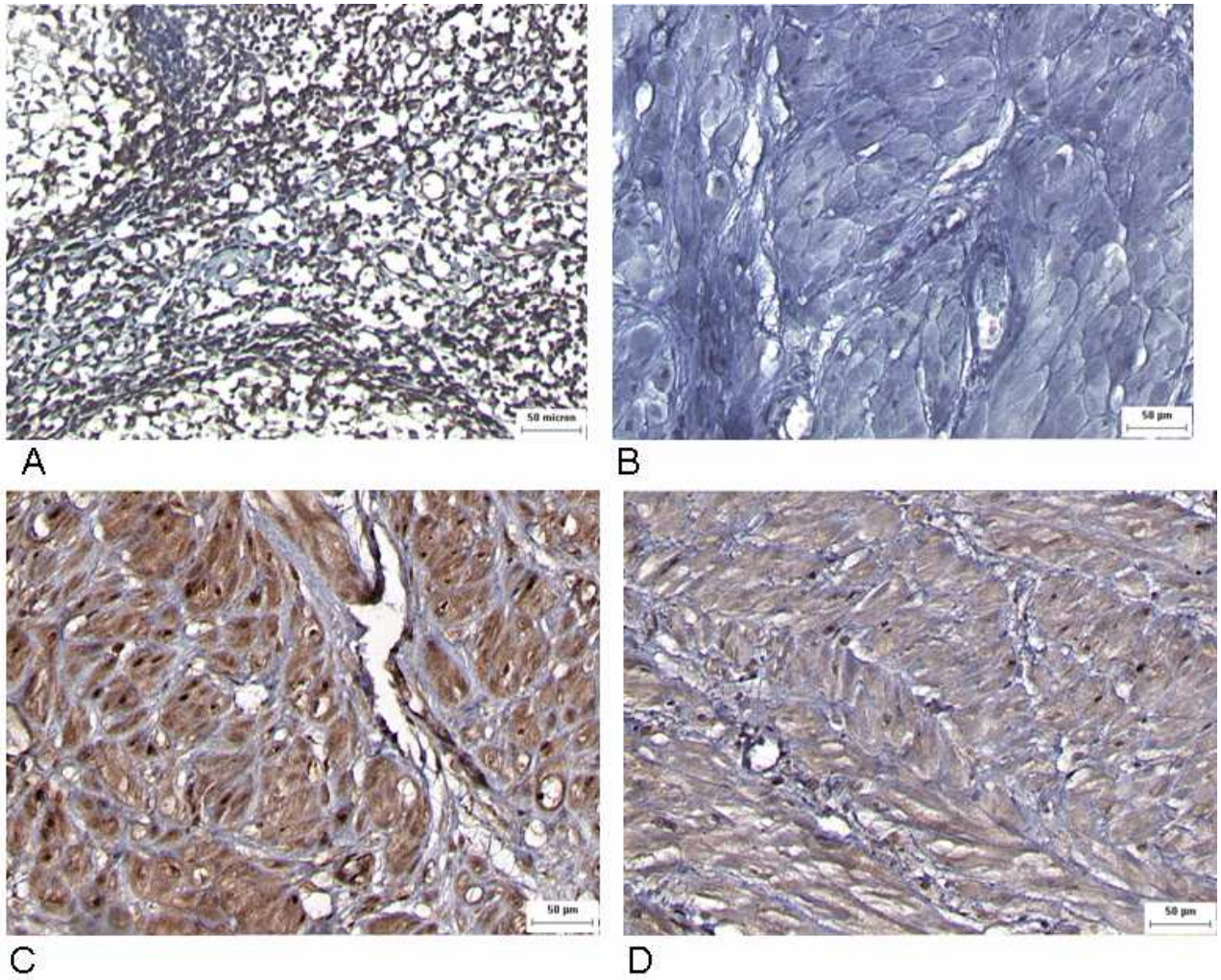

Figure 108 : IL1β staining at (x 20) for A) Tonsil positive control, B) Myometrium negative control, C) A placebo group example from STOPPIT samples and D) A progesterone group example from STOPPIT tissue.

IL6

The positive control using tonsil showed pale staining and the negative control showed blue staining only. There was faint staining of myocytes with IL6 and a gradient of staining was evident within the myometrial sections (Figure 109). Myocytes were generally stained brown with their darker nuclei present within the cell. The surrounding stroma again, stained blue with little expression. There was also staining in areas within blood vessels or adjacent to the blood vessel.

IL8

The positive control using tonsil showed dark staining and the negative control showed blue staining only. For the myometrial samples there was a gradient of tissue staining evident within some of the sections. Myocytes showed expression of IL8 with darker brown nuclei evident within the cells (Figure 110). Some myocytes showed expression which was less uniform in appearance but this may have been due to the gradient of staining present throughout. Generally, blood vessels and the endothelial lining stained brown giving evidence of expression. The surrounding connective tissue and stroma was blue with less expression evident however, there were a population of cells staining within the stroma. This may well have been a white cell population of cells but further confirmation of this would be required. Some of the progesterone samples showed very pale staining or non stained samples (one in labour sample, one not in labour).

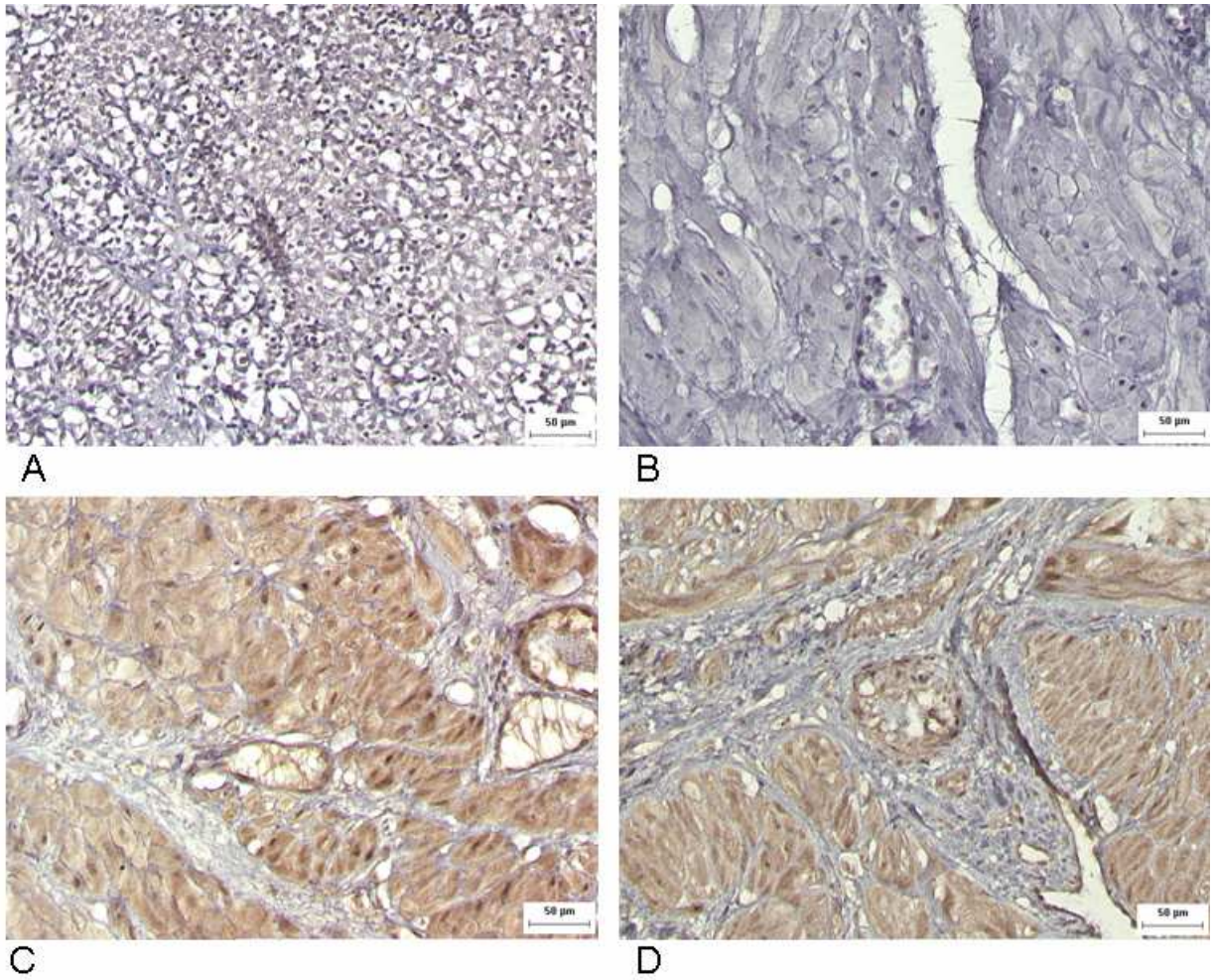

Figure 109 : IL6 staining at (x 20) A) Tonsil positive control, B) Myometrium negative control, C) A placebo group example from STOPPIT samples and D) A progesterone group example from STOPPIT tissue.

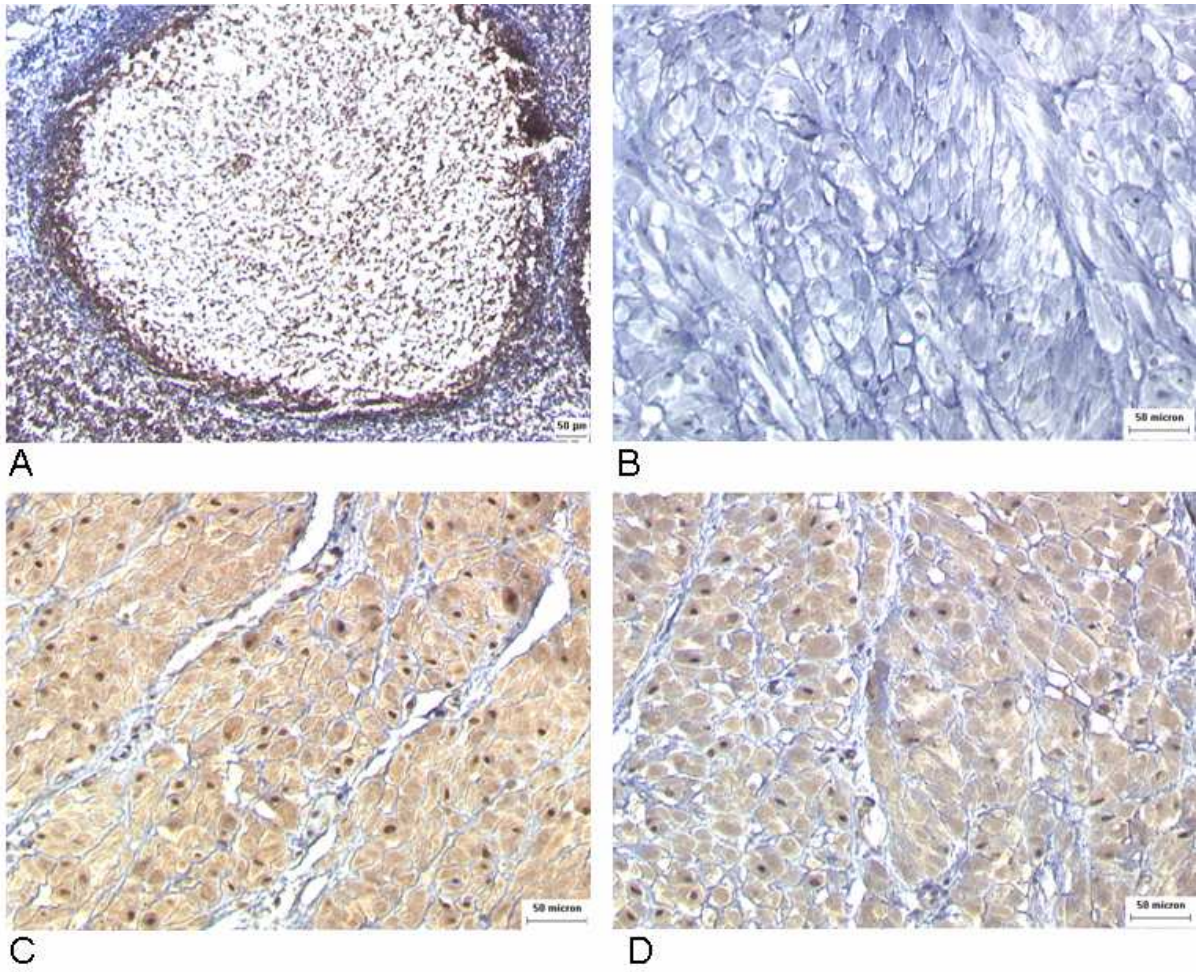

Figure 110 : IL8 staining (x 10) for A) Tonsil positive control and (x 20) for B) Myometrium negative control, C) A placebo group example from STOPPIT samples and D) A progesterone group example from STOPPIT tissue.

TNF-α

The positive control showed clear evidence of staining and the negative control stained blue only. Staining throughout was generally moderate however there was a gradient of staining evident and some non- uniform patterns. Myocytes generally stained brown with varying degrees of expression throughout the section and there were darker nuclei evident within the cell structure (Figure 111). The stroma tissue showed little expression with evidence of brown cells stained within indicating a possible white cell population.

CD 45

This is also known as the common leucocyte antigen and is present in all hematopoietic cells except erythrocytes and platelets. There was generally uniform staining however there was no expression around or within myocytes (Figure 112). There was some expression within the stroma and near blood vessels. No obvious difference was noted between the groups of placebo and progesterone and there was no statistical difference in the CD45 count $p>0.05$ (unpaired t-test).

Group	Mean Count	SEM	Number of fields observed
Placebo	3.00	0.22	240
Progesterone	2.86	0.40	120

Table 9 : CD 45 cell count with mean data from two independent observers shown. There was no statistical difference between the placebo and progesterone groups $p>0.05$ (unpaired t-test).

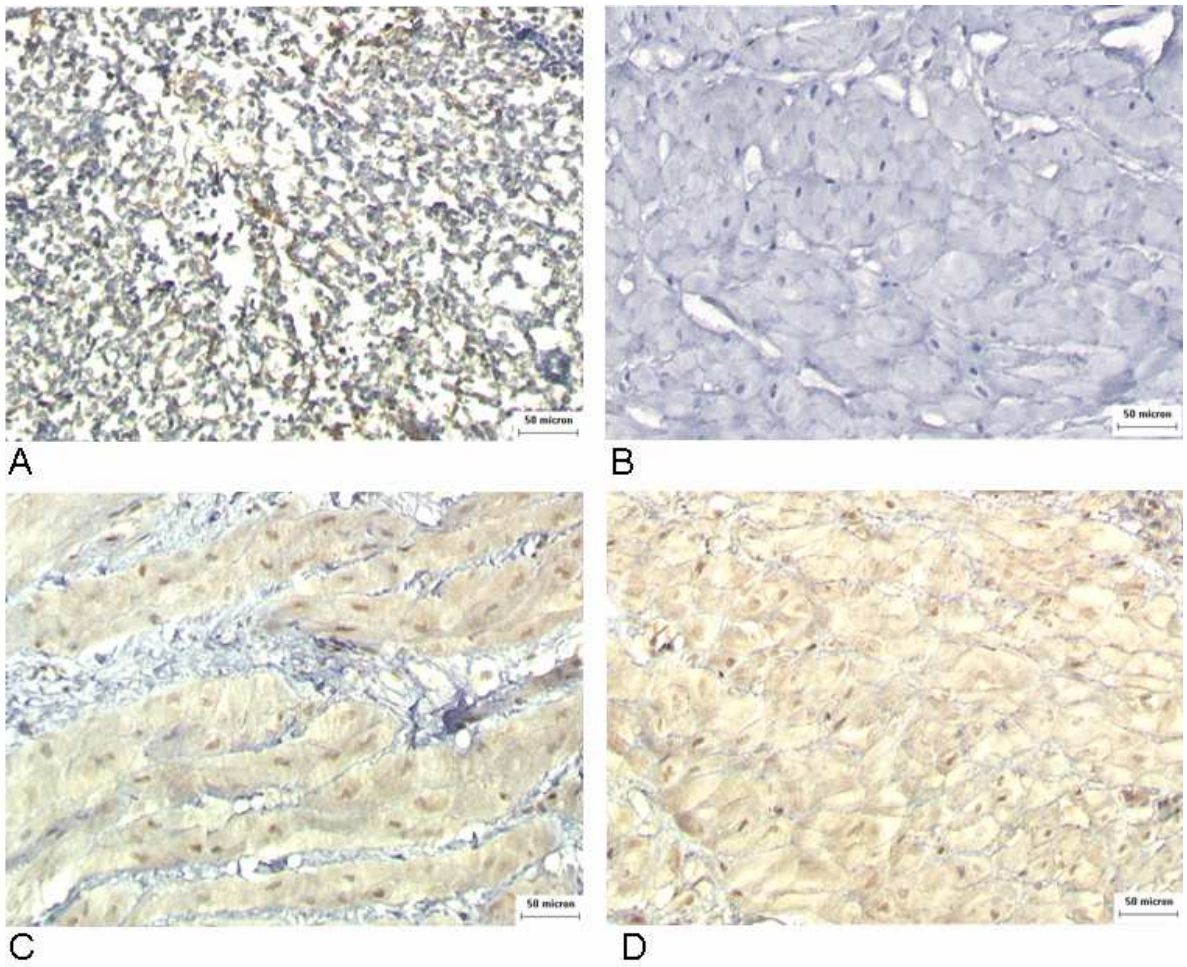

Figure 111 : TNF-α staining (x 20) for A) Tonsil positive control B) Myometrium negative control, C) A placebo group example from STOPPIT samples and D) A progesterone group example from STOPPIT tissue

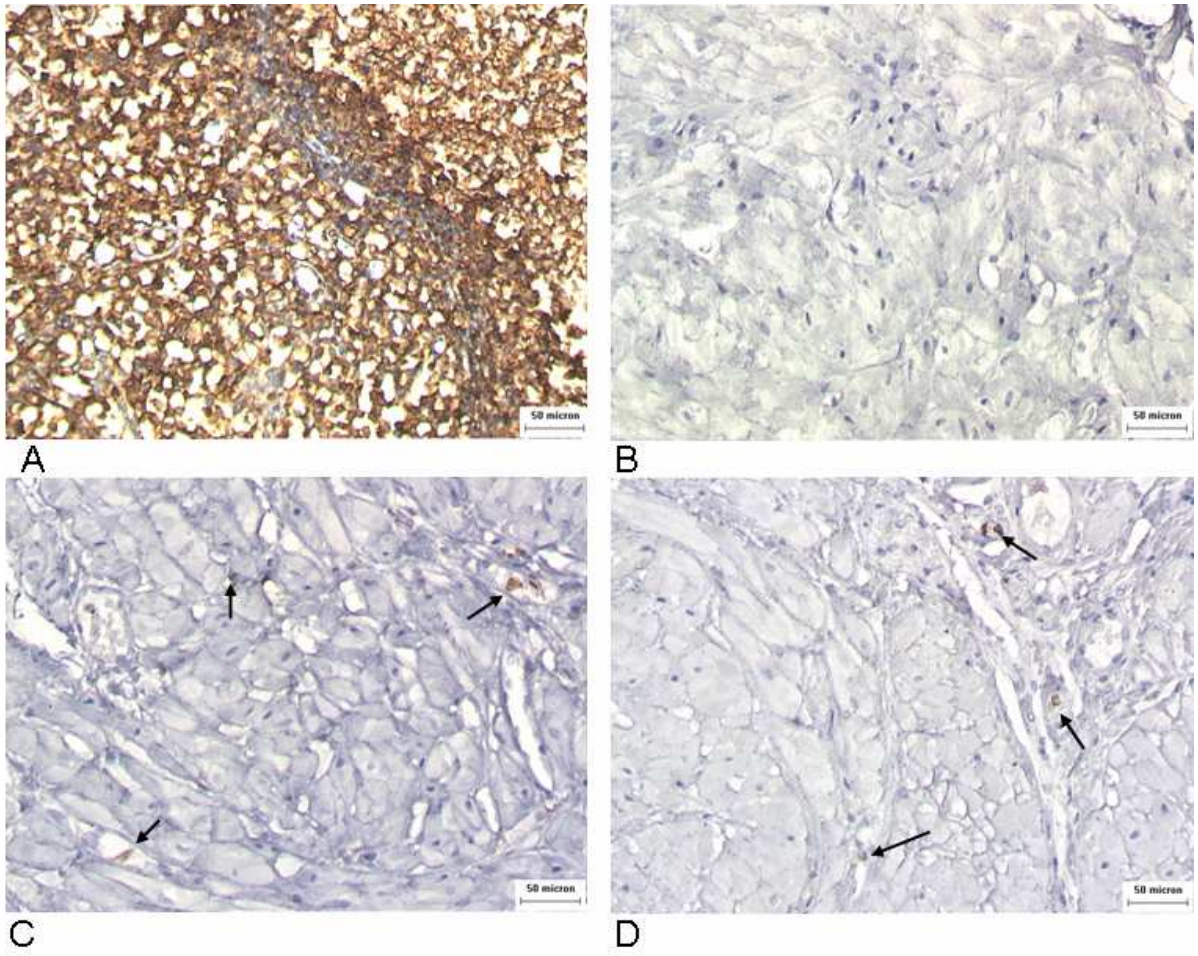

Figure 112 : CD45 (x 20) A) Tonsil positive control B) Myometrium negative control, C) A placebo group example from STOPPIT samples and D) A progesterone group example from STOPPIT tissue. Arrows indicate CD45 positive cells. There is no detectable difference between the placebo and progesterone staining for CD45

Western Blot

Connexin 43

Having identified no changes in the pro-inflammatory cytokines we selected to further investigate the contraction associated proteins. Time constraints prohibited an extensive investigation of the proteins from the STOPPIT samples and therefore we selected to look at connexin 43. The mRNA data had shown less expression within the progesterone treatment group both *in vitro* and *in vivo* and when compared to the control or placebo. We developed and optimised the antibody concentrations and protocol as discussed in chapter 2. Connexin 43 has a molecular weight of around 43 kilo Daltons. The intended loading control ß-actin has an identical molecular weight and therefore prior to protocol optimisation we elected to use a different loading control ß-tubulin which has a higher molecular weight of 55 kilo Daltons. This was in order to identify bands at the separate molecular weights. Our protocol however required that our initial blot was stripped and then probed again, for the loading control antibody. Tubulin is present in almost all eukaryotic cells and is used commonly as a housekeeping gene.

We were able to show an even distribution of protein on the initial gel electrophoresis (stained with Coomassie blue) and there was little difference between the extracted proteins from the myometrial samples (Figure 113). We were then able to clearly demonstrate the presence of connexin 43 with clear bands evident at around 43 KDa, (between the 37kDa and 50kDa molecular weight marker indicator). We also showed the presence of ß-tubulin clearly at 55 kDa however, samples one and three lacked expression of this. Although there was a clear difference in mRNA expression, less expression within the progesterone group compared to placebo, this did not translate into a protein effect. We detected no difference statistically between the density ratio for placebo versus progesterone $p>0.05$ t-test (Figure 115, Figure 116). Connexin 43 mRNA expression did not correlate with its protein expression when analysed with respect to the clinical grouping.

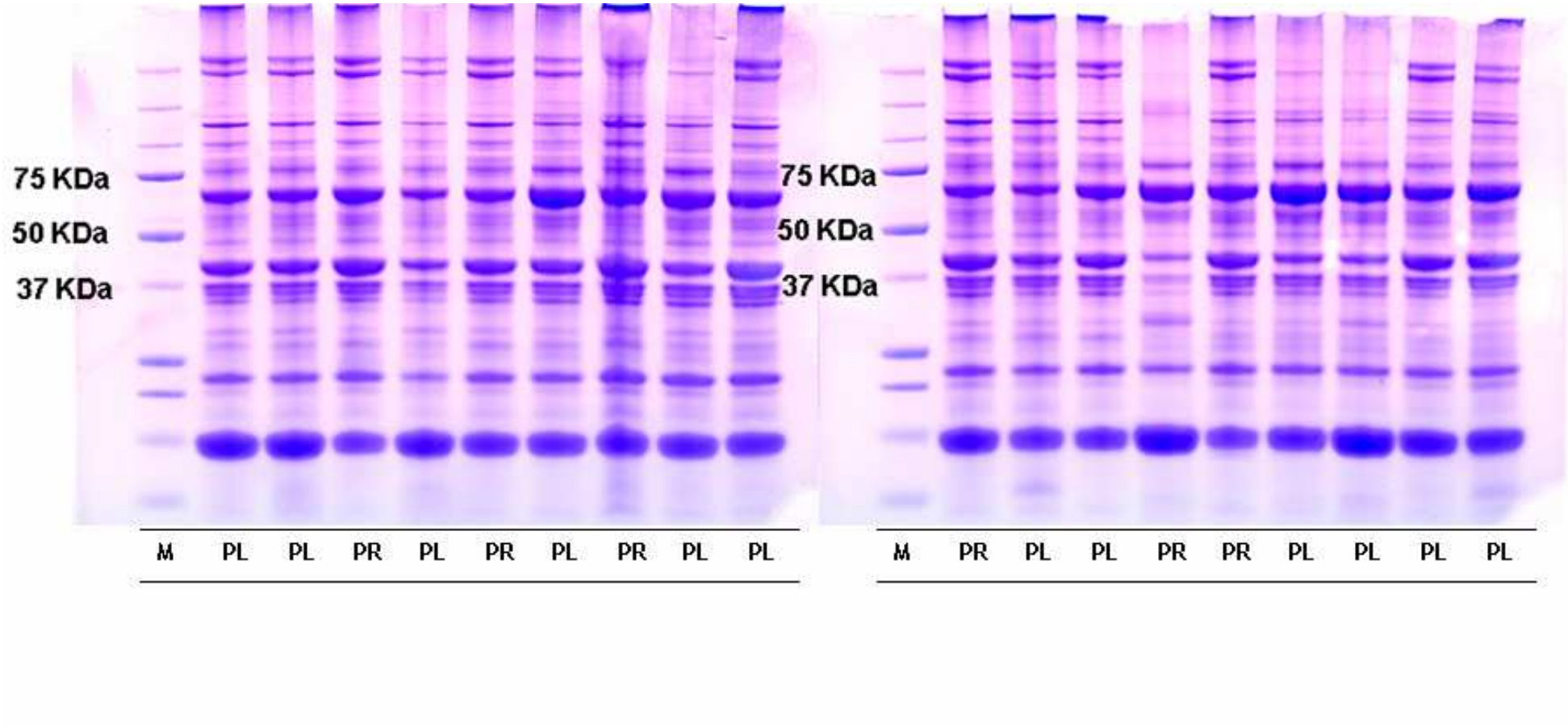

Figure 113 : Protein gel showing the spread for each protein extracted from the 18 STOPPIT samples. The molecular weight marker is indicated (M) and (PL) placebo, (PR) progesterone for each. This shows generally an even distribution amongst the specimens.

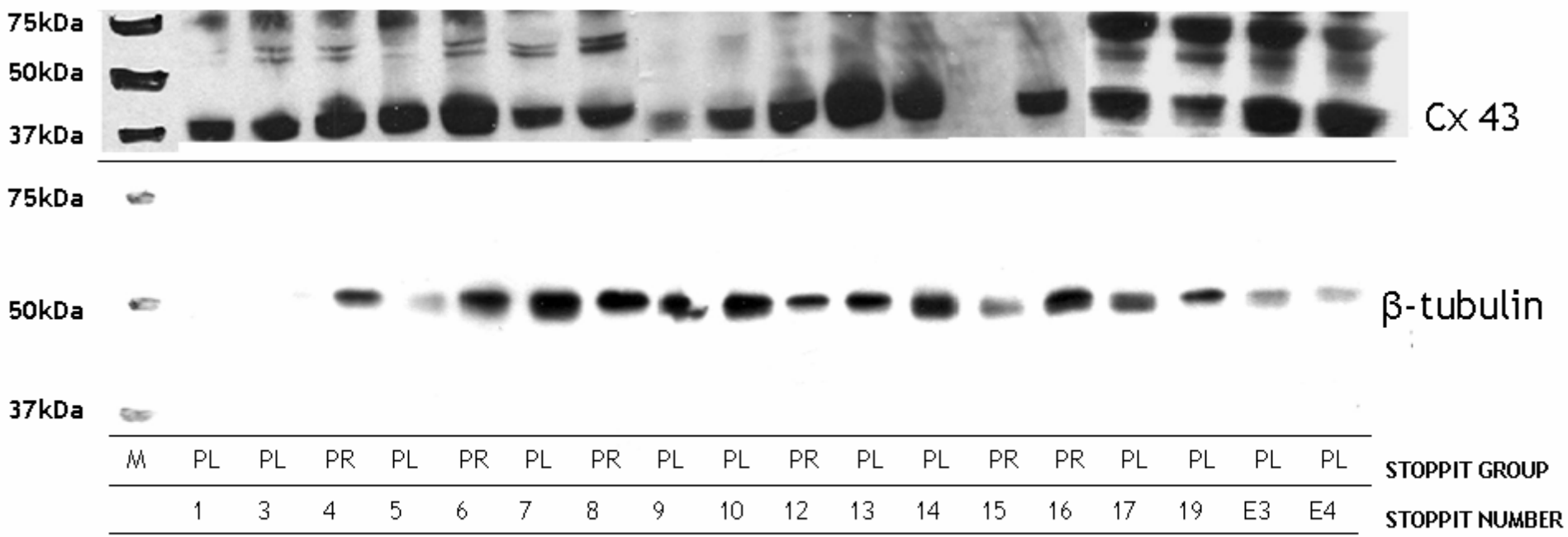

Figure 114 : Western Blot of connexin 43 and the loading control gene β-tubulin for all 18 STOPPIT myometrial samples. Samples 1 and 3 showed no expression of the housekeeping gene. Sample 15 showed no expression of connexin 43. The STOPPIT group and number given to each sample are indicated below.

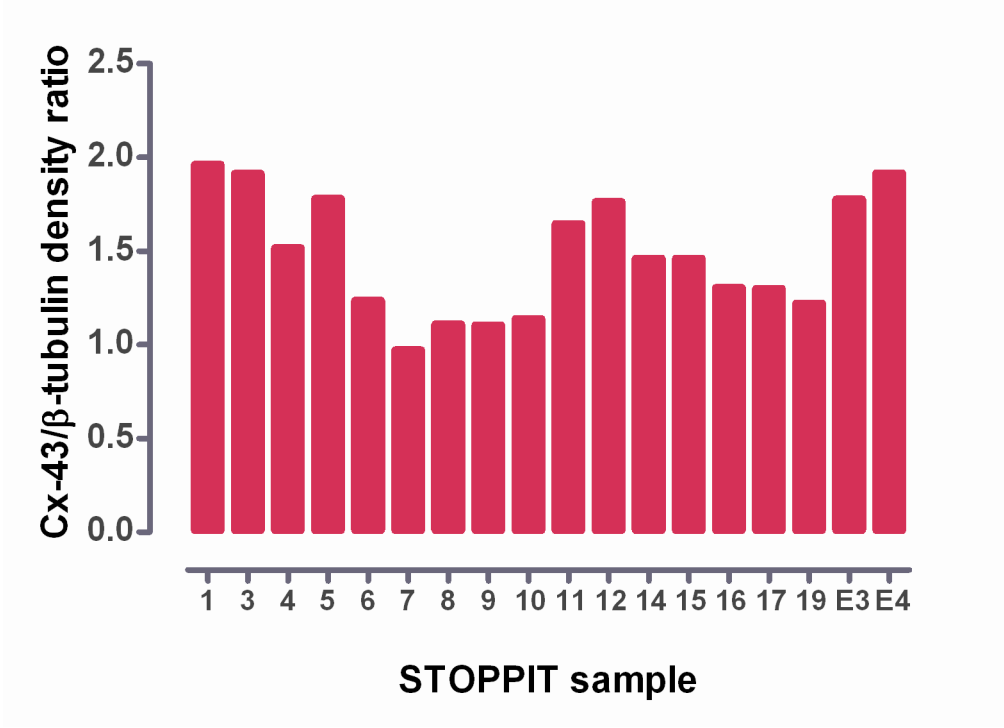

Figure 115 : Western analysis of connexin 43, indicating each STOPPIT myometrial sample relative to the control β-tubulin.

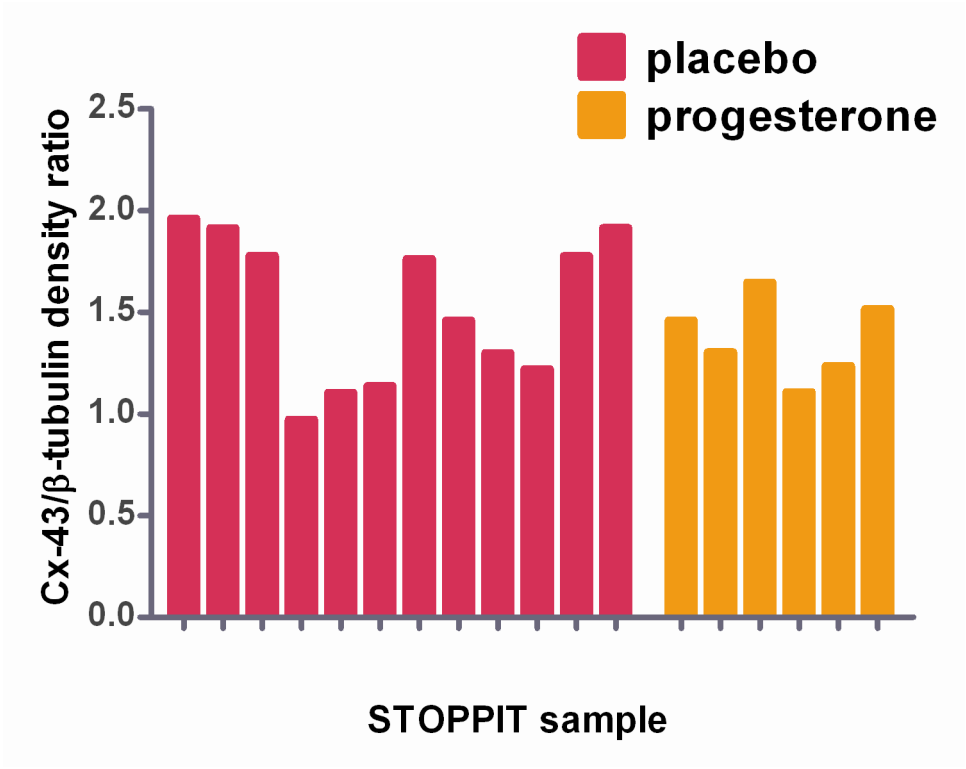

Figure 116 : Western analysis of connexin 43, indicating each STOPPIT myometrial sample and divided into STOPPIT groups. There was no difference in protein analysis between the placebo and progesterone group, $p>0.05$ (t-test).

Discussion

The impact of prolonged maternal progesterone on human myometrium: The myometrial effects of prolonged *in vivo* treatment with progesterone.

In this chapter we aimed to look at the myometrial effects of progesterone versus placebo *in vivo*. In addition to our *in vitro* data (chapter 4) our cohort of STOPPIT patients allowed us to examine possible *in vivo* effects.

New evidence published after this study was commenced suggests that the evidence to support the prevention of preterm delivery in twins is not as robust as in high risk singleton pregnancies (114). Our study however, enabled a valuable opportunity to obtain myometrial tissue from participating patients on either a progesterone treatment and with placebo controls, all randomised and blinded. We hoped to use this as a marker of progesterone treatment on myometrium *in vivo*.

Within the clinical trial, participants were randomised either to daily progesterone gel (90mg) (Crinone®) or placebo gel both administered vaginally by the participant and starting at 24 weeks + 0 days gestation. Patients were required to administer the gel at the same time each day and also asked to keep a diary of any symptoms or added information. They generally attended the antenatal clinic between every two to four weeks and were seen by a designated STOPPIT midwife. They were also contacted by telephone at regular intervals to ensure compliance and general wellbeing.

The mode of delivery in women participating in STOPPIT was left to the pregnant woman and her obstetrician providing her care. There is however a higher rate of caesarean section in twins, largely due to malpresentations or intrapartum complications (196). Within the STOPPIT cohort the caesarean section rate was approximately 67% at the start of substudy recruitment and of these sections around 70% were semi-elective and pre-labour. Within the study centres an average of 4 participants per month were randomised in Glasgow and 3 in Edinburgh. We calculated a loss rate of around 15 % (patients unwilling to donate a biopsy, having a vaginal delivery), but overall thought we would be

able to obtain fresh biopsies from around 45 women over the 17 month recruitment period. We also hoped that around 50% of these women would be on placebo/progesterone or within 2 weeks of stopping. We therefore assumed a sample size of approximately 20 in placebo and progesterone groups, the study therefore had a 95% power to show a difference in 1.2 standard deviations between the groups.

Unfortunately, we were unable to achieve this number of patients within our cohort, initially due to the practical difficulties of transporting samples in an adequate time frame from Edinburgh, to the place of all experimental equipment in Glasgow. There was also a higher loss rate and reduction in the recruitment numbers within Glasgow itself. As we were blinded at the time of sampling we were unable to determine the amount within each group we had obtained. We therefore elected to collect all consented patients within STOPPIT substudy whether they had an elective or emergency intrapartum caesarean section.

The gestational stage at which the myometrial biopsies were collected was generally at an interval greater than two weeks after finishing participating within the clinical trial i.e. a break of 14 days or more from the additional placebo or progesterone. As an unpredictable variable within this cohort it is possible therefore that some myometrial effects of supplementary progesterone have been lost. Any non-genomic action of progesterone would take minutes as opposed to hours (48) and therefore these effects are unlikely to have persisted. Genomic actions may well persist longer and therefore changes hypothesised in gene expression may still be evident within the samples. This time delay may well have implications for our data interpretation comparing the placebo and progesterone group.

Progesterone levels at term are already high, with higher levels detectable within the myometrium than maternal plasma (197). Early studies by Csapo indicated a potential progesterone deficiency in preterm labour (198) but these findings have not been reproduced and the mechanism of action for progesterone to prevent preterm labour remains unknown. It is not fully understood either how a supplementary dose of progesterone may prevent preterm labour when placenta and myometrial progesterone levels are already

high. Levels within the myometrium on supplementary progesterone may well be higher than serum levels due to uterine first pass metabolism and studies have shown after vaginal administration of progesterone, uterine tissue concentration has been found to exceed by more than 10-fold the levels achieved by systemic administration (199). This uterine first-pass effect may lead to myometrial changes if prolonged as in the STOPPIT cohort.

The numbers achieved within this study are small. This therefore, may underpower our study and we should interpret our results with caution. To carry out human *in vivo* studies is a difficult undertaking and therefore important to carry out when appropriate. Many *in vivo* studies prior to this have supported the idea that progesterone can influence the myometrium. Pinto et al found that large doses of progesterone (100- 200 mg bolus IV, almost twice as much as the placenta produces in 24 hours at term) given to women in labour at term was able to inhibit the frequency and intensity of uterine contractions. There cohort was also small but they were able to clearly demonstrate a non-genomic effect of progesterone *in vivo*. Our cohort of women is also small, but we have been able to demonstrate some changes within our myometrium between placebo and progesterone groups. We will discuss these in the following section.

This study demonstrated that the predominant isoform of NOS within our human myometrial samples was eNOS. This is consistent with other data which localised eNOS to myometrial smooth muscle cells (185) . Norman et al also demonstrated an up regulation of eNOS in pregnant myometrium versus non pregnant suggesting that this may play a role in myometrial quiescence. There was however, no change in NOS activity or expression comparing uterine tissues taken immediately before and after the onset of labour suggesting no regulatory involvement in the acute onset of parturition (200). Bartlett et al again showed the detection of mRNA for all three NOS isoforms however a high cycle number implied a low copy number. They went on to demonstrate that eNOS, iNOS and bNOS proteins were not expressed at detectable levels in myocytes of human myometrium throughout pregnancy (201). They concluded therefore that NO produced by the myometrium played an insignificant role in the regulation of uterine smooth muscle tone during pregnancy. This opposes data that endogenous nitric oxide plays a role in the control of myometrial contractility during pregnancy.

In rat uterine tissue, cells obtained from progesterone-primed uterine tissue presented an increase in the nitrite concentration concomitant with a decrease in the PGE production (202). NO production was compared in the rat uterus and cervix in another group and they observed that treatment with the antiprogestin onapristone suppressed uterine NO production while continuous administration of progesterone from day 19 of pregnancy increased NO production. We demonstrated no difference in MRNA expression for all iNOS and bNOS isoforms within the placebo versus progesterone groups. We observed within the elective caesarean group that there was less eNOS mRNA expression in the progesterone versus placebo group. Norman et al previously demonstrated that there was an elevated level of eNOS protein in preterm non-labouring samples compared with term non-labouring and non-pregnant tissue. Our cohort of women within the placebo group has a wider range of gestational ages and a greater proportion of this group were sampled from women at a more preterm gestation <250 days, suggesting this higher eNOS expression within the placebo group could be simply a gestational effect. If progesterone promoted myometrial relaxation by inducing eNOS production then an up regulation would have been predicted rather than our observation of the opposite. Within the myometrium treated with progesterone and as we have observed down regulation of eNOS a potential uterine relaxant we also see down regulation of mRNA expression for contraction associated proteins, connexin 26 and 43. We propose that a compensatory mechanism may well occur in myometrial tissue with supplementary progesterone reducing expression of contraction associated proteins fundamental to the mechanism of labour.

We observed no changes in CGRP or its receptor within the two groups. Dong et al concluded from their work that increased CGRP-receptors in myometrium, and resulting enhanced myometrial sensitivity to CGRP, may play a role in maintaining human myometrium in a quiescent state during pregnancy. Both Western blotting and mRNA analysis showed that CGRP-receptors are present in human myometrium, and that the expression of these receptors is increased during pregnancy and decreased during term labour (91). They also demonstrated that *in vitro* progesterone increased CGRP receptor expression in myometrial explants. Other groups have studied animal models with ovariectomised mice. They showed that levels of inhibition exerted by CGRP during the various stages of the estrous cycle and in response to steroid hormone

treatment correlated with the protein levels of CGRP-Receptor. The myometrial mRNA levels did not change significantly during the mouse estrous cycle or in response to hormone (progesterone) treatment, indicating that the regulation of CGRP-Receptor protein does not occur at the transcriptional level (203). In relation to labour itself another group demonstrated that parturition had no impact on maternal serum CGRP levels, and no significant correlation existed between plasma CGRP levels and cervical dilatation during labour (204). If levels of CGRP therefore are not implicated in the parturition process then this may explain why we observed no difference between our groups within both emergency and elective caesarean section cohort. This indicates that CGRP may not play a central role in either labour itself or the prevention of preterm labour.

We observed no difference within the groups for prostaglandin receptor subtype EP2. When analysis was restricted to non labouring samples there was a lower mRNA density within the progesterone group compared to placebo. Previously in animal models progesterone has been shown to increase EP2 mRNA expression. They concluded that the expression of relaxant EP2 receptors in the rat uterus increases with pregnancy and decreases with labour, and appeared to be progesterone dependent (133). Other data has shown that in humans within the upper uterine segment, EP2 was higher in term labour samples and lower in preterm labour samples compared to term non-labour (191). They suggest this does not fit with the theory of a contractile phenotype within the upper segment at term. These observations may indicate that the EP2 perhaps plays a role in controlling the timing of labour-onset possibly by maintaining quiescence until the excitatory influences become more dominant allowing a shift to a contractile phenotype. Again, our data also suggest a compensatory mechanism may exist or indeed a balance between uterine contractions promoters and uterine relaxants is present and in which progesterone may have a central role .Our data used lower segment samples and is not a comparison of labouring versus non-labouring however as a uterine relaxant the decrease within the progesterone group does not reflect an expected progesterone enhanced EP2 presence within myometrium. Again, this may be due to small sample numbers or indeed a gestational effect with a larger number of preterm samples within the placebo cohort.

Within our results targeting the pro-inflammatory cytokines we observed no differences within our mRNA gene data for IL1β, IL6 and IL8. Our immunohistochemistry data supports this and although we observed expression of pro- inflammatory cytokines within our myometrial samples both placebo and progesterone groups were stained in a similar manner. The CD45, common leucocyte antigen demonstrated that again, there were little differences within the two groups. The inflammatory process involved in parturition appears to involve complex signalling pathways and interactions between cervix and myometrium and there is gathering evidence to suggest that labour itself is an inflammatory process. Osman et al showed that parturition was associated with a significant increase in IL-1β, IL-6 and IL-8 mRNA expression in cervix and myometrium and their histological analysis demonstrated that leukocytes (predominantly neutrophils and macrophages) infiltrated the uterine cervix coincident with the onset of labour (24). There is also debate suggesting that term labour and preterm labour are not identical processes and may be triggered following different pathways or mechanisms. Such studies have shown within lower segment myometrium , the levels of IL-1β, IL-6 and IL-8 mRNA expression were significantly higher in term labour samples compared with preterm labour samples (205). Our data focuses on myometrium and then in addition the effect of progesterone. Progesterone has been demonstrated to suppress the immune response in early pregnancy (181). Progesterone has also been shown to decrease the production of IL-6 after LPS stimulation without altering the production of TNF-α or IL-10 in fetoplacental arteries using a placental explant model (206). We observed no such change or difference in our groups using progesterone specifically. Elovitz et al demonstrated that the progestogenic agent, medroxyprogesterone acetate, reduced COX-2, IL-1β and TNF-α mRNA expression in the myometrium of pregnant mice pretreated with LPS. These studies demonstrated that progestational agents decreased the incidence of inflammation-induced preterm parturition and medroxyprogesterone completely prevented preterm birth compared to progesterone which decreased the overall rate of preterm birth (207). They suggest that medroxyprogesterone may well have this additional immunosuppressive action due to its glucocorticoid receptor binding ability as well as progesterone receptor (208).

It is also difficult to determine within human studies antenatally which patients with preterm labour have pregnancies which are complicated by intrauterine

inflammation. Our cohort specifically has less patients that were preterm (before 250 days gestation) within the progesterone group and very few in labour making it difficult to make conclusions due to small numbers. Our decision to study the effects of progesterone *in vivo* and *in vitro*, rather than medroxyprogesterone acetate, was to specifically evaluate an agent that is currently used in clinical practice for the prevention of preterm birth. The type of progesterone may well have a specific interaction with the inflammatory cascade and this may explain why we observe little difference with progesterone compared with groups showing reduction in pro-inflammatory cytokines with other progestins.

We observed a lower expression in the progesterone treated group with the contraction associated proteins mRNA for both connexin 26 and 43 within the elective caesarean section cohort and connexin 26 within the total STOPPIT group. This is mirrored within our myometrial cell culture work where we also observed a lower expression of connexin 43 within our in vitro data when treated with progesterone (chapter 4). One suggestion is that this principal genomic mechanism by which progesterone represses myometrial contractility is by modulating the expression of genes encoding contraction associated proteins such as connexin 43 (48). Within animal models using rats, Lye et al demonstrated that connexin 43 levels were at there highest during delivery and were closely associated with the protein levels and appearance of gap junctions. The connexin 43 levels were positively regulated by oestrogen and negatively by progesterone during pregnancy (209, 210). There is evidence to support that in humans there is a correlation between increased cervical dilation or increased frequency of uterine contractions and increased area of gap junctions within the myometrium (86). The progesterone agonist, medroxyprogesterone acetate to treat causes a marked decrease in the levels of connexin 43 mRNA in uterine smooth muscle cultures (211). It is thought that connexin 43 is synthesised days prior to parturition but accumulates within the cytoplasm until labour when it is transported to the plasma membrane and forms part of the gap junction plaques present at the cell surface (89).

The assembly of gap junction protein into functional gap junction plaques is the last point in a stepwise process that begins with oestrogen-dependent expression of the connexin 43 gene and continues with synthesis of connexin 43 in the rough

endoplasmic reticulum and transport to the Golgi, followed by its trafficking to the plasma membrane and its assembly into functional gap junctions. Hendrix et al found that the trafficking of myometrial connexin 43 from the Golgi and assembly into gap junctions at the plasma membrane was suppressed in progesterone treated rats but suggested that it may be the trafficking that of to the plasma membrane which is important rather than its synthesis (90).It may well be the ratio of oestrogen to progesterone that plays a central role regulating gap junction formation rather than progesterone levels alone in human myometrium (212). Within our samples, although we demonstrate a lower expression of connexin 43 in the progesterone group we observed no detectable difference in the protein levels. If myometrial samples had been collected a later time point this may have shown a reduction in connexin 43 protein synthesis. The samples however were all collected at caesarean section and therefore at one single time point, which may not have allowed for the reduction in protein synthesis within the endoplasmic reticulum.

Although we see a lower expression of the connexins, contraction associated protein mRNA there is also lower expression of EP2 and eNOS mRNA. This potentially reflects a gestational effect however could suggest some compensatory recovery after progesterone administration. We saw no functional changes with the contractility data (chapter 2) and hypothesised that this may indicate loss of instant non-genomic effects of progesterone after withdrawal of administration. The longer genomic effects which may be sustained with loss of s could explain one mechanism of action of progesterone in the prevention of preterm labour. It is also possible that this does not demonstrate a long-term detrimental impact on myometrial function with down regulation of these endogenous inhibitors alongside the down regulation of contraction associated proteins, s. This may suggest a functional and compensatory recovery occurs after progesterone administration.

The samples importantly were all obtained from twin pregnancies and uterine stretch is thought to have a key role in the onset of preterm labour. Mechanical stretch of the myometrium has biological consequences and may alter gap junction expression and responsiveness to inflammatory stimuli. Multiple pregnancy compared to singleton pregnancies however showed no difference in

expression of prostaglandin E_2 receptors, or gap junction proteins within myometrium (213).

Events that take place prior to and that are involved in parturition will interact with steroid hormones. If the functional progesterone withdrawal is central to a normal labour process then it may well play a part in preterm labour. The causes of preterm labour, whether inflammatory or idiopathic, may not have identical processes or sequence of events triggering parturition. In multiple pregnancies the mechanism may differ due to stretch mechanisms. Parturition will therefore occur when all the stimulatory influences reach a threshold which can overcome the uterine quiescence maintained by progesterone. It is likely therefore that contraction associated proteins are one such mechanism necessary for labour. There is good evidence to show that progesterone has an inhibitory effect on connexin 43, a major component of myometrial gap junctions. Gap junction formation is essential for developing and maintaining synchronous contractions within the uterus, a fundamental process within parturition.

The STOPPIT trial showed that progesterone, administered vaginally, does not prevent preterm birth in women with twin pregnancy. However, different pathophysiological mechanisms may account for the apparent difference in efficacy of progesterone in high risk singleton and twin pregnancy. The aetiology of preterm labour in twin pregnancy and spontaneous preterm birth is highly unlikely to have identical mechanisms of action. Although risk factors such as inflammatory processes, uterine overdistension or cervical disorders may have different pathological initiating factors, it is possible that a degree of overlap occurs once parturition processes have commenced. Factors such as increase in pro-inflammatory cytokines or gap junction proteins may be common to all parturition processes despite their initiating triggers. This may therefore explain why, although progesterone appears not to prevent preterm labour in twins we can still identify modifying mechanisms of action such as a reduction in the expression of connexin 43. Additional information on whether these changes are reproducible within singleton pregnancies and patients on 17OHPC as opposed to vaginal progesterone will confirm or refute this hypothesis.

Progesterone clearly has both non-genomic and genomic properties and maintains pregnancy and promotes relaxation through a combination of these.

More evidence is required to confirm our findings and further sampling of patients participating in clinical trials of progesterone to prevent preterm labour would be of extreme benefit. We would urge research groups carrying out trials of progesterone to prevent preterm labour to further investigate their study population's myometrium, membranes and placenta where possible. As evidence builds around progesterone and its mechanism of action, our understanding of appropriate clinical regimes will be maximised.

CHAPTER 6

Final discussion and future work

Conclusions

There are evidently myometrial effects of progesterone and these appear to be both non genomic and genomic. The purpose of this thesis was to investigate the role of progesterone on human myometrium both *in vitro* and *in vivo*.

In selecting pregnant patients both undergoing routine elective caesarean section and then within the STOPPIT study on progesterone or placebo treatment we had a significant number of myometrial biopsies on which to test our hypotheses. In view of the limitations within animal studies due to fundamental differences in our reproductive pathways our focus on human myometrium is an important one.

We hypothesised that progesterone has a direct inhibitory effect on spontaneous myometrial contractility, induces increased sensitivity to tocolytic agents and decreases sensitivity to contractile agonists such as oxytocin. These data were presented in chapter 3 of this thesis.

We found that progesterone albeit at high concentrations, exerted consistent, rapid and sustained inhibition of the amplitude of spontaneous myometrial contractions *in vitro* however, this affect was not achieved through the principal potassium channels. In contrast, 17OHPC did not exert any inhibitory effect on myometrial activity *in vitro*. We found, at the selected pharmacological doses used *in vitro* that progesterone did not increase sensitivity to tocolytic agent's nifedipine, ritodrine or levcromakalim with respect to spontaneous contractions. There was no decrease in sensitivity to the uterotonins oxytocin. Lastly, from our STOPPIT patient cohort we demonstrated no difference between the progesterone and placebo groups in either spontaneous contractility, response to tocolytics as above or response to oxytocin. One main conclusion of this arm of the study is that *in vivo* progesterone therapy to prevent pre-term labour does not appear to modify contractility *ex vivo*.

We have suggested that these differences between our *in vivo* and *in vitro* data may be due to the non genomic actions of progesterone requiring active progesterone therapy. This was lacking within the STOPPIT progesterone cohort. Whether progesterone treatment *in vivo* would manifest changed in contractility *ex vivo* if examined during the treatment remains to be determined.

The mechanism of action of progesterone both in inhibiting myometrial contractility and in preventing preterm birth therefore continues to warrant further investigation.

We then hypothesised that progesterone has inhibitory effects on endogenous uterine stimulants, stimulatory effects on endogenous uterine relaxants, induces upregulation of endogenous receptors that inhibit uterine contractions and inhibits contraction associated proteins. These data regarding the myometrial effects of progesterone *in vitro* were presented in chapter 4.

We demonstrated that administration of progesterone but not 17OHPC for up to 24 hours *in vitro* does not appear to modify uterine stimulants such as cytokines, COX-2 or endogenous uterine relaxants such as nitric oxide synthetases and prostaglandin dehydrogenase. Progesterone but not 17OHPC inhibited production of gap junction component connexin 43, an important contraction associated protein that is widely implicated in the parturition process. This modification of contraction associated protein is in agreement with other literature presented on human myometrial data *in vitro* (4) .

Lastly we hypothesised that progesterone *in vivo* has inhibitory effects on endogenous uterine stimulants, stimulatory effects on endogenous uterine relaxants, induces upregulation of endogenous receptors that inhibit uterine contractions and inhibits contraction associated proteins. These data are presented in chapter 5.

The STOPPIT trial concluded that progesterone, administered vaginally, does not prevent preterm birth in women with twin pregnancy. The STOPPIT cohort of patients we identified however, were actively receiving either progesterone or placebo between 24 and 34 weeks gestation on a daily basis. We used these patients as a potential example of the myometrial effects of progesterone *in vivo* with a placebo treated control group. Despite the negative findings of the trial with respect to preterm labour, these patients still may have displayed changes of the myometrium with progesterone administration. Since data are extremely limited on the in vivo effects of progesterone in humans we feel our data are an important addition. Although there are limitations within our data such as time delay between last progesterone dose and delivery and small numbers we acknowledge this. Nevertheless, this was a valid way of attempting

to further understand and ascertain myometrial differences from *in vivo* progesterone therapy. The data in chapter 5 highlights some important areas which are mirrored within the *in vitro* data. Prolonged maternal administration of progesterone appears to inhibit expression of gap junction components 26 and connexin 43 in myometrium. Connexin 43 importantly, was also modified *in vitro* within the progesterone treated arm. Uterine contractile inhibitors eNOS and EP2 were also unexpectedly, down regulated within the progesterone group. The s, as we have previously stated play an essential role in regulating synchronous myometrial contractions. If progesterone has been of benefit in those at risk of preterm labour with a history of spontaneous preterm birth, it is possible therefore that this is by stopping the development of these synchronous contractions whilst on progesterone therapy. The mechanism of preterm birth in twin pregnancy may be more related to stretch and pressure mechanisms which potentially modify prostaglandin synthesis via COX-2 (214) or inflammatory mediators such as IL8 (44). However, *ex-vivo* assessment of the functional impact of this does not demonstrate a long-term inhibitory impact on myometrial function with down regulation of endogenous inhibitors such as eNOS and EP2. This suggests a functional and compensatory recovery occurs after progesterone administration.

It is also important to understand the background to the functional progesterone withdrawal whereby the relaxatory actions mediated through PR-B are inhibited via a change in the PR-A to PR-B ratio. If normal myometrium can change and develop into a contractile phenotype towards term mediated via the loss of progesterone quiescence then early inappropriate progesterone withdrawal may also be important in the pathogenesis of preterm labour. This was out with the aims and hypotheses of this thesis however many groups such as Mesiano et al are researching this area in greater detail.

Progesterone clearly has both non-genomic and genomic properties as demonstrated in this thesis and throughout the literature. Progesterone maintains pregnancy and promotes relaxation through a combination of these however its mechanism of action in the prevention of preterm labour is still unclear. We would suggest that modification of gap junction components such as connexin 43 may be one of the main myometrial effects of progesterone in humans.

As evidence builds around progesterone and its mechanism of action, our understanding of appropriate clinical regimes in the prevention of preterm labour will be maximised. Clinical trials so far have shown that progesterone may be of benefit in women with a history of spontaneous preterm birth and, or a short cervix. It does not appear to be of benefit in twins (3) . This highlights that the underlying mechanisms of preterm birth have a multifactorial aetiology and the mechanism of preterm birth in twins is unlikely to be identical to the mechanism in women with a history of spontaneous preterm birth. The role of progesterone therefore may be limited to a subpopulation within those classified "at risk" of preterm birth.

In summary, this thesis has highlighted that although our understanding of the myometrial effects of progesterone is advancing there is still a lack of data from *in vivo* studies. Progesterone is potentially the most important reproductive hormone, involved at every stage from conceptus to birth. Its effects on the myometrium are only one aspect of the whole parturition process but critical to the timing, co-ordination and physiology of birth.

Future Research

This work represents further insights into the myometrial effects of progesterone. However there are clearly further avenues for future research based upon these findings.

Due to time limitations within this study we were unable to carry out full Western blot protocols to determine the protein analysis of the remaining connexin 26, as well as the other endogenous uterine stimulants and endogenous uterine relaxants. Further immunohistochemistry of the connexin 26 and 43 within the STOPPIT cohort would also be of benefit, providing a more complete analysis of the samples and therefore a more complete analysis of the data.

If progesterone withdrawal and oestrogen activation initiate parturition then it may also be of benefit to examine myometrium *in vitro* cell culture in a concentration-response manner with combinations of these steroid hormones at different ratios.

One of the main findings of our study was the reduction in mRNA expression of gap junction components connexin 26 and 43. Father assessment of these contraction associated proteins within the human myometrium in non-labouring and labouring as well as preterm non-labouring and preterm labouring would be a useful avenue to pursue. There is some evidence that expression of connexin 43 in the human pregnancy myometrium increases with the onset of labour (86). Evidence of this within preterm samples may support the theory that spontaneous preterm labour is related to an early upregulation of myometrial gap junctions. Cells within the vast majority of human tissues communicate directly through these clustered arrays of gap junctions and there is an apparent genetic component with autosomal dominant or recessive mutations of the genes linked to other medical conditions such as hearing loss and a skin diseases (215). There a proposed genetic component of preterm labour and this raises the possibility of one such possible genetic abnormality.

If the connexins are implicated in preterm labour then this raises the possibility of gap junctions and connexins as pharmacological targets. They allowing rapid intercellular communication and synchronisation of coupled cell activities, playing a critical role in many signalling processes, including co-ordinated

cardiac and smooth muscle contractions, neuronal excitability, neurotransmitter release, insulin secretion and epithelial electrolyte transport, No natural toxin or specific inhibitor of the connexins has been identified yet and most agents also affect other ionic channels and receptors. Future research, may further clarify gap junction physiology and the role of myometrial gap junctions as a potential pharmacological target in the prevention of preterm labour.

It is evident that research surrounding progesterone to prevent preterm labour is ongoing and evolving every year. Current trials such as the OPPTMUM study "Does progesterone prophylaxis to prevent preterm labour improve outcome?" which is a UK-based randomised trial to determine if progesterone prophylaxis to prevent preterm labour improves outcome has started recruiting in 2009. It is aimed at women at high risk of preterm labour and uses natural progesterone, 200mg daily from 22 – 34 weeks gestation, compared to placebo as its regime. The question posed is does progesterone improve obstetric outcome by lengthening pregnancy and thus reducing the incidence of preterm delivery before 34 weeks gestation. It also focuses on neonatal morbidity and mortality as well as longer term childhood cognitive function. These research aims are worthy and necessary in building a clearer picture as suggested by Tita et al on progesterone regimes, categories of women who will benefit from progesterone and longer term child outcomes (3). We would also suggest that in addition to our study that all preterm labour progesterone clinical trials consider obtaining funding and ethical approval to look at the myometrial effects of progesterone. National rates of caesarean section are now over 20 % in England and Wales (216). All studies of progesterone *in vivo* are likely to have a number of patients that require caesarean section for clinical reasons and we have shown that sampling the myometrium is relatively simple and easy. With the appropriate consent further examples of this may provide advances in our understanding of the mechanisms of action of progesterone. It would be of benefit to see more conclusive evidence that progesterone modifies the myometrium *in vivo* and not only myometrium but also the placental tissues. It is also possible that as progesterone has been shown to be of benefit in high risk patients with previous preterm labour, changes seen within myometrial gap junction components may be more evident in myometrium from patients in which there is a clinical benefit from progesterone.

The nature of the cascade of events culminating in labour and preterm labour is a complex subject. Therapeutic advances and greater understanding of the mechanism of preterm birth may be possible through human myometrial studies and in particular progesterone and its mechanism of action.

Appendix

Contents

This appendix contains figures referenced in chapter 5. These are scatter plot graphs of STOPPIT data containing gene mRNA expression results against gestational age in pregnancy.

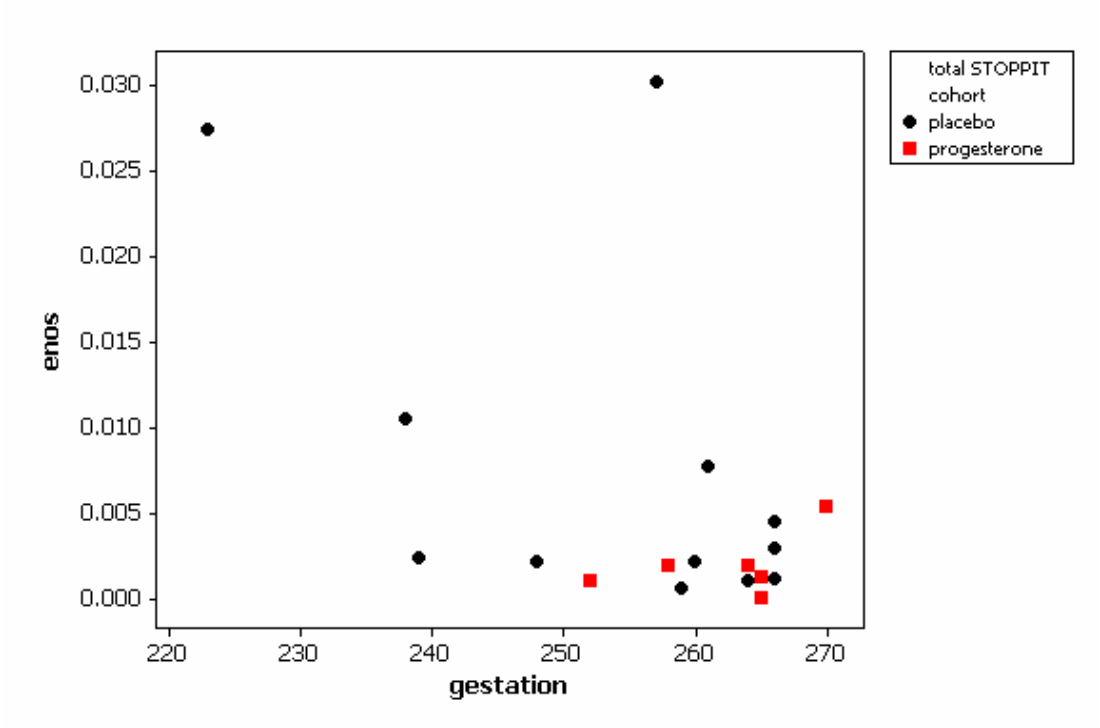

Figure 117: This graph shows enos gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

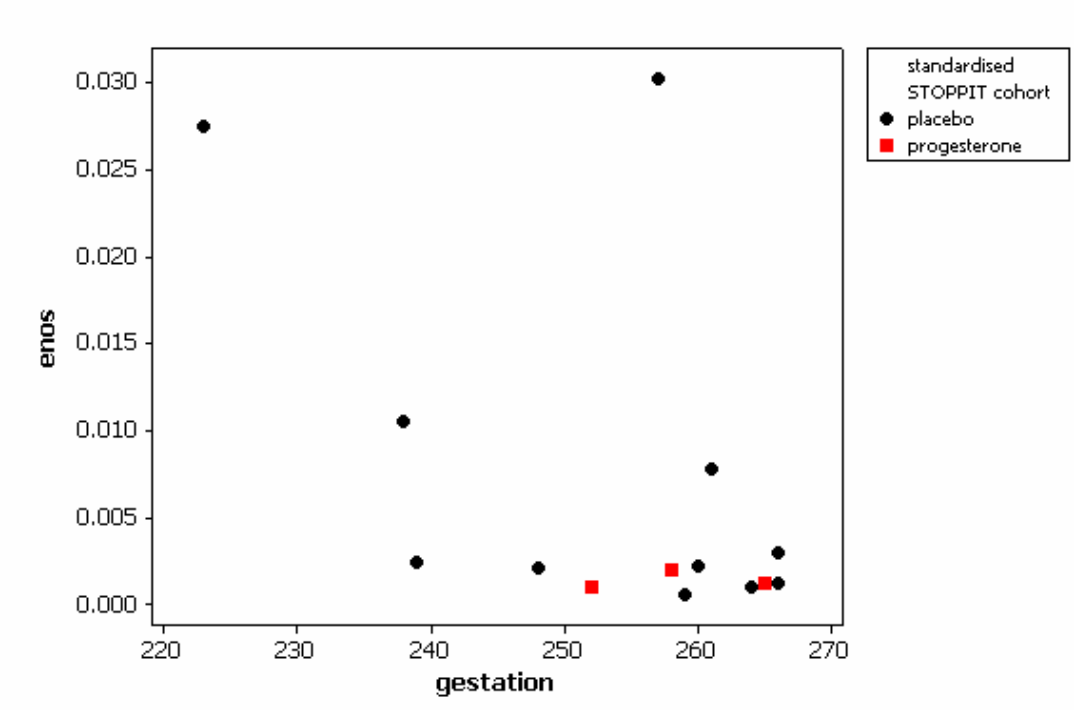

Figure 118 : This graph shows enos gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

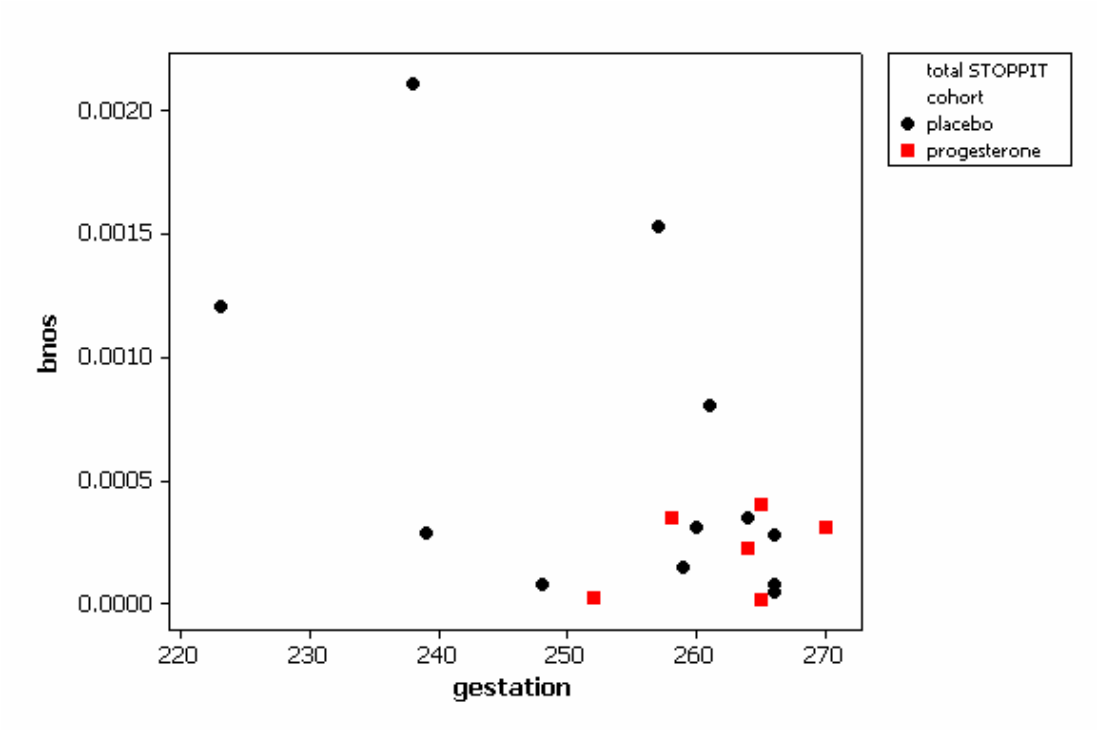

Figure 119 : This graph shows bnos gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

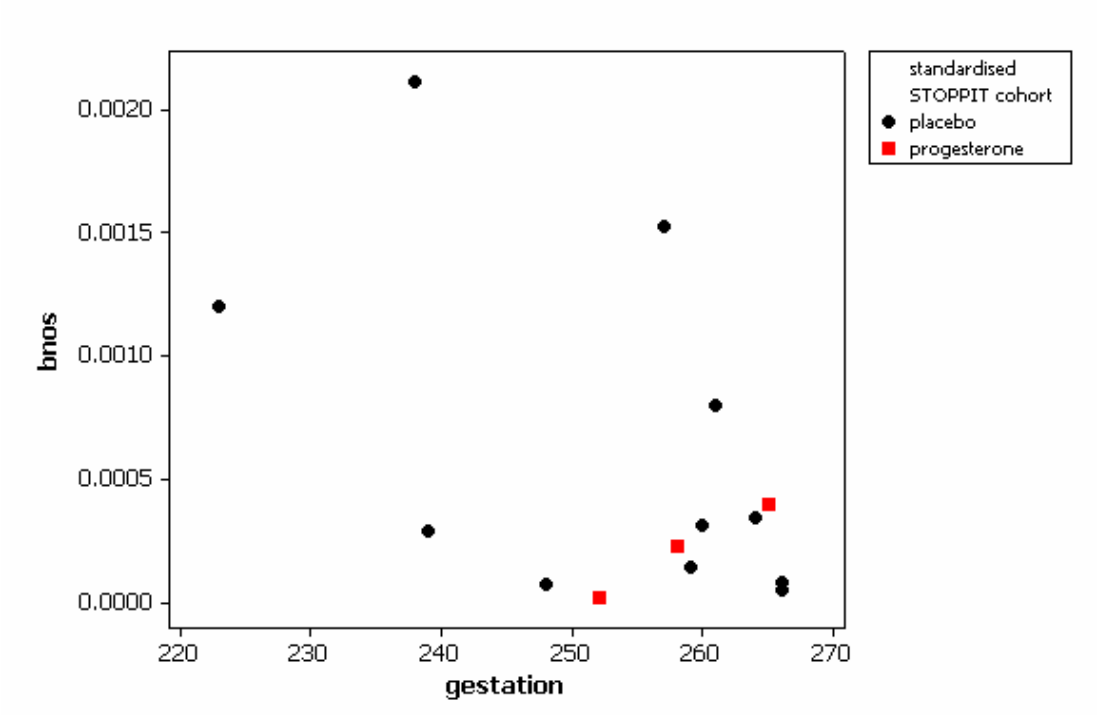

Figure 120 : This graph shows bnos gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

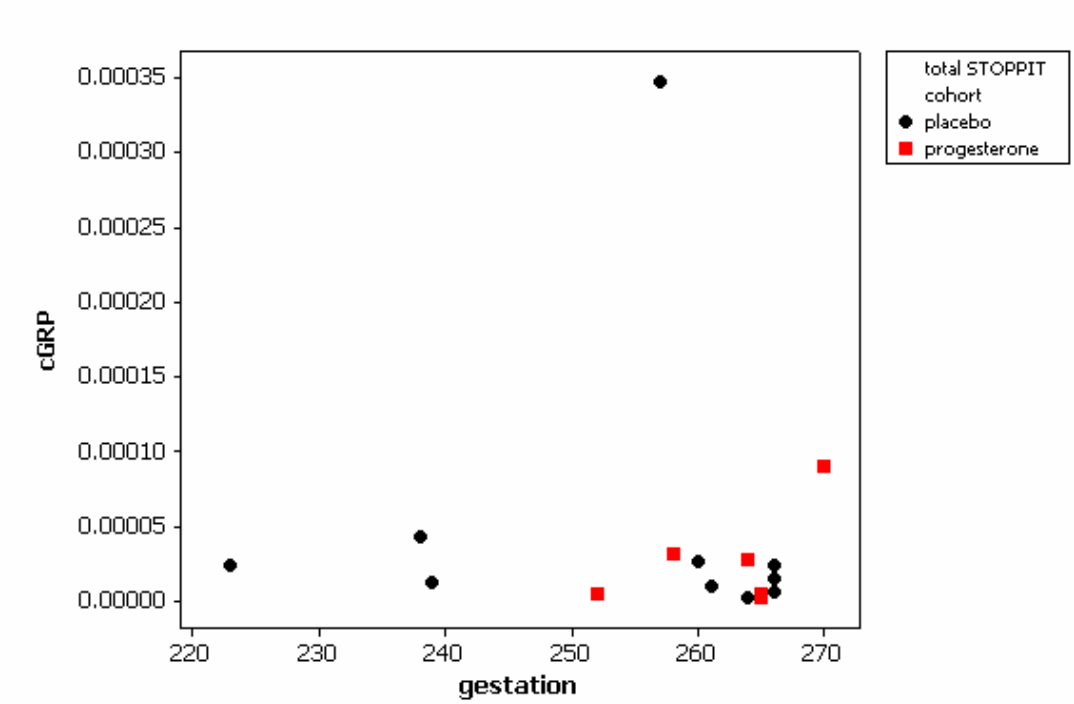

Figure 121 : This graph shows cGRP gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

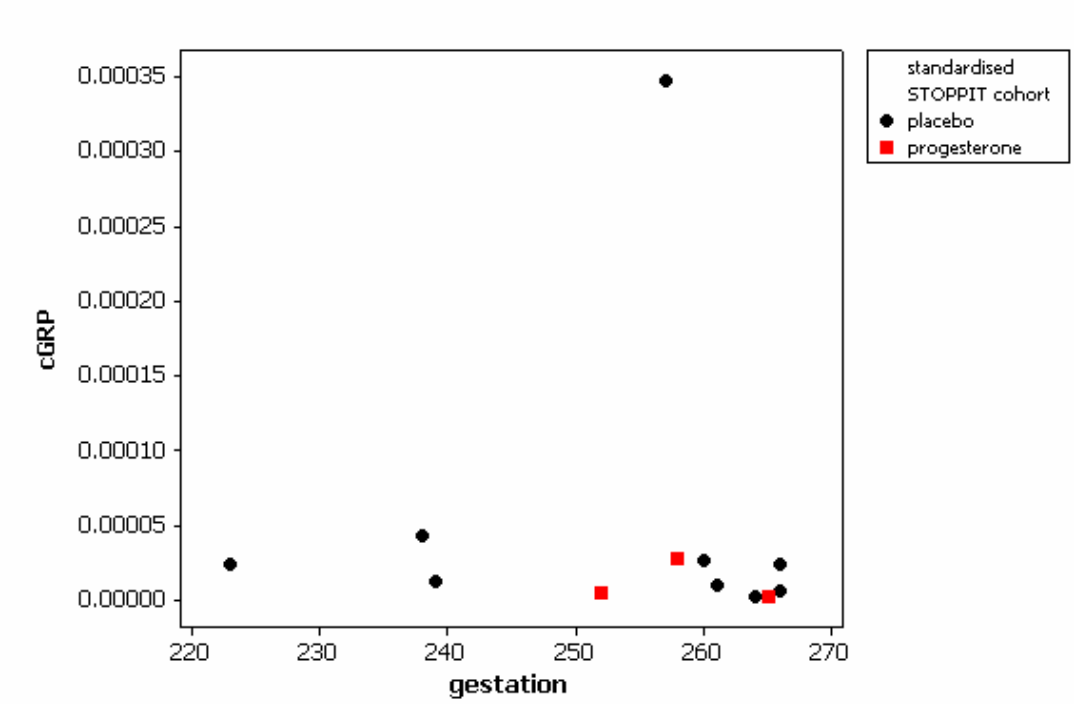

Figure 122 : This graph shows cGRP gene mRNA expression levels, relative to 18S.Satndardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

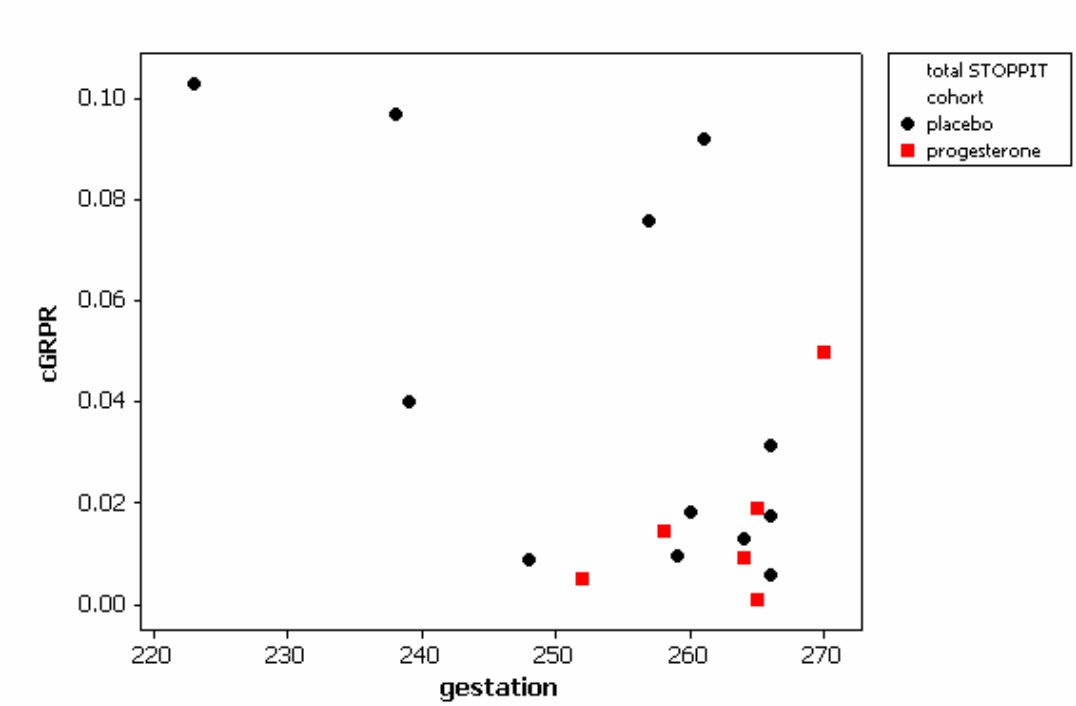

Figure 123 : This graph shows cGRPR gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

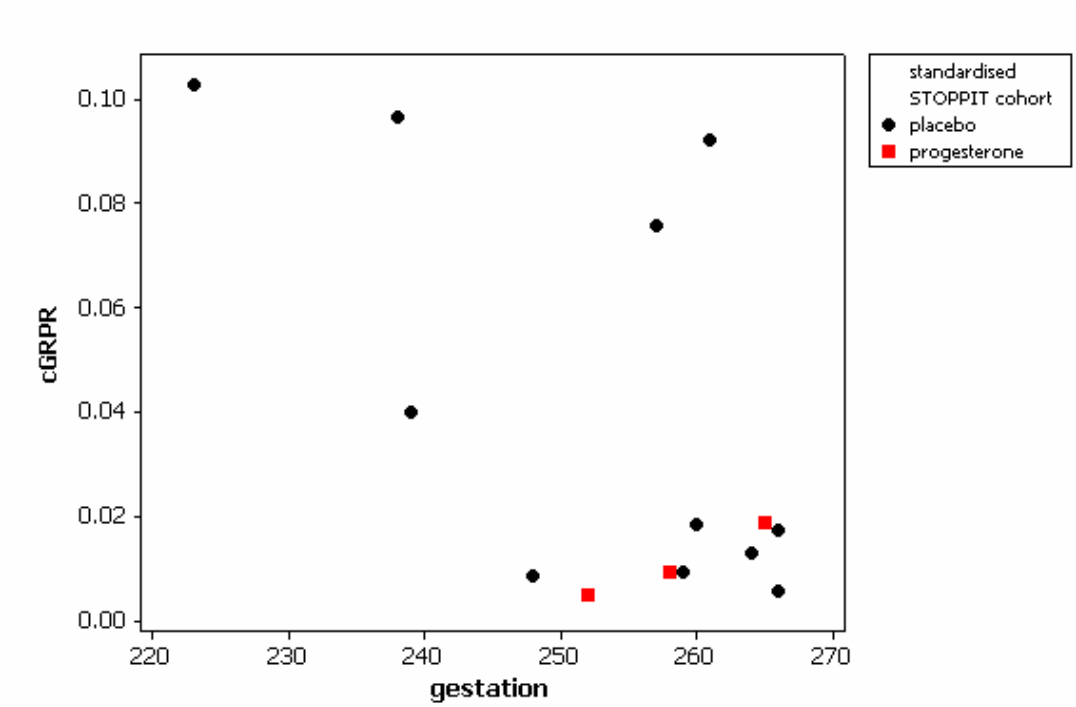

Figure 124 : This graph shows cGRPR gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

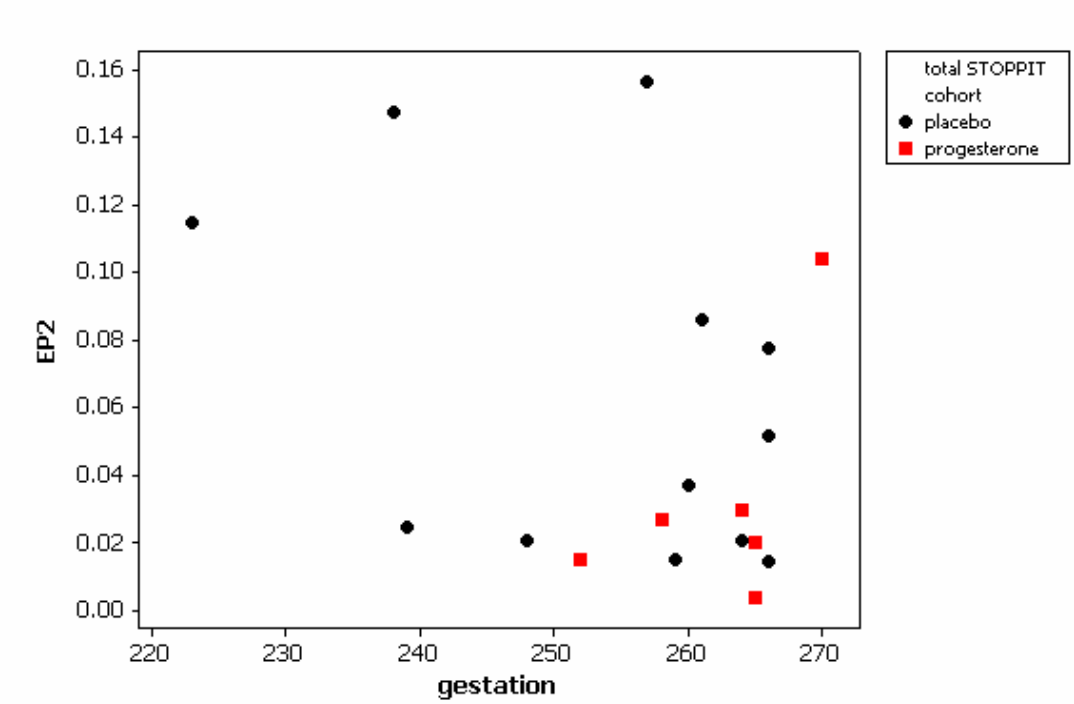

Figure 125 : This graph shows EP2 gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

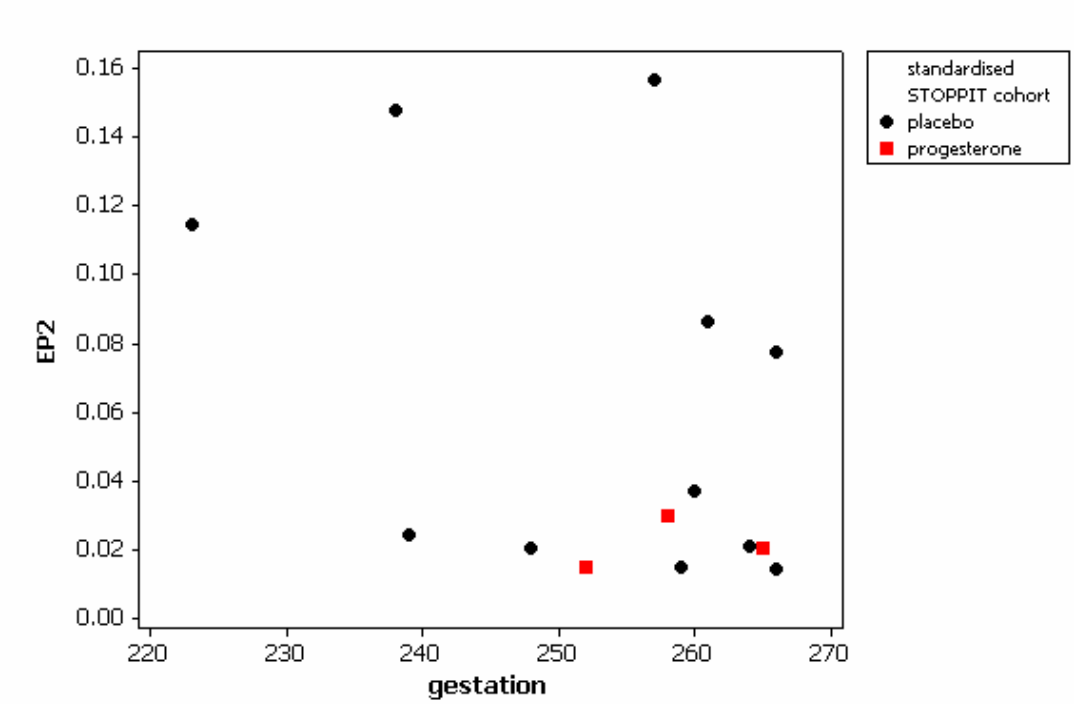

Figure 126 : This graph shows EP2 gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

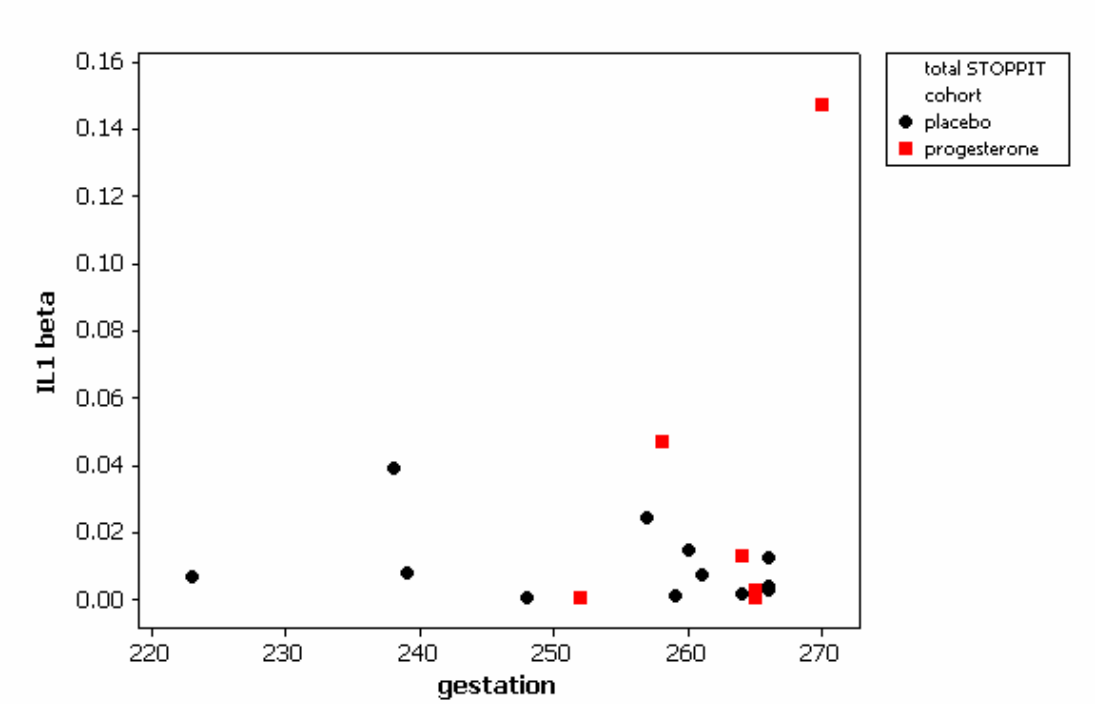

Figure 127 : This graph shows IL1β gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

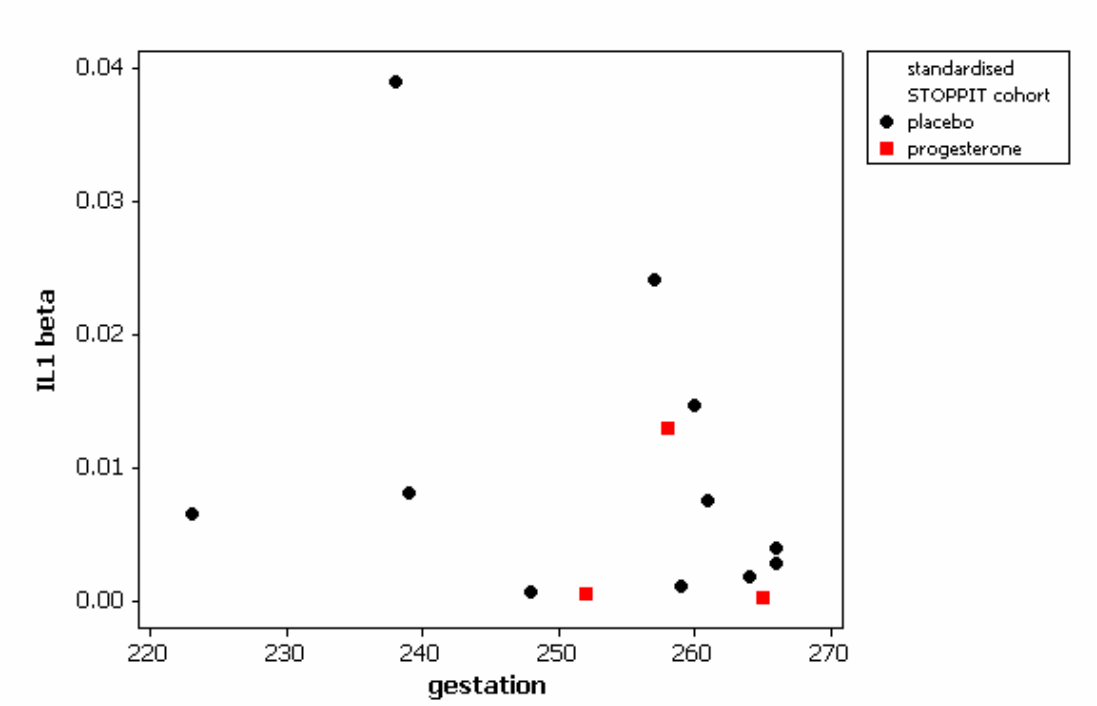

Figure 128 : This graph shows IL1β gene mRNA expression levels, relative to 18S. Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

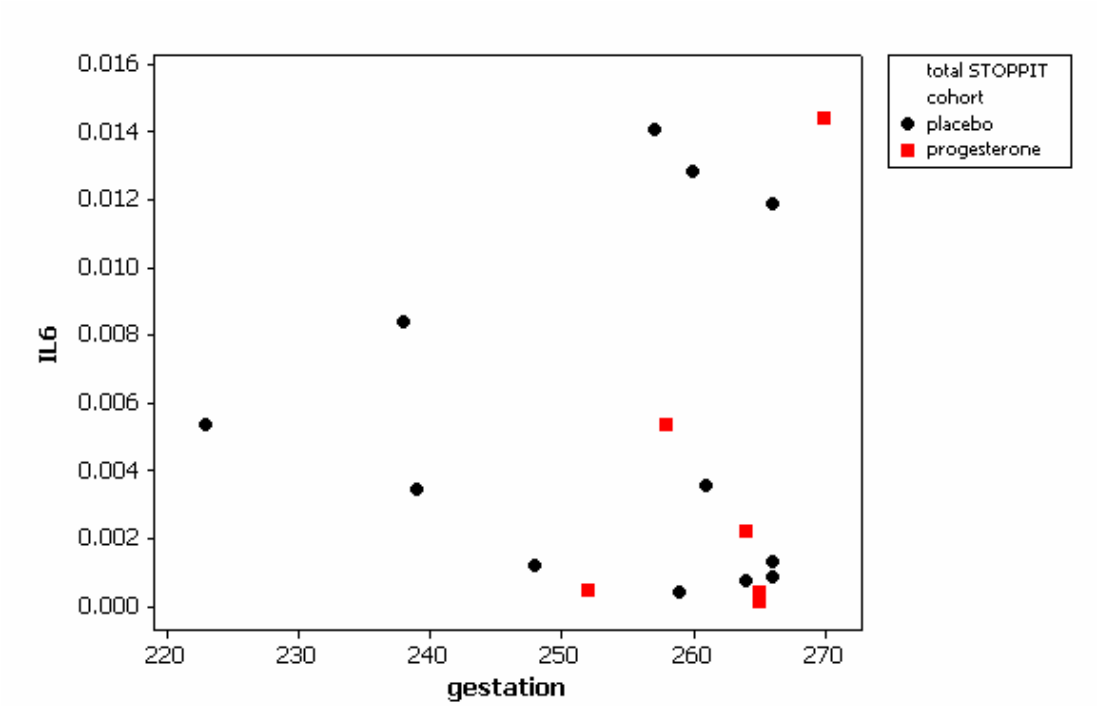

Figure 129 : This graph shows IL6 gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

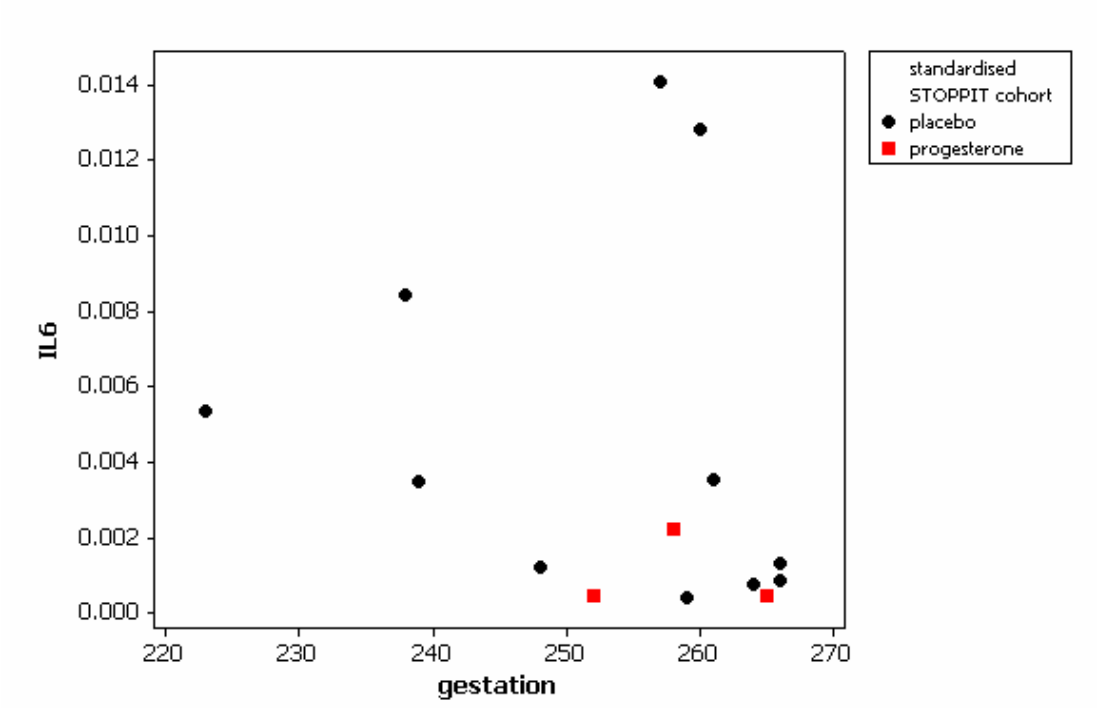

Figure 130 : This graph shows IL6 gene mRNA expression levels, relative to18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect. All progesterone samples were sampled at a gestation >250 days (35+5).

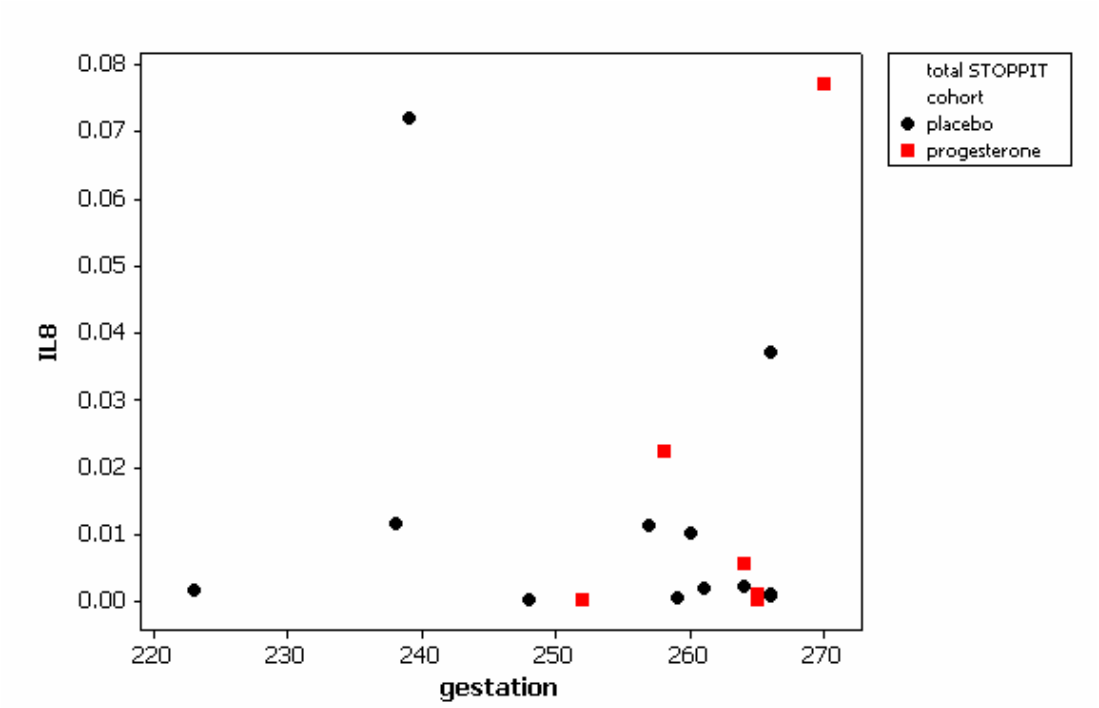

Figure 131 : This graph shows IL8 gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect.

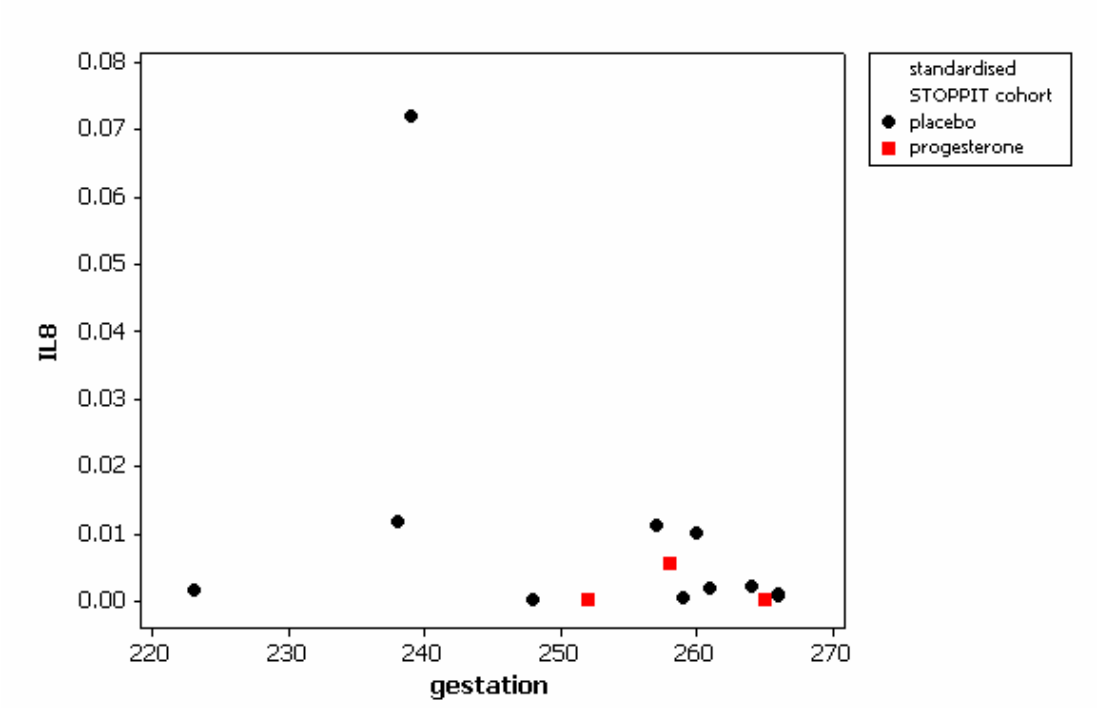

Figure 132 : This graph shows IL8 gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect.

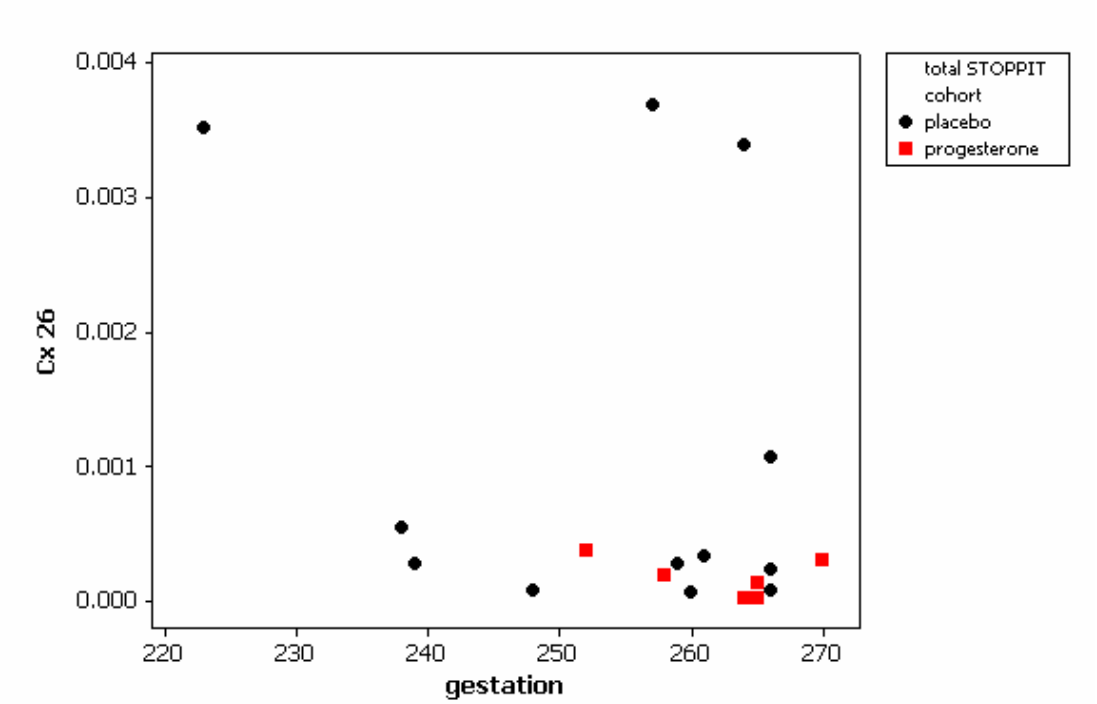

Figure 133 : This graph shows Cx 26 gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect

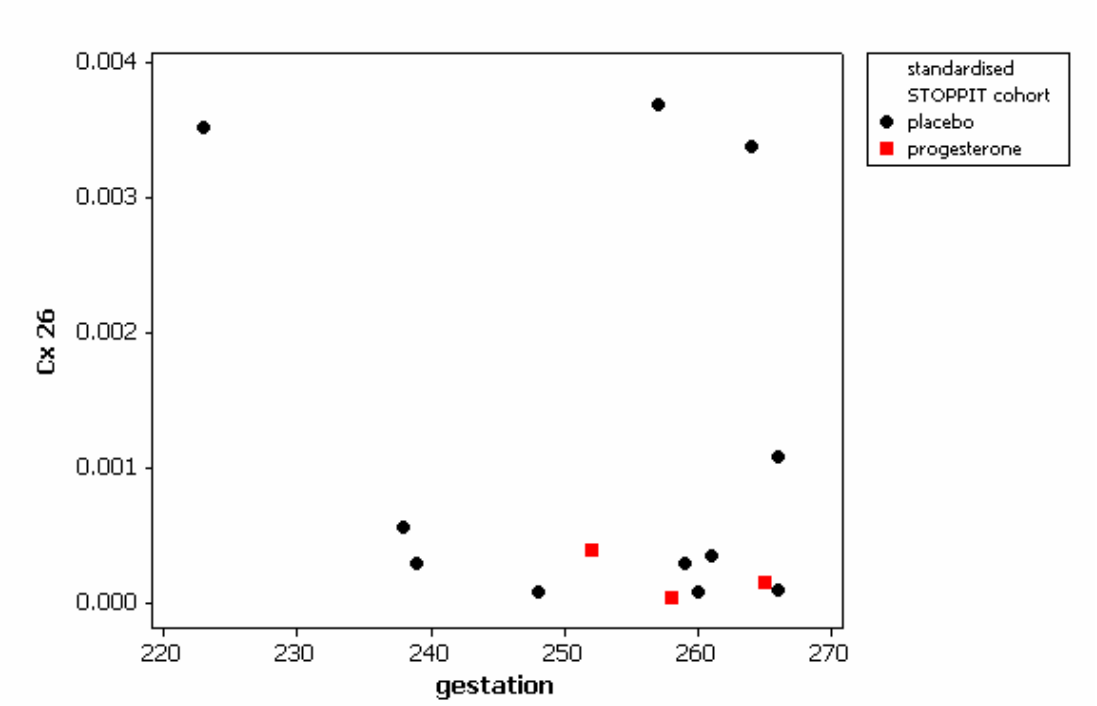

Figure 134 : This graph shows Cx 26 gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect.

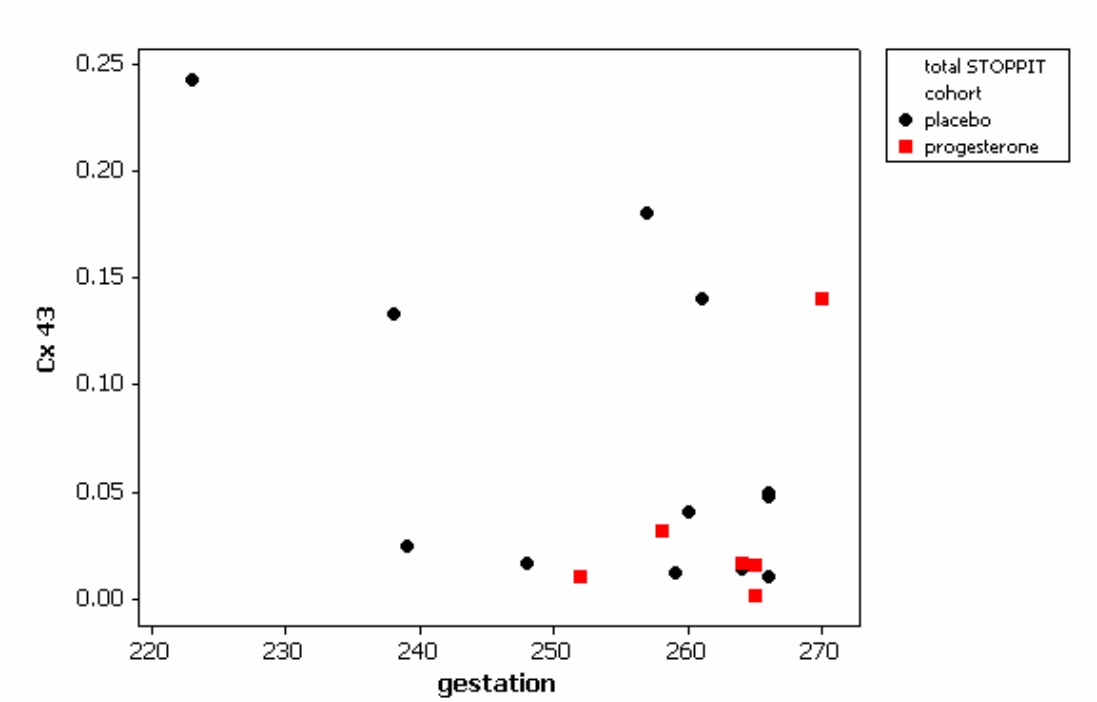

Figure 135 : This graph shows Cx 43 gene mRNA expression levels, relative to 18S.Total STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect

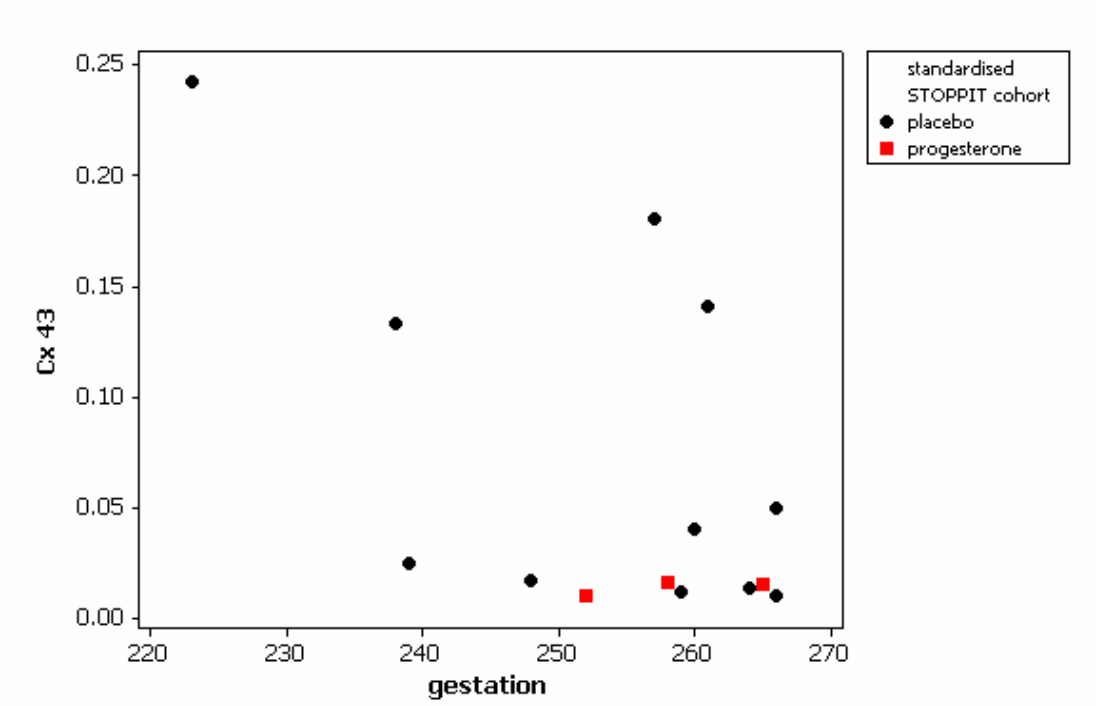

Figure 136 : This graph shows Cx 43 gene mRNA expression levels, relative to 18S.Standardised STOPPIT cohort values have been plotted with gestation in order to ascertain if gestation may have had an overall effect.

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Publications

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Inhibition of human myometrial contractility by progesterone does not operate via certain potassium channels

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Short title: Myometrial contractility and progesterone.

Précis: Progesterone but not 17α-hydroxyprogesterone caproate inhibits spontaneous myometrial contractions *in vitro* and not via specific potassium channels.

ABSTRACT

Objectives: Recent clinical trials have demonstrated a beneficial effect of supplementation with progesterone to prevent preterm labor. We aimed to determine the effects of progesterone treatment *in vitro* and *in vivo* and 17α-hydroxyprogesterone caproate (17OHPC) *in vitro* on myometrial contractions.

Methods: Myometrial strips were taken from women undergoing cesarean delivery at term. We also obtained myometrial biopsies from women participating in a clinical trial of progesterone to prevent preterm labor in twins (STOPPIT). After establishment of spontaneous contractions, strips were exposed to progesterone or 17OHPC. Separate strips were exposed to oxytocin and tocolytics alone and in combination with progesterone. Potassium channel blockers were added in conjunction with progesterone. STOPPIT samples were used to compare the effects of *in vivo* progesterone and placebo. We measured amplitude, frequency and activity integral of contractions.

Results: Maximum inhibition of contraction amplitude was 93±2% and 67±14% for progesterone at 30 μM and vehicle (70% ethanol), respectively, $p<0.05$. 17OHPC did not exert an inhibitory effect. Water soluble progesterone exerted a maximal inhibitory effect on amplitude of contractions of 82±10% at 100 μM, $p<0.05$. The inhibitory effect of progesterone was unaffected by potassium channel blockers. There was no difference between *in vivo* placebo and progesterone-treated groups in either amplitude or frequency of contractions, nor was there any difference in the response to oxytocin or the tocolytic drugs.

Conclusions: Progesterone exerts rapid inhibition of the amplitude of myometrial contractions *in vitro* but 17OHPC does not. The action of progesterone does not appear to operate via potassium channels nor does it lead to enhancement of the activity of certain tocolytic drugs.

INTRODUCTION

Preterm birth is the single biggest cause of perinatal morbidity and mortality with rates of preterm birth rising worldwide. The UK now has the highest rate of premature birth in Europe (2) with 5.3% of overall births in Scotland occurring spontaneously before 37 weeks gestation (1). It has long been established that progesterone is a pre-requisite for a successful pregnancy outcome, with functional withdrawal of progesterone now thought to underlie human labor. Consistent with this theory, recent clinical trials have demonstrated a beneficial effect of supplementation with either progesterone or the synthetic progestin, 17 α-hydroxyprogesterone caproate (17OHPC), in the prevention of preterm labor (104, 111). However, progesterone is not uniformly effective in preventing preterm labor and at present its principal mode of action on myometrium is unknown (48).

Progesterone and its related progestins have both an indirect and direct, concentration-dependent relaxant effect on spontaneous myometrial contractile activity (98, 105, 106). Progesterone is thought to modulate potassium channel activity. Potassium channels are widely expressed on the myocyte membrane surface (129); specifically adenosine triphosphate sensitive-potassium channels ($K_{ATP)}$, BK_{Ca} (large conductance channel), IK_{Ca} (intermediate conductance channel), K_V (voltage-operated channel) and SK_{Ca} (small conductance channel) are all present in pregnant myometrium (128). Additionally, levcromakalim, a K_{ATP} opener, has been shown to exert a concentration-dependent inhibition of spontaneous myometrial activity *in vitro* (64, 92).Calcium channel blockers such as nifedipine have also been used widely as tocolytic agents to eliminate myometrial contractions *in vivo* (55). The opening of potassium channels or closure of calcium channels by progesterone could either hyperpolarise or prevent depolarisation of the cell respectively and would thereby potentially directly inhibit contractile activity. In addition to these immediate effects on the myometrium, progesterone may also sensitise the uterus to tocolytics. A clinical trial using vaginal progesterone reported that patients responded

more favourably to β-mimetics tocolysis when pretreated with progesterone compared to placebo (104), despite no difference in the incidence of threatened preterm labor in the two groups. In vitro progesterone has been shown to sensitise myometrium to the relaxant effects of the β_2-agonist, ritodrine (99).

The purpose of this study was therefore to determine: 1) the effects of in vitro progesterone and its synthetic analogue, 17OHPC, on spontaneous myometrial contractility in vitro 2) whether in vitro progesterone exerts its myometrial effects via specific potassium channels, and 3) whether in vitro progesterone enhances activity of other tocolytic drugs.

MATERIALS AND METHODS

Subjects and Preparation of tissues

We recruited 84 women undergoing elective prelabor cesarean section between 37 and 41 weeks gestation from three maternity hospitals within Scotland. The project was approved by North Glasgow University Hospitals Research Ethics Committee reference number 05/S0705/18. All patients gave written informed consent to participate. At operation, a myometrial biopsy 2cm long, 1cm wide and 1cm thick was taken from the mid-upper lip of the lower segment cesarean section incision into the myometrium. This was done following delivery of the baby and prior to routine clinical administration of Syntocinon 5 IU. Myometrial biopsies were immediately placed in a buffered Krebs solution (NaCl 133 mM, KCl 4.7 mM, glucose 11.1 mM, $MgS0_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.2 mM, TES 10 mM), stored at 4°C and used within 12 hours of collection.

In the laboratory, strips of myometrium 15mm long, 2mm wide and 2mm deep were cut, secured with silk and placed under isometric conditions with a 20 milliNewton (mN) resting tension. When suspended in this manner strips developed rhythmic activity which stabilised within 90 minutes to 3 hours. Once stable activity had developed with the amplitude and frequency having less than 5% variation between contractions then we were added drugs as relevant for each experiment. Each biopsy from a participant was divided into identical strips. One a strip from each participant was used to test each drug and a further strip was used as a control strip. Additionally multiple drugs were tested in parallel from each participant. No participant provided more than one strip for each drug (or control). Contractility was recorded via a tension transducer (FT03, Grass Technologies, Slough, UK) attached to one end of the strip which was connected to a data acquisition system (PowerLab ML870/P, Chart, version 3.6, all AD Instruments). Each tissue strip was suspended in a separate 10ml organ bath filled with Krebs-Henseleit solution, gassed with 95% O_2/5% CO_2, and maintained at 37 ^{o}C.

Contractile analysis

The amplitude of spontaneous contraction was taken as the mean amplitude of 3 consecutive contractions recorded prior to the addition of drug. Stable activity was defined as a series of at least 3 contractions reaching the same amplitude and frequency after the 2-hour equilibration period. The response to drug was defined as the effect on contraction at the time point of interest and recorded in milliNewtons (mN). Frequency was recorded in contractions h^{-1} by measuring the interval between the peaks of two consecutive contractions occurring at the time point of interest. For time point analysis, contraction amplitude and frequency were recorded following the development of stable rhythmic activity, immediately prior to addition of drug (time 0) and at 30 minute periods thereafter for 4 hours. An integral measure of contractile activity was determined by the area under the tension curve for each contraction.

Progesterone, 17OHPC experiments and water soluble progesterone

Progesterone (10 nM-100 µM final bath concentrations) was added at 20-minute intervals to reach cumulative concentrations of 10 nM, 100 nM, 1 µM etc. Amplitude and frequency of contractions were recorded at the end of each time period. Similar experiments were then carried out with 17OHPC. Both progesterone and 17OHPC were dissolved in a 70% ethanol vehicle with subsequent dilutions in Krebs. For progesterone and 17OHPC a parallel series of time matched experiments were carried out using ethanol (70%) vehicle control diluted in Krebs as appropriate. As will be seen from the results, the ethanol vehicle inhibited myometrial contraction, thus interfering with the actions of progesterone itself. Accordingly, a water soluble progesterone contained within a cyclodextrin was also examined which required no vehicle control as it was dissolved in Krebs solution. We subsequently tested the effects of the cyclodextrin compound which was used to make the progesterone water soluble on myometrial contractions.

In order to assess the effects of progesterone for periods greater than 20 minutes, time course experiments were carried out. A single dose of water soluble progesterone, to reach a bath concentration of 100 µM, was added after the equilibration period. Thereafter the amplitude and frequency were recorded at 30 minute intervals over a period of 4 hours.

Potassium channel experiments

In view of the inhibitory effect of ethanol at high concentrations, these experiments were conducted with only water soluble progesterone. Each of the potassium channel blocking agents, glibenclamide 1.5 µM (blocks K_{ATP} channels), charybdotoxin 100 nM (blocks IK_{Ca}, BK_{Ca} and K_v channels), iberiotoxin 100 nM (blocks BK_{Ca} channels) and apamin 100 nM (blocks SK_{Ca} channels) were added to myometrial strips when spontaneous rhythmic activity had stabilised and progesterone was added after 20 minutes. Concentration-

response curves were then carried out adding water soluble progesterone as described above. Time matched controls were included for all agents and a DMSO vehicle control was also included for glibenclamide. Time course experiments were also repeated with progesterone 100 µM after treatment with each potassium channel blocking agent.

In vitro progesterone incubation with oxytocin, levcromakalim, nifedipine and ritodrine

We performed concentration-response curves to examine the effects on myometrial activity of oxytocin (which stimulates myometrial contractions in concentrations of 10 pM-10 µM), levcromakalim (a K_{ATP} channel opener, 100 pM -10 µM), nifedipine (a calcium channel blocker, 100 pM-10 µM) and ritodrine (a β_2-agonist, 0.01 µM-1 mM) all added to cumulatively to achieve final bath concentrations at these ranges, on separate strips from patients, $n \geq 6$. We also constructed parallel concentration-response curves on tissues incubated for 60 min with progesterone 1 µM .

Drugs and solutions

All concentrations stated are final bath concentrations. All drugs were obtained from Sigma (Poole) except for iberiotoxin and charybdotoxin which were obtained from Latoxan (France). All drugs were dissolved in saline except levcromakalim (DMSO solvent), oxytocin (acetic acid), nifedipine (ethanol), progesterone (70% ethanol), 17OHPC (70% ethanol) and glibenclamide (DMSO).The maximum concentration reached with ethanol was 0.2% of the total organ bath volume for all experiments except the progesterone (70% ethanol). This reached higher ethanol concentrations and consequentially we used alternative progesterone. Water soluble progesterone, Sigma (Poole) is contained within a cylclodextrin (Sigma) compound. This enables hydrophobic molecules to be incorporated into the cavity of the cylclodextrin by displacing water, and

when the water soluble complex is dissolved in a larger volume of aqueous solvent the process is reversed thereby releasing the molecule into the solvent.

Statistical analysis

A reduction in the magnitude of the amplitude contractions was expressed as a percentage inhibition (mean ± SEM) of the contraction obtained immediately before the first addition of the drug. Statistical analysis was carried out using one-way analysis of variance with either Bonferroni correction or Kruskal-Wallis test for non parametric data or unpaired t test, for parametric data (Graph Pad Prism, Minitab). N is defined as the number of patients and myometrial strip number is stated separately. A value of $p< 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of progesterone, 17OHPC and water soluble progesterone

Following equilibration for 2 hours, myometrial strips contracted in a rhythmic manner (amplitude 46±1 mN, frequency 7.7±0.1 contractions h^{-1}) for >4hours (figures 1a,1d), data obtained from 64 strips from 15 patients. Progesterone dissolved in ethanol (10 nM- 30 μM) produced a concentration-dependent inhibitory effect on the amplitude of myometrial contractions (figure 1a, b). The vehicle 70% ethanol, at appropriate dilutions, also exerted an inhibitory effect on the amplitude of contractions. Maximum inhibition of amplitude was 93±2% and 67±14% for 30 μM progesterone and appropriate vehicle. 17OHPC also exerted an inhibitory effect on contraction amplitude but this was not significantly different from the vehicle (figure 1c).

Given the demonstrable inhibitory effect of the ethanol vehicle at high concentrations on myometrial contractions, we used water soluble progesterone (which does not contain ethanol) for all subsequent experiments. The cyclodextrin compound encapsulating the

progesterone was tested alone, with 6 myometrial biopsies and had no effect on either the amplitude or frequency of myometrial contractions (data not shown). Time course experiments showed that water soluble progesterone (100 μM) exerted a 42±5% inhibition of contractile amplitude 30 minutes after addition to the bath, and reached a maximal inhibition of 82±10% at 1 hour (figure 1d). Water soluble progesterone also exerted a concentration-dependent inhibitory effect on amplitude (max of 82±10% at 100 μM (figure 1e). Water soluble progesterone generally had no effect on the frequency of contractions (figure 1f); but in some cases a small rise was seen. Overall the activity integral (area under the curve) showed a clear decrease with progesterone compared with control (figure 1g).

Effects of potassium channel blockers on responses to progesterone

Levcromakalim, a K_{ATP} channel opener, produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous contractions. These effects were antagonised by the K_{ATP} channel blocker, glibenclamide (1.5 μM) (figure 2a, b). In contrast, glibenclamide had no effect on the progesterone-induced inhibition of myometrial contractility (figure 2c, d). Charybdotoxin 10 nM (which blocks IK_{Ca}, BK_{Ca} and K_v channels), iberiotoxin 100 nM (which blocks BK_{Ca} channels) and apamin 100 nM (which blocks SK_{Ca} channels) failed to affect the ability of progesterone to inhibit myometrial contractility (figure 3 a-f).

Effects of progesterone 1 μM incubation in vitro on oxytocin and tocolytics

Previous work has suggested sensitisation to tocolytics by progesterone (0.01 μM) in vitro (99). As expected, oxytocin (10 pM-10 μM) produced a concentration-dependent increase in both amplitude and frequency of myometrial contractions. Levcromakalim (100 pM -10 μM), nifedipine (100 pM-10 μM) and ritodrine (0.01 μM-1 mM) each produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous

contractions. A short 60 minute water soluble progesterone (1 μM) incubation did not alter spontaneous amplitude or frequency of myometrial contractions. This progesterone incubation also failed to alter the concentration-dependent stimulatory effect of oxytocin or concentration-dependent inhibitory effects of levcromakalim, nifedipine or ritodrine $n \geq 10$ (figure 4 a-d).

DISCUSSION

Our study clearly demonstrates that progesterone, albeit at high concentrations, exerts consistent, rapid and sustained inhibition of the amplitude of spontaneous myometrial contractions *in vitro* - consistent with effects observed in clinical studies of acute administration of progesterone at term (127). In contrast, 17OHPC did not exert any inhibitory effect on myometrial activity over and above that seen with its vehicle (70% ethanol). However, the concentrations of progesterone required to inhibit contractions *in vitro* (100 μM) are unlikely to be achieved with therapeutic doses of progesterone *in vivo*. However, it may not be the absolute concentration of progesterone that is important in vivo, given that it has been suggested that patterns of change as opposed to absolute concentrations of progesterone and other steroid hormones may be the important factor in affecting myometrial contractility (157).

Our observation that 17OHPC had no independent inhibitory effect confirms previous work from other groups(158). Despite this, a number of clinical trials administering intramuscular injections of 17OHPC have been successful in preventing preterm delivery in high risk women. Sexton et al suggests that this positive clinical outcome may well be mediated through long-term genomic pathways which cannot be reproduced under organ bath conditions (158). We were unable to test this as we were only able to conduct experiments for 8 hours in vitro before myometrial contractility began to decline (presumably due to cell death and lack of energy substrates in the tissue). It is possible therefore that 17OHPC requires either prolonged incubation (which cannot be tested in

vitro) or the contribution of other tissues or molecules that are present in vivo to exert an effect. It also remains unclear as to whether 17OHPC is active itself or requires metabolic activation. One suggested pathway of activation is hydrolysis of 17 OHP by plasma and tissue esterases, but a recent study showed that this does not occur in vitro, suggesting again either different effects in vivo or alternative mechanisms of metabolism (159). Progesterone and 17OHPC may potentially act upon different receptors in vitro and in vivo. For example, 17OHPC has recently been found to have lower affinity than progesterone for the progesterone PR receptor (160). These differences may account for the greater efficacy of progesterone compared with 17OHPC on contractility *in vitro*.

In this study, the speed of the progesterone response suggests a non-genomic effect. However by use of selective inhibitors, we clearly demonstrate that progesterone does not exert its inhibition of myometrial contraction via a range of potassium channels, including $K_{ATP,}$ BK_{Ca}, IK_{Ca} and SK_{Ca}, despite a key role for them in contractile responses. Alternative pathways in which progesterone may exert an effect could be via sodium or chloride channels. There is some evidence to suggest that sodium channels may be up regulated in myometrium at late gestation (167) but the distinct roles of specific sodium channels is not clearly understood. Ion channels may well have regulatory pathways related to myometrial function but whether progesterone utilises them in its mechanism to prevent preterm labor remains unclear.

There are several non-genomic pathways by which progesterone may inhibit myometrial contractility in vitro. We consider the most likely to be those operating through the cell membrane progesterone receptors α, β and γ, which themselves are similar to G-protein coupled receptors (109) . An alternative non-genomic pathway of progesterone action is interaction with gamma amino butyric acid A ($GABA_A$) receptors. These receptors appear to be important in the emerging effects that progesterone has on the brain (97, 217). Additionally, initial studies of rat myometrium $GABA_A$ antagonists prevented progesterone induced inhibition of contractions, suggesting that progesterone might mediate its action

via the GABA receptor (107). These results could not be replicated in more recent studies of human myometrium. A prior study found that relaxation produced by the above steroids was not blocked by the $GABA_A$ receptor antagonists, picrotoxin or bicuculline, but was reversed by calcium, they suggest that blockade of calcium influx appeared to be responsible this relaxation (108).

Thus the potential role of GABA receptors in the mechanism of action of progesterone in inhibiting myometrial contractions remains controversial, with progesterone's main genomic action in maintaining uterine quiescence is considered to result via the PR-B receptor (48, 77).

A previous study has reported enhanced responses to β-sympathomimetics following both in vivo and in vitro treatment with progesterone (104). However, we did not see an enhanced effect of any tocolytic, including levcromakalim (a K_{ATP} channel opener), nifedipine (a calcium channel blocker) and ritodrine (a β_2- agonist) in our in vitro progesterone groups. This is in contrast to previous work carried out by Chanrachakul et al, where they demonstrated an enhancement of the tocolytic effect of ritodrine using a lower concentration of progesterone (0.01 μM) in vitro (99). Their experimental design involved using oxytocin-induced contractions as opposed to spontaneous contractions in our study. Additionally, they used progesterone dissolved in ethanol (against appropriate ethanol controls) in contrast to our own study where we used progesterone in cyclodextrin. The differences between the Chanrachakul study and our own are hard to explain unless progesterone's effect in enhancing the tocolytic effects of ritodrine require either ethanol or stimulation with oxytocin to become apparent. Their experimental design involved using oxytocin-induced contractions as opposed to spontaneous contractions in our study. Lastly although a potential weakness of our study may be the use of lower segment myometrial biopsies, others have previously shown no difference between upper and lower segment biopsies in terms of myometrial contractility *in vitro* (147) .

Progesterone has been demonstrated to suppress the immune response in early pregnancy (181). Elovitz et al demonstrated that the progestational agent, medroxyprogesterone acetate, reduced COX-2, IL-1β and TNF-α mRNA expression in the myometrium of pregnant mice pretreated with LPS. These studies demonstrated that progestational agents decreased the incidence of inflammation-induced preterm parturition and medroxyprogesterone completely prevented preterm birth compared to progesterone which decreased the overall rate of preterm birth (207).Although it is tempting to speculate that this inhibition of production of inflammatory genes may be part of the mechanism of action by which progesterone affects myometrial contractility, again this would be a genomic effect that would take longer than the rapid effects on contractility that we observed with progesterone.

In conclusion we have clearly demonstrated that progesterone but not 17OHPC inhibits myometrial contractility in vitro, however, this affect is not achieved through the principal potassium channels. Further we show that progesterone does not enhance the inhibitory response of tocolytics with respect to spontaneous contractions. The mechanism of action of progesterone both in inhibiting myometrial contractility and in preventing preterm birth therefore continues to warrant further investigation.

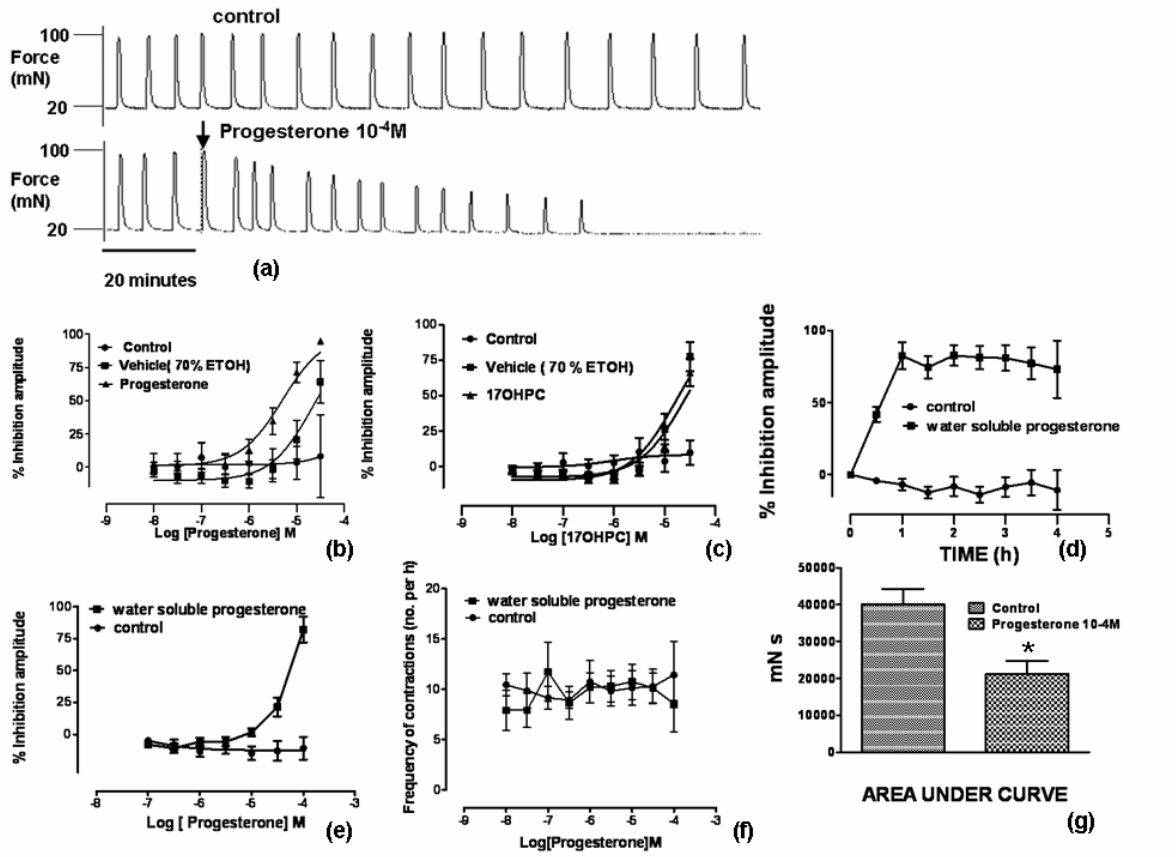

Figure 1(a) Contractility trace showing the stability of rhythmic contraction of myometrial strips and inhibition of activity by progesterone (100μM). Concentration-response curves showing the effects of progesterone and the appropriate dilution of vehicle (70% ethanol) n≥9 (b) and 17OHPC n≥6(c). Time course assessment (d) of a maximal concentration water soluble progesterone,100μM over 4 hours on the amplitude of contractions. Concentration-response curves for amplitude of contractions (e) and frequency of contractions (f) both for water soluble progesterone n≥5. Activity integral assessment of control versus water soluble progesterone (g) *$p<0.05$ n≥6.

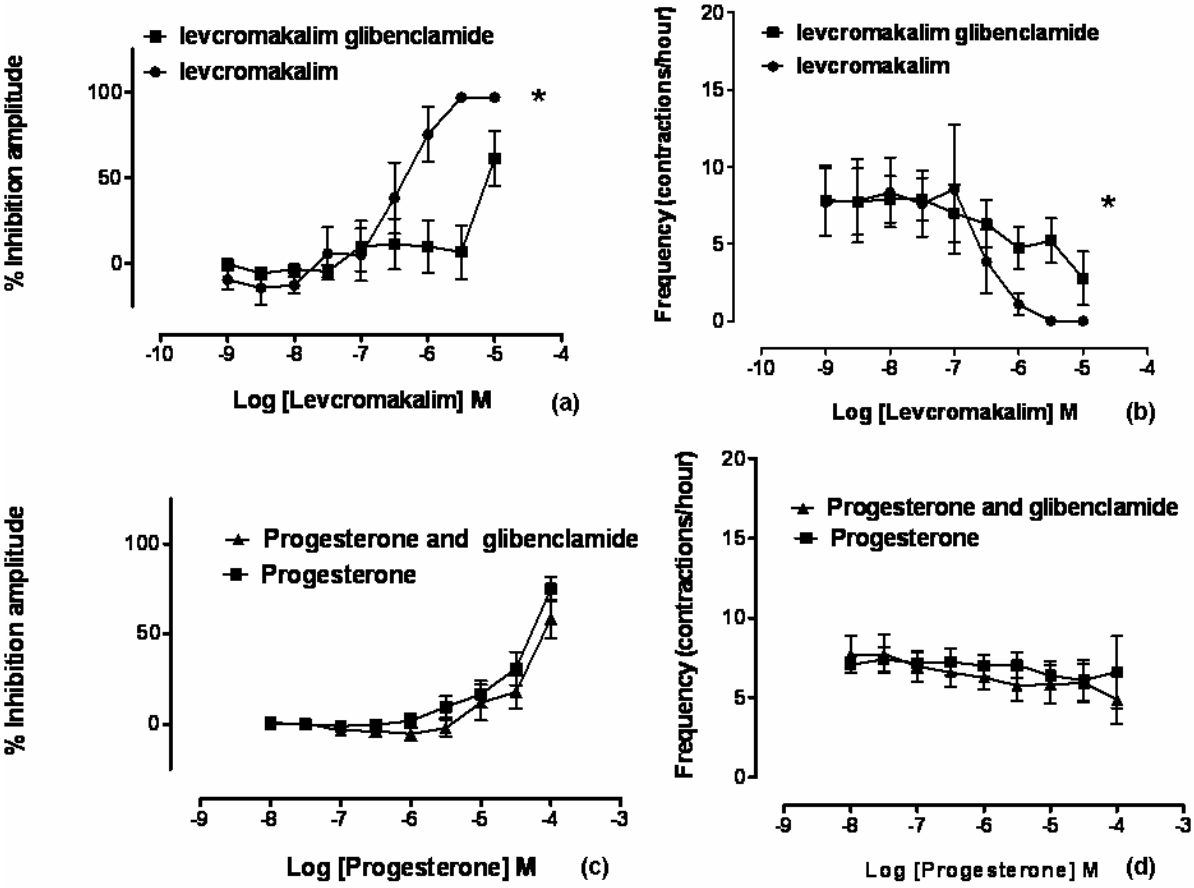

Figure 2. Concentration-response curves showing the effects of levcromakalim and progesterone on amplitude (a and c) and frequency (b and d) of myometrial contractions. The effects of glibenclamide (1.5 µM) on the actions of levcromakalim and progesterone are also shown n≥7.The right shift in the curve is evident, * $p<0.05$ indicates a difference between the response of contractile amplitude and frequency levcromakalim , and in the presence of its antagonist glibenclamide (ANOVA).

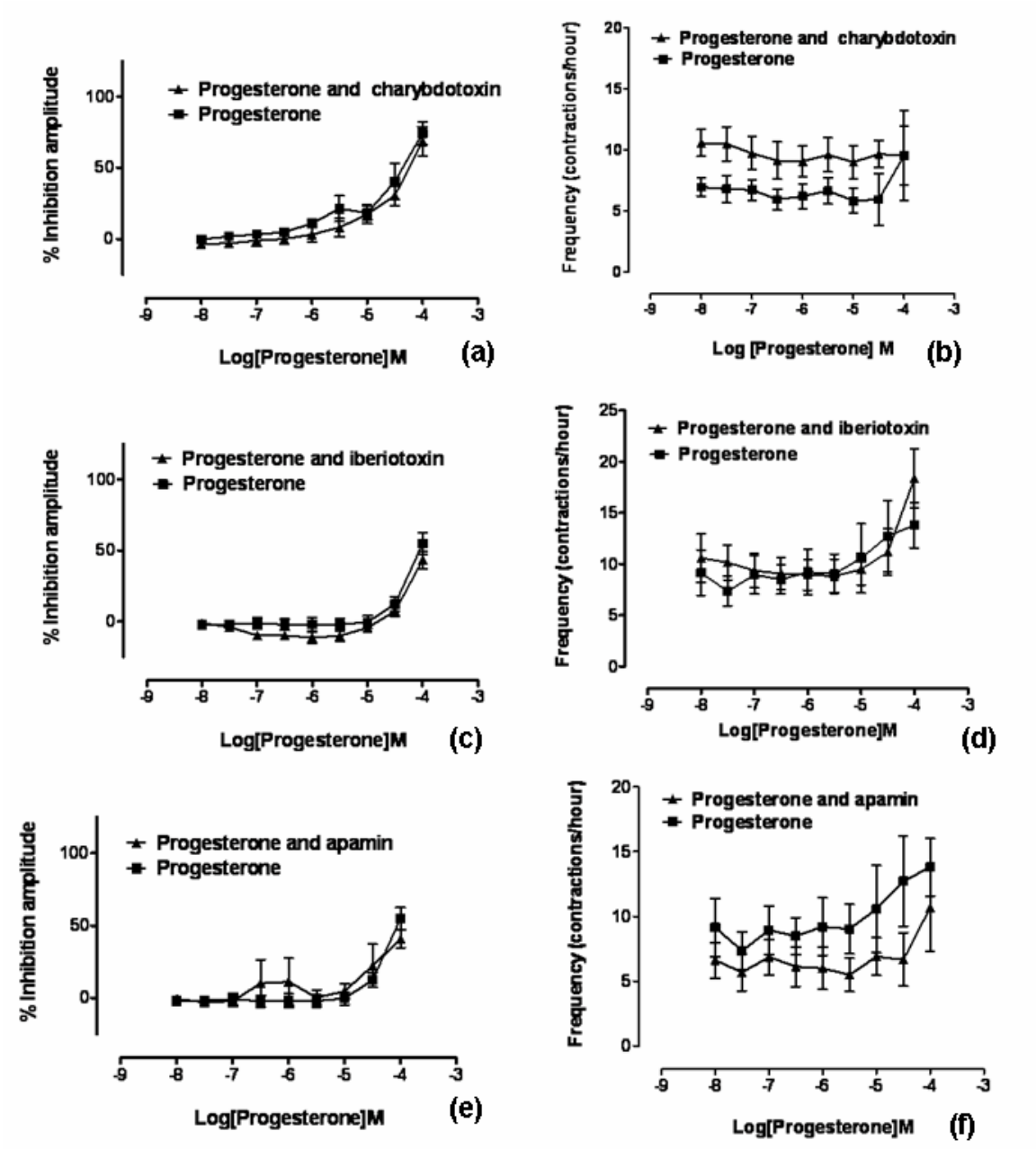

Figure 3. Concentration-response curves showing how changes in amplitude and frequent of myometrial contractions induced by progesterone are influenced by the presence of charybdotoxin n≥6 (a, b), iberiotoxin n≥6 (c, d) and apamin n≥6 (e, f). There was no significant difference in the response of contractions to progesterone in the presence of any of the potassium channel agents tested (ANOVA).

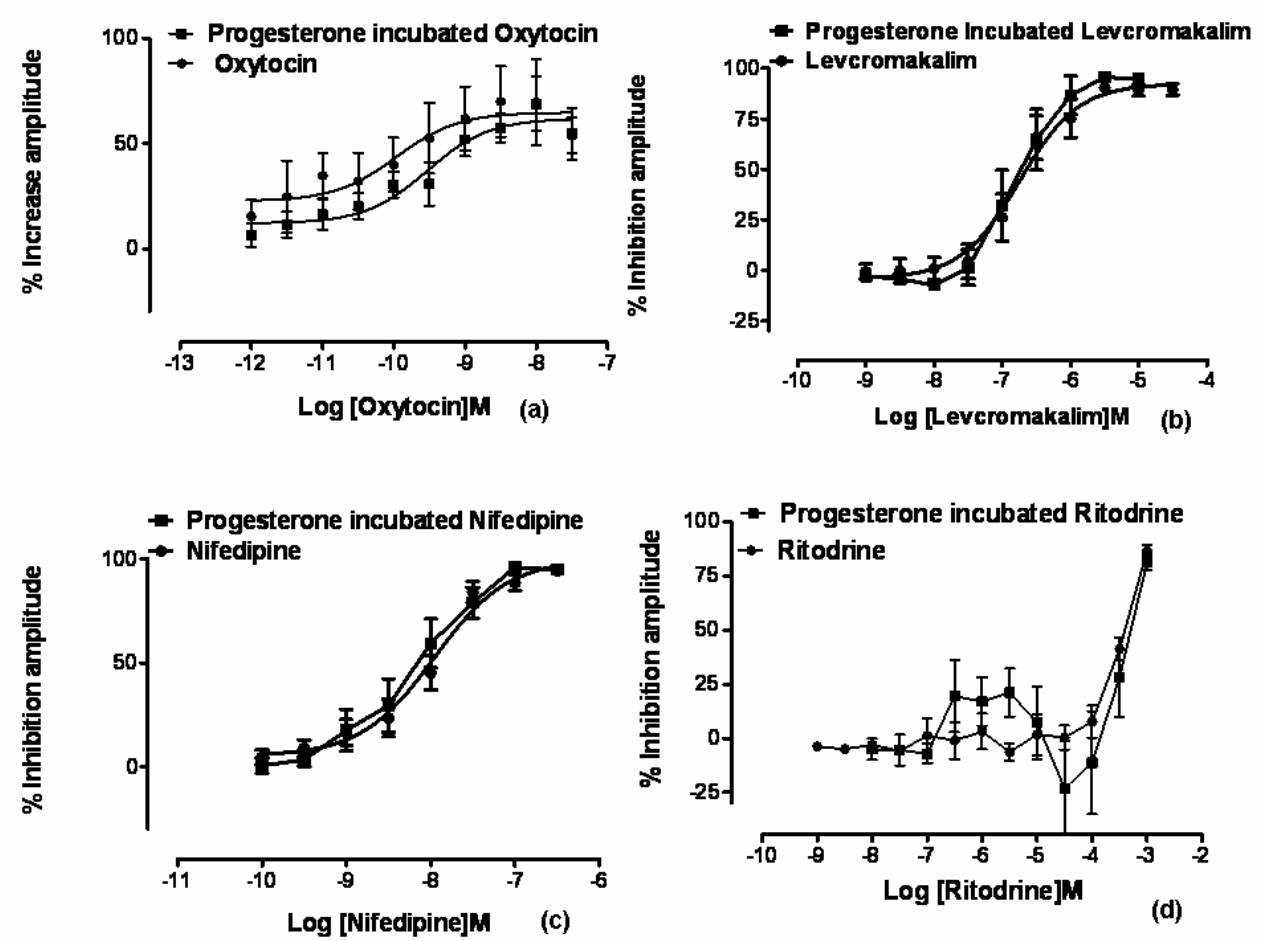

Figure 4. Concentration-response curves showing the effects of oxytocin (a), levcromakalim (b), nifedipine (c) and ritodrine (d) on the amplitude of myometrial contractility *in vitro* following incubation with progesterone 1 µM n≥6 . There was no significant difference in any of the drugs tested after the incubation with progesterone (ANOVA).

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Inhibition of human myometrial contractility by progesterone does not operate via certain potassium channels

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Short title: Myometrial contractility and progesterone.

Précis: Progesterone but not 17α-hydroxyprogesterone caproate inhibits spontaneous myometrial contractions *in vitro* and not via specific potassium channels.

ABSTRACT

Objectives: Recent clinical trials have demonstrated a beneficial effect of supplementation with progesterone to prevent preterm labor. We aimed to determine the effects of progesterone treatment *in vitro* and *in vivo* and 17α-hydroxyprogesterone caproate (17OHPC) *in vitro* on myometrial contractions.

Methods: Myometrial strips were taken from women undergoing cesarean delivery at term. We also obtained myometrial biopsies from women participating in a clinical trial of progesterone to prevent preterm labor in twins (STOPPIT). After establishment of spontaneous contractions, strips were exposed to progesterone or 17OHPC. Separate strips were exposed to oxytocin and tocolytics alone and in combination with progesterone. Potassium channel blockers were added in conjunction with progesterone. STOPPIT samples were used to compare the effects of *in vivo* progesterone and placebo. We measured amplitude, frequency and activity integral of contractions.

Results: Maximum inhibition of contraction amplitude was 93±2% and 67±14% for progesterone at 30 μM and vehicle (70% ethanol), respectively, $p<0.05$. 17OHPC did not exert an inhibitory effect. Water soluble progesterone exerted a maximal inhibitory effect on amplitude of contractions of 82±10% at 100 μM, $p<0.05$. The inhibitory effect of progesterone was unaffected by potassium channel blockers. There was no difference between *in vivo* placebo and progesterone-treated groups in either amplitude or frequency of contractions, nor was there any difference in the response to oxytocin or the tocolytic drugs.

Conclusions: Progesterone exerts rapid inhibition of the amplitude of myometrial contractions *in vitro* but 17OHPC does not. The action of progesterone does not appear to operate via potassium channels nor does it enhance the activity of certain tocolytic drugs.

INTRODUCTION

Preterm birth is the single biggest cause of perinatal morbidity and mortality with rates of preterm birth rising worldwide. The UK now has the highest rate of premature birth in Europe (2) with 5.3% of overall births in Scotland occurring spontaneously before 37 weeks gestation (1). It has long been established that progesterone is a pre-requisite for a successful pregnancy outcome, with functional withdrawal of progesterone now thought to underlie human labor. Consistent with this theory, recent clinical trials have demonstrated a beneficial effect of supplementation with either progesterone or the synthetic progestin, 17 α-hydroxyprogesterone caproate (17OHPC), in the prevention of preterm labor (104, 111). However, progesterone is not uniformly effective in preventing preterm labor and at present its principal mode of action on myometrium is unknown (48).

Progesterone and its related progestins have both an indirect and direct, concentration-dependent relaxant effect on spontaneous myometrial contractile activity (98, 105, 106). Progesterone is thought to modulate potassium channel activity. Potassium channels are widely expressed on the myocyte membrane surface (129); specifically adenosine triphosphate sensitive-potassium channels ($K_{ATP)}$, BK_{Ca} (large conductance channel), IK_{Ca} (intermediate conductance channel), K_V (voltage-operated channel) and SK_{Ca} (small conductance channel) are all present in pregnant myometrium (128). Additionally, levcromakalim, a K_{ATP} opener, has been shown to exert a concentration-dependent inhibition of spontaneous myometrial activity *in vitro* (64, 92).Calcium channel blockers such as nifedipine have also been used widely as tocolytic agents to eliminate myometrial contractions *in vivo* (55). The opening of potassium channels or closure of calcium channels by progesterone could either hyperpolarise or prevent depolarisation of the cell respectively and would thereby potentially directly inhibit contractile activity. In addition to these immediate effects on the myometrium, progesterone may also sensitise the uterus to tocolytics. A clinical trial using vaginal progesterone reported that patients responded more favourably to β-mimetics tocolysis when pretreated with progesterone compared to

placebo (104), despite no difference in the incidence of threatened preterm labor in the two groups. In vitro progesterone has been shown to sensitise myometrium to the relaxant effects of the β_2-agonist, ritodrine (99).

The purpose of this study was therefore to determine: 1) the effects of in vitro progesterone and its synthetic analogue, 17OHPC, on spontaneous myometrial contractility in vitro 2) whether in vitro progesterone exerts its myometrial effects via specific potassium channels, and 3) whether in vitro progesterone enhances activity of other tocolytic drugs.

MATERIALS AND METHODS

Subjects and Preparation of tissues

We recruited 84 women undergoing elective prelabor cesarean section between 37 and 41 weeks gestation from three maternity hospitals within Scotland. The project was approved by North Glasgow University Hospitals Research Ethics Committee reference number 05/S0705/18. All patients gave written informed consent to participate. At operation, a myometrial biopsy 2cm long, 1cm wide and 1cm thick was taken from the mid-upper lip of the lower segment cesarean section incision into the myometrium. This was done following delivery of the baby and prior to routine clinical administration of Syntocinon 5 IU. Myometrial biopsies were immediately placed in a buffered Krebs solution (NaCl 133 mM, KCl 4.7 mM, glucose 11.1 mM, $MgS0_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.2 mM, TES 10 mM), stored at 4°C and used within 12 hours of collection.

In the laboratory, strips of myometrium 15mm long, 2mm wide and 2mm deep were cut, secured with silk and placed under isometric conditions with a 20 milliNewton (mN)

resting tension. When suspended in this manner strips developed rhythmic activity which stabilised within 90 minutes to 3 hours. Once stable activity had developed with the amplitude and frequency having less than 5% variation between contractions then we were added drugs as relevant for each experiment. Each biopsy from a participant was divided into identical strips. One a strip from each participant was used to test each drug and a further strip was used as a control strip. Additionally multiple drugs were tested in parallel from each participant. No participant provided more than one strip for each drug (or control). Contractility was recorded via a tension transducer (FT03, Grass Technologies, Slough, UK) attached to one end of the strip which was connected to a data acquisition system (PowerLab ML870/P, Chart, version 3.6, all AD Instruments). Each tissue strip was suspended in a separate 10ml organ bath filled with Krebs-Henseleit solution, gassed with 95% O_2/5% CO_2, and maintained at 37 ^{o}C.

Contractile analysis

The amplitude of spontaneous contraction was taken as the mean amplitude of 3 consecutive contractions recorded prior to the addition of drug. Stable activity was defined as a series of at least 3 contractions reaching the same amplitude and frequency after the 2-hour equilibration period. The response to drug was defined as the effect on contraction at the time point of interest and recorded in milliNewtons (mN). Frequency was recorded in contractions h^{-1} by measuring the interval between the peaks of two consecutive contractions occurring at the time point of interest. For time point analysis, contraction amplitude and frequency were recorded following the development of stable rhythmic activity, immediately prior to addition of drug (time 0) and at 30 minute periods thereafter for 4 hours. An integral measure of contractile activity was determined by the area under the tension curve for each contraction.

Progesterone, 17OHPC experiments and water soluble progesterone

Progesterone (10 nM-100 µM final bath concentrations) was added at 20-minute intervals to reach cumulative concentrations of 10 nM, 100 nM, 1 µM etc. Amplitude and frequency of contractions were recorded at the end of each time period. Similar experiments were then carried out with 17OHPC. Both progesterone and 17OHPC were dissolved in a 70% ethanol vehicle with subsequent dilutions in Krebs. For progesterone and 17OHPC a parallel series of time matched experiments were carried out using ethanol (70%) vehicle control diluted in Krebs as appropriate. As will be seen from the results, the ethanol vehicle inhibited myometrial contraction, thus interfering with the actions of progesterone itself. Accordingly, a water soluble progesterone contained within a cyclodextrin was also examined which required no vehicle control as it was dissolved in Krebs solution. We subsequently tested the effects of the cyclodextrin compound which was used to make the progesterone water soluble on myometrial contractions.

In order to assess the effects of progesterone for periods greater than 20 minutes, time course experiments were carried out. A single dose of water soluble progesterone, to reach a bath concentration of 100 µM, was added after the equilibration period. Thereafter the amplitude and frequency were recorded at 30 minute intervals over a period of 4 hours.

Potassium channel experiments

In view of the inhibitory effect of ethanol at high concentrations, these experiments were conducted with only water soluble progesterone. Each of the potassium channel blocking agents, glibenclamide 1.5 µM (blocks K_{ATP} channels), charybdotoxin 100 nM (blocks IK_{Ca}, BK_{Ca} and K_v channels), iberiotoxin 100 nM (blocks BK_{Ca} channels) and apamin 100 nM (blocks SK_{Ca} channels) were added to myometrial strips when spontaneous rhythmic activity had stabilised and progesterone was added after 20 minutes. Concentration-response curves were then carried out adding water soluble progesterone as described

above. Time matched controls were included for all agents and a DMSO vehicle control was also included for glibenclamide. Time course experiments were also repeated with progesterone 100 µM after treatment with each potassium channel blocking agent.

In vitro progesterone incubation with oxytocin, levcromakalim, nifedipine and ritodrine

We performed concentration-response curves to examine the effects on myometrial activity of oxytocin (which stimulates myometrial contractions in concentrations of 10 pM-10 µM), levcromakalim (a K_{ATP} channel opener, 100 pM -10 µM), nifedipine (a calcium channel blocker, 100 pM-10 µM) and ritodrine (a β_2-agonist, 0.01 µM-1 mM) all added to cumulatively to achieve final bath concentrations at these ranges, on separate strips from patients, $n \geq 6$. We also constructed parallel concentration-response curves on tissues incubated for 60 min with progesterone 1 µM .

Drugs and solutions

All concentrations stated are final bath concentrations. All drugs were obtained from Sigma (Poole) except for iberiotoxin and charybdotoxin which were obtained from Latoxan (France). All drugs were dissolved in saline except levcromakalim (DMSO solvent), oxytocin (acetic acid), nifedipine (ethanol), progesterone (70% ethanol), 17OHPC (70% ethanol) and glibenclamide (DMSO).The maximum concentration reached with ethanol was 0.2% of the total organ bath volume for all experiments except the progesterone (70% ethanol). The progesterone (70% ethanol) would have reached higher concentrations than appropriate for organ bath experiments and consequentially we used alternative progesterone. Water soluble progesterone, Sigma (Poole) is contained within a cylclodextrin (Sigma) compound. This enables hydrophobic molecules to be incorporated into the cavity of the cylclodextrin by displacing water, and when the water

soluble complex is dissolved in a larger volume of aqueous solvent the process is reversed thereby releasing the molecule into the solvent.

Statistical analysis

A reduction in the magnitude of the amplitude contractions was expressed as a percentage inhibition (mean ± SEM) of the contraction obtained immediately before the first addition of the drug. Statistical analysis was carried out using one-way analysis of variance with either Bonferroni correction or Kruskal-Wallis test for non parametric data or unpaired t test, for parametric data (Graph Pad Prism, Minitab). N is defined as the number of patients and myometrial strip number is stated separately. A value of $p< 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of progesterone, 17OHPC and water soluble progesterone

Following equilibration for 2 hours, myometrial strips contracted in a rhythmic manner (amplitude 46±1 mN, frequency 7.7±0.1 contractions h^{-1}) for >4hours (figures 1a,1d), data obtained from 64 strips from 15 patients. Progesterone dissolved in ethanol (10 nM- 30 μM) produced a concentration-dependent inhibitory effect on the amplitude of myometrial contractions (figure 1a, b). The vehicle 70% ethanol, at appropriate dilutions, also exerted an inhibitory effect on the amplitude of contractions. Maximum inhibition of amplitude was 93±2% and 67±14% for 30 μM progesterone and appropriate vehicle. 17OHPC also exerted an inhibitory effect on contraction amplitude but this was not significantly different from the vehicle (figure 1c).

Given the demonstrable inhibitory effect of the ethanol vehicle at high concentrations on myometrial contractions, we used water soluble progesterone (which does not contain ethanol) for all subsequent experiments. The cyclodextrin compound encapsulating the

progesterone was tested alone, with 6 myometrial biopsies and had no effect on either the amplitude or frequency of myometrial contractions (data not shown). Time course experiments showed that water soluble progesterone (100 μM) exerted a 42±5% inhibition of contractile amplitude 30 minutes after addition to the bath, and reached a maximal inhibition of 82±10% at 1 hour (figure 1d). Water soluble progesterone also exerted a concentration-dependent inhibitory effect on amplitude (max of 82±10% at 100 μM (figure 1e). Water soluble progesterone generally had no effect on the frequency of contractions (figure 1f); but in some cases a small rise was seen. Overall the activity integral (area under the curve) showed a clear decrease with progesterone compared with control (figure 1g).

Effects of potassium channel blockers on responses to progesterone

Levcromakalim, a K_{ATP} channel opener, produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous contractions. These effects were antagonised by the K_{ATP} channel blocker, glibenclamide (1.5 μM) (figure 2a, b). In contrast, glibenclamide had no effect on the progesterone-induced inhibition of myometrial contractility (figure 2c, d). Charybdotoxin 10 nM (which blocks IK_{Ca}, BK_{Ca} and K_v channels), iberiotoxin 100 nM (which blocks BK_{Ca} channels) and apamin 100 nM (which blocks SK_{Ca} channels) failed to affect the ability of progesterone to inhibit myometrial contractility (figure 3 a-f).

Effects of progesterone 1 μM incubation in vitro on oxytocin and tocolytics

Previous work has suggested sensitisation to tocolytics by progesterone (0.01 μM) in vitro (99). As expected, oxytocin (10 pM-10 μM) produced a concentration-dependent increase in both amplitude and frequency of myometrial contractions. Levcromakalim (100 pM -10 μM), nifedipine (100 pM-10 μM) and ritodrine (0.01 μM-1 mM) each produced a concentration-dependent inhibition of the amplitude and frequency of spontaneous

contractions. A short 60 minute water soluble progesterone (1 μM) incubation did not alter spontaneous amplitude or frequency of myometrial contractions. This progesterone incubation also failed to alter the concentration-dependent stimulatory effect of oxytocin or concentration-dependent inhibitory effects of levcromakalim, nifedipine or ritodrine $n \geq 10$ (figure 4 a-d).

DISCUSSION

Our study clearly demonstrates that progesterone, albeit at high concentrations, exerts consistent, rapid and sustained inhibition of the amplitude of spontaneous myometrial contractions *in vitro* - consistent with effects observed in clinical studies of acute administration of progesterone at term (127). In contrast, 17OHPC did not exert any inhibitory effect on myometrial activity over and above that seen with its vehicle (70% ethanol). However, the concentrations of progesterone required to inhibit contractions *in vitro* (100 μM) are unlikely to be achieved with therapeutic doses of progesterone *in vivo*. However, it may not be the absolute concentration of progesterone that is important in vivo, given that it has been suggested that patterns of change as opposed to absolute concentrations of progesterone and other steroid hormones may be the important factor in affecting myometrial contractility (157).

Our observation that 17OHPC had no independent inhibitory effect confirms previous work from other groups(158). Despite this, a number of clinical trials administering intramuscular injections of 17OHPC have been successful in preventing preterm delivery in high risk women. Sexton et al suggests that this positive clinical outcome may well be mediated through long-term genomic pathways which cannot be reproduced under organ bath conditions (158). We were unable to test this as we were only able to conduct experiments for 8 hours in vitro before myometrial contractility began to decline (presumably due to cell death and lack of energy substrates in the tissue). It is possible therefore that 17OHPC requires either prolonged incubation (which cannot be tested in

vitro) or the contribution of other tissues or molecules that are present in vivo to exert an effect. It also remains unclear as to whether 17OHPC is active itself or requires metabolic activation. One suggested pathway of activation is hydrolysis of 17 OHP by plasma and tissue esterases, but a recent study showed that this does not occur in vitro, suggesting again either different effects in vivo or alternative mechanisms of metabolism (159). Progesterone and 17OHPC may potentially act upon different receptors in vitro and in vivo. For example, 17OHPC has recently been found to have lower affinity than progesterone for the progesterone PR receptor (160). These differences may account for the greater efficacy of progesterone compared with 17OHPC on contractility *in vitro*.

In this study, the speed of the progesterone response suggests a non-genomic effect. However by use of selective inhibitors, we clearly demonstrate that progesterone does not exert its inhibition of myometrial contraction via a range of potassium channels, including $K_{ATP,}$ BK_{Ca}, IK_{Ca} and SK_{Ca}, despite a key role for them in contractile responses. Alternative pathways in which progesterone may exert an effect could be via sodium or chloride channels. There is some evidence to suggest that sodium channels may be up regulated in myometrium at late gestation (167) but the distinct roles of specific sodium channels is not clearly understood. Ion channels may well have regulatory pathways related to myometrial function but whether progesterone utilises them in its mechanism to prevent preterm labor remains unclear.

There are several non-genomic pathways by which progesterone may inhibit myometrial contractility in vitro. We consider the most likely to be those operating through the cell membrane progesterone receptors α, β and γ, which themselves are similar to G-protein coupled receptors (109) . An alternative non-genomic pathway of progesterone action is interaction with gamma amino butyric acid A ($GABA_A$) receptors. These receptors appear to be important in the emerging effects that progesterone has on the brain (97, 217). Additionally, initial studies of rat myometrium $GABA_A$ antagonists prevented progesterone induced inhibition of contractions, suggesting that progesterone might mediate its action

via the GABA receptor (107). These results could not be replicated in more recent studies of human myometrium. A prior study found that relaxation produced by the above steroids was not blocked by the $GABA_A$ receptor antagonists, picrotoxin or bicuculline, but was reversed by calcium, they suggest that blockade of calcium influx appeared to be responsible this relaxation (108).

Thus the potential role of GABA receptors in the mechanism of action of progesterone in inhibiting myometrial contractions remains controversial, with progesterone's main genomic action in maintaining uterine quiescence is considered to result via the PR-B receptor (48, 77).

A previous study has reported enhanced responses to β-sympathomimetics following both in vivo and in vitro treatment with progesterone (104). However, we did not see an enhanced effect of any tocolytic, including levcromakalim (a K_{ATP} channel opener), nifedipine (a calcium channel blocker) and ritodrine (a β_2- agonist) in our in vitro progesterone groups. This is in contrast to previous work carried out by Chanrachakul et al, where they demonstrated an enhancement of the tocolytic effect of ritodrine using a lower concentration of progesterone (0.01 μM) in vitro (99). Their experimental design involved using oxytocin-induced contractions as opposed to spontaneous contractions in our study. Additionally, they used progesterone dissolved in ethanol (against appropriate ethanol controls) in contrast to our own study where we used progesterone in cyclodextrin. The differences between the Chanrachakul study and are own are hard to explain unless progesterone's effect in enhancing the tocolytic effects of ritodrine require either ethanol or stimulation with oxytocin to become apparent. Their experimental design involved using oxytocin-induced contractions as opposed to spontaneous contractions in our study. Lastly although a potential weakness of our study may be the use of lower segment myometrial biopsies, others have previously shown no difference between upper and lower segment biopsies in terms of myometrial contractility *in vitro* (147) .

Progesterone has been demonstrated to suppress the immune response in early pregnancy (181). Elovitz et al demonstrated that the progestational agent, medroxyprogesterone acetate, reduced COX-2, IL-1β and TNF-α mRNA expression in the myometrium of pregnant mice pretreated with LPS. These studies demonstrated that progestational agents decreased the incidence of inflammation-induced preterm parturition and medroxyprogesterone completely prevented preterm birth compared to progesterone which decreased the overall rate of preterm birth (207).Although it is tempting to speculate that this inhibition of production of inflammatory genes may be part of the mechanism of action by which progesterone affects myometrial contractility, again this would be a genomic effect that would take longer than the rapid effects on contractility that we observed with progesterone.

In conclusion we have clearly demonstrated that progesterone but not 17OHPC inhibits myometrial contractility in vitro, however, this affect is not achieved through the principal potassium channels. Further we show that progesterone does not enhance the inhibitory response of tocolytics with respect to spontaneous contractions. The mechanism of action of progesterone both in inhibiting myometrial contractility and in preventing preterm birth therefore continues to warrant further investigation.

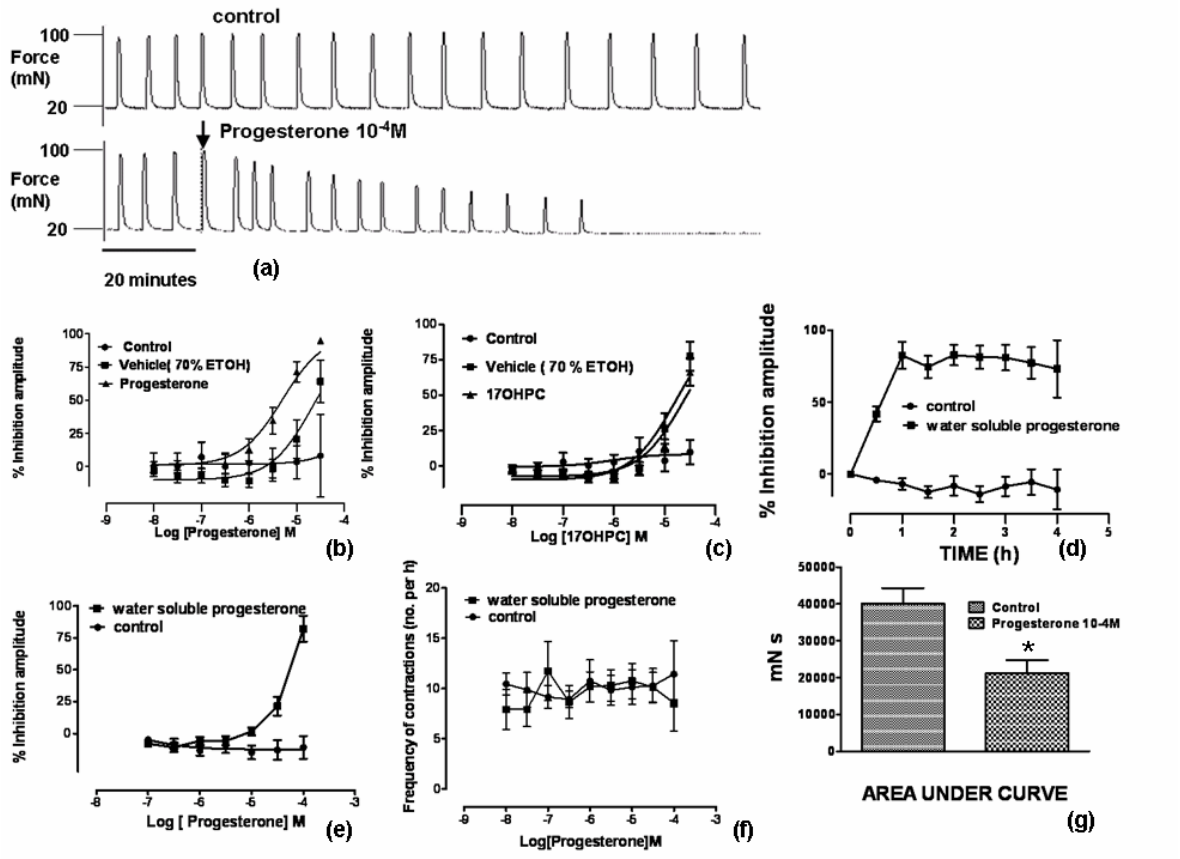

Figure 1(a) Contractility trace showing the stability of rhythmic contraction of myometrial strips and inhibition of activity by progesterone (100μM). Concentration-response curves showing the effects of progesterone and the appropriate dilution of vehicle (70% ethanol) n≥9 (b) and 17OHPC n≥6(c). Time course assessment (d) of a maximal concentration water soluble progesterone,100μM over 4 hours on the amplitude of contractions. Concentration-response curves for amplitude of contractions (e) and frequency of contractions (f) both for water soluble progesterone n≥5. Activity integral assessment of control versus water soluble progesterone (g) *$p<0.05$ n≥6.

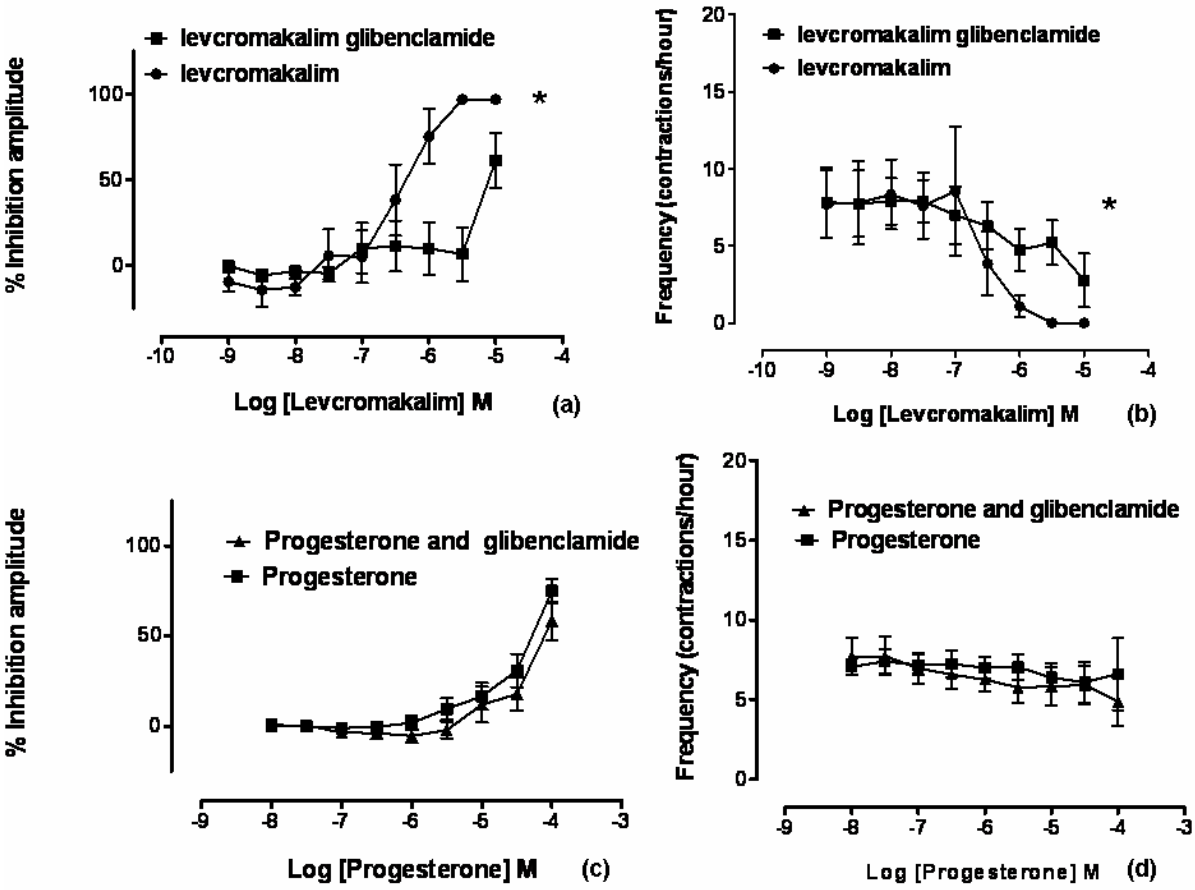

Figure 2. Concentration-response curves showing the effects of levcromakalim and progesterone on amplitude (a and c) and frequency (b and d) of myometrial contractions. The effects of glibenclamide (1.5 µM) on the actions of levcromakalim and progesterone are also shown n≥7.The right shift in the curve is evident, * $p<0.05$ indicates a difference between the response of contractile amplitude and frequency levcromakalim , and in the presence of its antagonist glibenclamide (ANOVA).

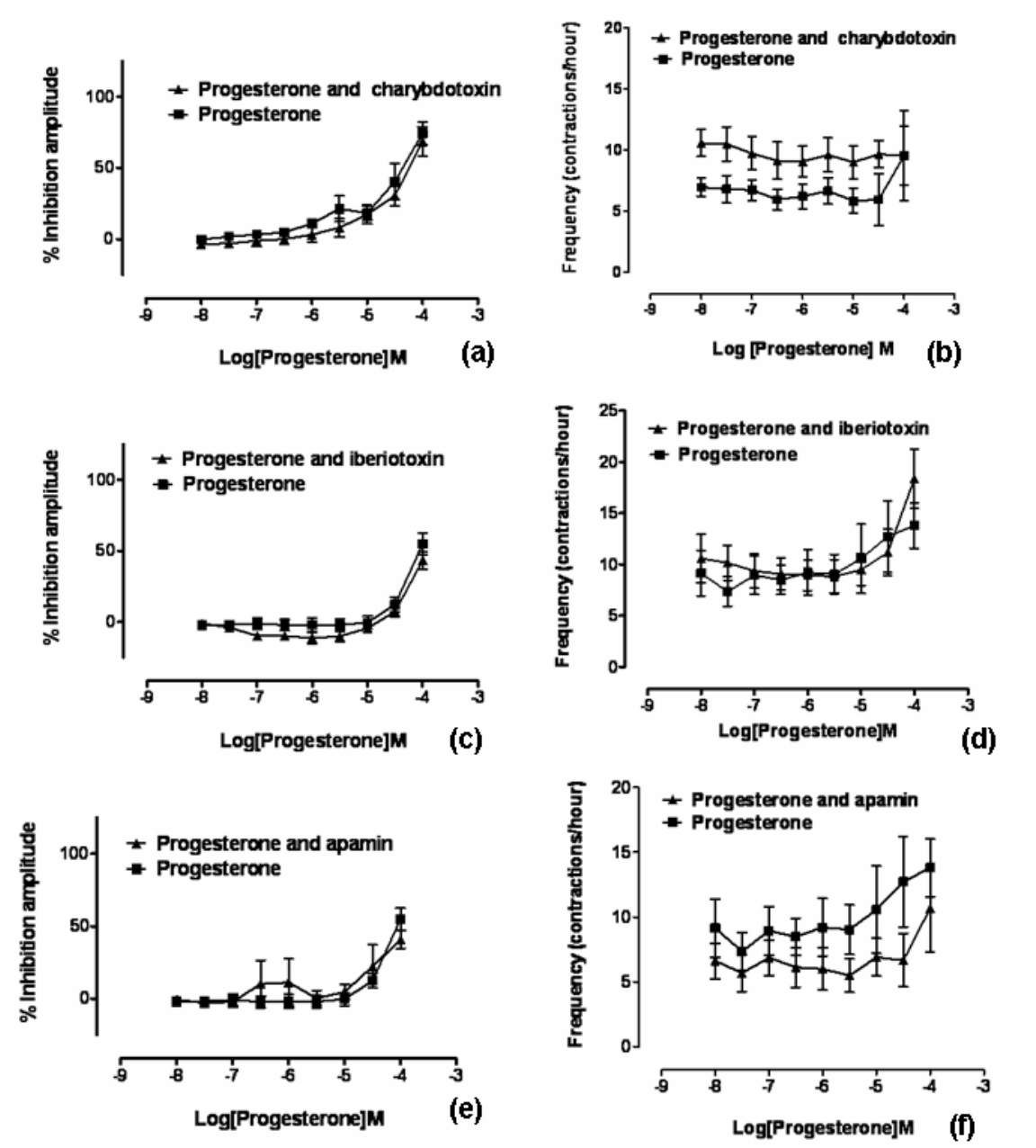

Figure 3. Concentration-response curves showing how changes in amplitude and frequent of myometrial contractions induced by progesterone are influenced by the presence of charybdotoxin n≥6 (a, b), iberiotoxin n≥6 (c, d) and apamin n≥6 (e, f). There was no significant difference in the response of contractions to progesterone in the presence of any of the potassium channel agents tested (ANOVA).

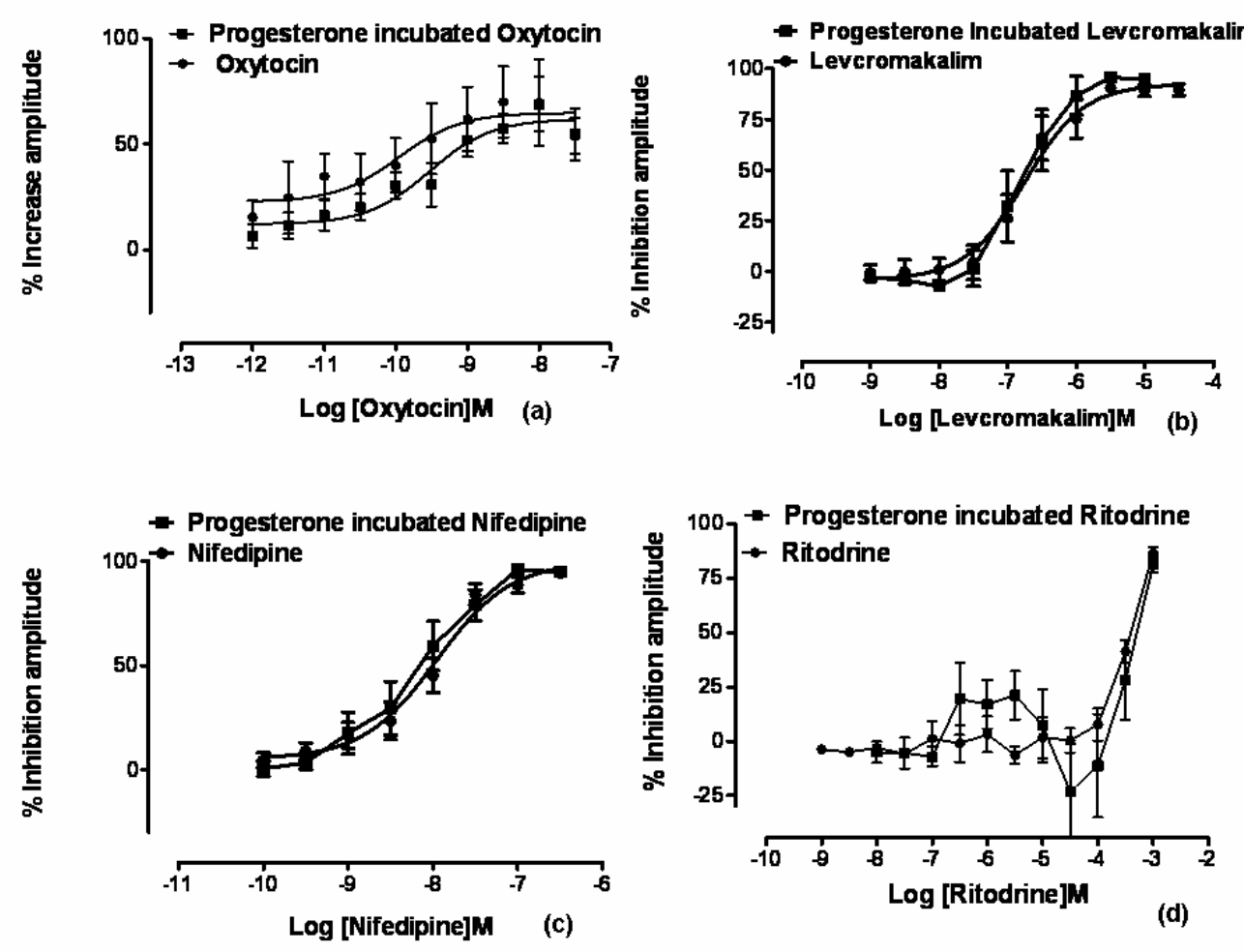

Figure 4. Concentration-response curves showing the effects of oxytocin (a), levcromakalim (b), nifedipine (c) and ritodrine (d) on the amplitude of myometrial contractility *in vitro* following incubation with progesterone 1 μM n≥6 . There was no significant difference in any of the drugs tested after the incubation with progesterone (ANOVA).

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