Contractile Properties of Multiple Pregnancy Myometrium

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Abstract

Multiple pregnancies are associated with higher rates of perinatal mortality than singleton pregnancies. This is due to the higher rates of preterm delivery and its associated complications. However it is not understood why there is a difference in gestation between multiple pregnancies and singleton, and what mechanism is responsible for this.

This thesis explores the possible different myometrial properties between multiple pregnancies and singletons, and the effect of progesterone on the uterine contractility by placing myometrial strips onto a force transducer, which recorded the trace reflecting the contractility of the strip. In a physiological saline solution there were no significant differences between the myometrial contractility properties of multiple pregnancies and singletons.

The effect of progesterone showed a dose response relationship in singleton and twin myometrium as when incremental doses of progesterone were applied, greater amounts of reduction in force was exhibited. It was found that there were significant differences between twin and singleton myometrium at 10, 100μM progesterone with there being a significantly greater effect in singleton myometrium. This reduced effect in twins may explain why there was no effect found to prevent preterm birth in twins (Norman et al 2008), and suggests that a higher dose may have more beneficial in twins

A possible explanation for this difference in non genomic action could be due to the greater quantities of stretch applied to twin myometrium earlier on in pregnancy, and thus reducing the expression of the progesterone membrane receptor. This possible theory was explored by applied stretch on myometrial strips in 100μM progesterone. Although the paired stretched samples displayed a smaller response to progesterone, this was however not significant.

Preterm delivery could also be affected by the maternal diet, as poor maternal nutrition leads to poor intrauterine growth, and is more likely to be preterm. A literature search was performed for studies that focused on maternal nutrition, and weight gain. It was found that early weight gain was associated with larger birthweights, and that total weight gain should be directed by maternal Body Mass Index (BMI), however much work is still required to explore how much weight gain is required, due to successful varying suggestions. It became apparent that when dietary supports (e.g. dieticians) were used, there were smaller rates of

preterm delivery and preterm birth. Yet it was hard to clarify if this success was down to the dietary interventions or the content of the diet itself.

This work has shown that twin and singleton myometrium respond differently to progesterone which plays a key role in uterine quiescence. This difference in response may be the underlying trigger for preterm labour and that to improve the rates of preterm delivery we also need to think about the mother's diet as well.

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Abbreviations

AC	Adenyl Cyclase
ART	Assisted Reproductive Technology
AUC	Area under the curve (force integral)
AV	Arterio-venous anatomoses
BKca-	Large conductance calcium sensitive potassium channels
BMI	Body Mass Index
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CEMACH	Confidential Enquiry of Maternal and Child Health
Ca^{2+}	Calcium ions
cGMP	Cyclic guanosine monophosphate
COX-2	Cyclo-oxygenase 2
CX	Connexin
FSH	Follicle stimulating hormone
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulphonic acid
IKca	Intermediate conductance calcium sensitive potassium channels
IL-8	Interleukin 8
IOM	Institute of Medicine
IP_3	Inositol 1,4,5-triphosphate
IVF	In vitro fertilisation
K^+	Potassium ions
L-Type	Long lasting calcium channels
MAPK	Mitogen activated protein kinase
MLCK	Myosin Light Chain Kinase
mPR	Membrane progesterone receptor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ions
NK-Kb	Nuclear factor –Kb
ONS	Office of National Statistics
PKA	Protein Kinase A
PMCA	Ca^{2+} ATPase transporter
PRA	Progesterone nuclear receptor A

PRB	Progesterone nuclear receptor B
RNA	Ribonucleic acid
RU486	Mifepristone
SKca	Short conductance calcium sensitive potassium channels
SR	Sarcoplasmic reticulum
TRAP	Twin Reversed Arterial Perfusion
TTTS	Twin to twin transfusion syndrome
T-type	Transient calcium channels

Chapter 1: Introduction

1.1 Abstract

The uterus undergoes great stretch and expansion as a result of the growing fetus and placenta and the accumulation of amniotic fluid through pregnancy. The mechanism that initiates contractions and why these occur early in women who deliver preterm are still largely uncertain, despite increased knowledge on uterine structure and physiology of contractions. This chapter will discuss the general anatomy and physiology of the uterus and then focus on myometrial contractility, with special emphasis on multiple pregnancies and their associated complications, and on current tocolytic drugs with an overview of their actions.

One of main aims of this work was to explore the actions of progesterone, therefore its role in labor is discussed as well as its molecular interactions in the uterus.

This chapter will then conclude with the specific aims of the thesis.

1.2 The uterus

For many centuries it was believed that the uterus was a multi-chambered organ, and moved around the body, but it was not until the renaissance that dissection became legal and the great minds of Leonardo da Vinci and Vesalius used their artistic and scientific skills to lay the foundations of our knowledge today. It was found that the uterus was anchored in the pelvis by ligaments, and that it was a one chambered organ with its own blood supply and nervous system.

1.2.1 Gross anatomy and embryology

The human uterus in a sexually mature non pregnant female is 7.5 cm by 5 cm, and is found posterior to the bladder and anterior to the pouch of Douglas. In the majority of women it is anteverted, with 10% having a retroverted uterus where the fundus is anterior to the cervix. Anteriorly, the uterus is covered by the peritoneum which reflects the bladder surface, and forms a fold in-between the bladder and the uterus known as the uterovesical fold. Posterior to the uterus the peritoneum forms another fold separating the uterus from the rectum and sigmoid colon called the recto-uterine pouch or more widely known as the pouch of Douglas.

The uterus is supported weakly by the anterior and posterior ligaments, with the broad ligament enveloping the uterus as well as the fallopian tubes, blood vessels and nerve endings. The round ligament also extends from the anterior surface of the uterus from below and anterior of the lateral cornua to the pelvic side wall. This becomes thickened in pregnancy and stretches. The strongest support comes from the utero-sacral ligaments that extend posteriorly to the sacrum, and the transverse cervical ligaments.

The uterus is a highly anatomised organ, and blood can be redirected easily. The source of the uterine blood supply is the common iliac artery, which divides into the external and internal arteries, with the internal artery becoming the uterine artery and the ovarian arteries. The uterine artery divides anteriorly and posteriorly becoming the anterior arcuate artery and the posterior arcuate artery (Bannister 1995).

The uterus is made up of the body, isthmus and the cervix. There are three layers of the uterus: the serosal layer of peritoneum, myometrium and the endometrium. The myometrium is made up of smooth muscle cells, which are responsible for the uterus contracting in labour, and the endometrium is a highly vascularised glandular lining that is shed during menstruation.
It develops from the paramesonephric duct and by 10 weeks the uterine tubes have formed (Mitchell 2005).

1.2.2 The structure of the myometrium

The myometrium is made up of two muscle layers, an outer longitudinal layer (stratum subserosum) and an interior circular layer (stratum supravasculare). The outer layer consists of a network of smooth muscle bundles whose size and number are regulated by steroid hormones and the distension of the uterus due to the expanding fetus during pregnancy. The myocytes vary from 5 to 100µm in diameter, 300 to 600µm in length, and have an average volume of 21000mm^3.
The myocytes consist of a thick myosin filament (15nm), a thick actin filament (6-8nm) and intermediate filaments of desmin, and vimentin (10nm), as well as microtubules. These smooth muscle cells are myogenic, and are able to spontaneously depolarize without requiring stimulation from a hormonal or neurological source (Garfield 2007).

1.2.3 Membrane potential

The resting membrane potential of the myometrium is largely determined by the membrane conductance to potassium ions (K^+). It varies in pregnancy from around -40 to -50 mV in early pregnancy, becoming more negatively charged in mid pregnancy to -60 mV and then finally depolarizing somewhat to around -45mV at term. This potential difference occurs due to a more negatively charged intracellular environment created by a higher extracellular concentration of positive ions such as calcium (Ca^{2+}), sodium (Na^+) (Sanborn 2002), and maintained by an efflux of intracellular K^+ ions (Brainard 2007). The extracellular concentration of Ca^{2+} is about 10^{-3}M, and 10^{-7}M in the intracellular fluid (Kawarabayashi 1994).

When the membrane depolarizes to around -40 mV it reaches the threshold value for voltage-activated long lasting (L type) calcium channels to open and due to the concentration and electrical gradients, calcium ions influx into the cell (Mathew et al 2004).
There are two types of calcium channels: transient, and long lasting. The transient (T-type) is rapidly inactivated and has a single channel conductance of 12pS while the L type calcium channels has a conductance of 29pS and is slowly inactivated (Kawarabayashi 1994).
Sodium ions help maintain tone, with two channels Na 2.1 and Nav2.3 found in the human myometrium, yet a high percentage are inactivated at the resting potential, and their full role in the myometrium is unknown (Brainard 2007).

1.2.4 Potassium ions and repolarization

In order for the cell membrane to repolarise it has to reverse the disruption of the cell membrane potential by using an efflux of positively charged potassium ions. During this time of repolarization an action potential cannot be started. There are a variety of different potassium channels that allow the potassium ions to leave the cell; Ca^{2+} sensitive, voltage-gated, adenosine triphosphate (ATP) sensitive channels.

The Ca^{2+} sensitive channels can be further subdivided into three groups: large conductance (BKca), intermediate (IKca) , and small (SKca) channels, with the BKca having the predominant role in the human myometrium. BKca channels are sensitive to voltage as well as calcium. They have a basic structure of S1-S6 transmembrane domains plus an additional four hydrophobic domains (S7-S10) forming a carboxy terminus. It is composed of α and β subunits, calcium sensitivity is increased when both subunits are expressed as a result of the β subunit, as a more negative voltage is required to open the channel. BKca are activated as a result of increased intracellular calcium levels (Khan et al 2001). Glucocorticoids and beta oestradiol upregulate the expression of BKca, which is encoded for by the signal gene KCNMA1 (Benkusky 2002, Xie1998). Isoforms of BKca channels have been shown to increase their expression in labor: these are less sensitive to calcium and voltage, and have an additional 44 amino acids in the first intracellular loop (Brainard 2007).

Voltage gated channels have a similar basic structure as Ca^{2+} sensitive channels with six transmembrane domains (S1-S6) yet have an additional pore forming hairpin loop. S4 is positively charged, and is sensitive to membrane voltage changes.

ATP sensitive channels are a type of inward recitifiers, having two membrane spanning regions M1 and M2, either side of a hairpin loop. however they play a small role in repolarization (Khan et al 2001). The predominant isoform found in the myometrium is Kir6.1, and it is inhibited by intracellular ATP and stimulated by magnesium adenosine diphosphate (Brainard 2007).
Calcium efflux mechanisms are also activated, in order for the cell to stabilise and reduce the intracellular calcium concentrations. Calcium efflux occurs via two transporters : Ca^{2+} ATPase (PMCA) 70% total efflux and Na^{+}-Ca^{+} exchanger 30% efflux.

The plasma membrane Ca^{2+} ATPase (PMCA) pumps Ca^{2+} out of the cell at the expense of ATP, and also counter-transports a proton potentially changing intracellular pH. It has four isoforms with PMCA 1&4 located in the uterus, with PMCA 4 having a predominant role (Moller et al 1996). The isoforms have ten membrane spanning regions, and the amino acid sequence is 80-90% homologous. PMCAs are regulated by calmodulin, protein kinase C as well as cGMP. PMCA 4 is mostly triggered by calmodulin and has the least basal activity.

The Na+-Ca+ exchanger is an electrogenic antiporter located on the plasma membrane. It exchanges three extracellular sodium ions for one intracellular calcium ion. The Na^{+} concentration gradient is maintained by a Na/K ATPase (Na+ pump). The sodium-calcium exchanger has nine membrane domains, and 98 amino acids (Floyd et al 2007).

1.2.5 Myometrial contractility

When calcium ions enter the cell they form a complex with a protein called calmodulin. This complex activates the enzyme Myosin Light Chain Kinase (MLCK). The now activated enzyme causes the phosphorylation of serine 19 on the myosin head, leading to cross bridges forming between myosin and actin and ultimately resulting in contraction of the myocyte (Wray 2007). See figure 1.1

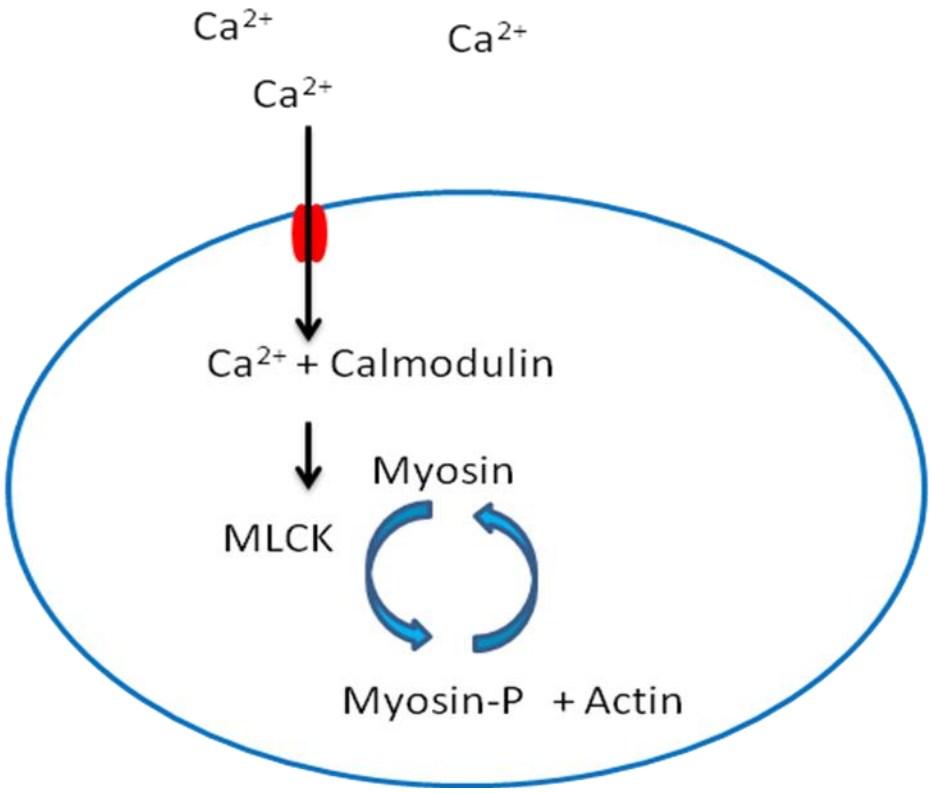

Figure 1.1: The mechanism of contraction (adapted from Wray 2007)

In order for the muscle to relax the MLCK pathway needs to be reversed and the myosin-actin bond broken. Myosin phosphatase dephosphorylates the myosin light chain inhibiting the cross bridge cycling. Calcium voltage gated channels also now close due to decreasing calcium levels, and this subsequently causes dissociation of the Ca^{2+}-calmodulin complex, and leading to inactivation of MLCK (Wray 2007).

1.2.6 The role of the sacroplasmic reticulum

The sarcoplasmic reticulum (SR) is an internal source of calcium, and occupies 5-7% of the cell volume. A Ca-ATPase pump on its membrane enables it to accumulate calcium from the cytoplasm and this also helps maintain a low resting Ca^{2+} in the cytoplasm. Many agonists allow the calcium to be released and help increase the force of the contraction by making it more readily available to form calmodulin complexes. For example, oxytocin binds to receptors on the cell membrane thereby activating phospholipase C. This produces inositol 1,4,5,-triphosphate (IP3) from phosphatidylinositol (3,4,5)-triphosphate. On the SR membrane there are IP_3 gated release channels, which when activated cause calcium to be

released into the cytoplasm (Wray 2007). There are also Ca-gated receptors (ryanodine receptors) on the SR membrane but their role in the uterus appears to be non-functional.

1.2.7 The role of cyclic adenosine monophosphate (cAMP) and progesterone

Intracellular cAMP enhances muscle relaxation through the cAMP-dependent protein kinase A (PKA) pathway. PKA is activated by cAMP when it binds to the regulatory units. The activated PKA causes phosphorylation of myosin light chain kinase, decreasing its activity and thus reducing the amount of phosphorylated myosin and thus contraction.

cAMP is produced when G protein coupled receptors interact with an agonist producing adenyl cyclase (AC). AC converts ATP to cAMP. AC has nine difference isoforms, and is found in low levels in all human tissues, and contains a NH_2 and has two hydrophobic domains consisting of 6 transmembrane helices separating two 40kDa cytoplasmic domains. The formation of cAMP is reversed by the enzyme phosphodiesterase breaking cAMP to 5 adenosine monophosphate (Price et al 2001).

Progesterone has been found to increase the accumulation of cAMP within the myometrium, and the concentration was reduced when progesterone was removed (Fu 1998). This rise in cAMP occurs as a result of the inhibition of phosphodiesterase (Gellerson et al 2009). *In vitro* work using myometrial samples taken from women in labor has also shown that cAMP is more sensitive to progesterone at 300μmol, while oestrogen at a concentration of 250μmol has no effect (Kofinas 1990).

1.2.8 Gap junctions

The transport of ions and molecules required for cell coupling, communication and survival are aided by gap junction proteins called connexins (CX) that form intercellular pathways helping molecules to move from cell to cell. Mammalian studies have found that when there was a gestation-dependent increase, there was an increase in the conduction capabilities between cells and that there was an increase in quantity at preterm and term labour (Blanks et al 2007).
The connexin believed to have the most impact on the myometrium is CX-43, and work in knockout mice found that if absent there was a delay in delivery (Doring et al 2006).

1.3 Current tocolytics and preterm birth

Current guidelines from the Royal College of Obstetricians and Gynaecologists (RCOG) recommend the use of tocolytics in apparent preterm labor to buy time to allow corticosteroids to improve fetal lung maturity or to facilitate *in utero* transfer. Currently there are no guidelines to support long term use of tocolytics (e.g. over one week). The guidelines do not advocate tocolytic use if there is any suspicion of infection.

Over the choice of drug to use, the guidelines state:

'If a tocolytic drug is to be used, ritodrine no longer is the best choice. Atosiban or nifedipine appear preferable as they have fewer adverse effects and seem to have comparable effectiveness. Atosiban is licensed for this usage in the UK but nidedipine is not.' (RCOG 2008)

1.3.1 Atosiban

Atosiban is an oxytocin receptor antagonist, and therefore competes with oxytocin for receptor sites in the decidua and myometrium (Blumenfeld et al 2009). Oxytocin is a hormone produced by the posterior pituitary gland, and increases the force and frequency of uterine contractions by causing receptors in uterine myocytes to open and cause a calcium influx thus stimulating contractions. This effect was augmented in multiple pregnancies (Turton et al 2009).

1.3.2 Ritodrine

Ritodine is an example of a beta agonist and causes intracellular cyclic AMP levels to increase and inactivates MLCK, by binding to beta 2 adrenergic receptors on the myocyte cell membrane. By inactivating the intracellular MLCK, it prevents the phosphorylation of calmodulin (Blumenfeld et al 2009). Ritodrine was widely used in the past although it had frequent side effects, including palpitations, tremor, nausea, headache, and even chest pain. Pulmonary oedema was a rare but very serious adverse event (RCOG 2008).

1.3.3 Magnesium sulphate

Magnesium sulphate works by inhibiting the voltage gated calcium channels, and MLCK. It is widely used in America, despite little evidence for its efficacy. A Cochrane review accessing the usefulness of magnesium sulphate for the prevention of preterm birth found it to be ineffective (Crowther 2002), although a more recent Cochrane review found it to have neuroprotective properties, reducing the risk of cerebral palsy (Doyle et al 2009). For the mother there is a risk of magnesium toxicity which, if serious can lead to respiratory depression and even cardiac arrest.

1.3.4 Calcium channel blockers

Calcium channel blockers (e.g. nifedipine) cause the relaxation of smooth muscles by inhibiting voltage dependent calcium channels thus preventing the re-uptake of calcium (Blumenfeld et al 2009). Nifedipine is not currently licensed in the United Kingdom for prevention of preterm delivery, although it is used, and one third of women will experience side effects, such as headaches. The optimal dose has yet to be defined. A Cochrane review found that calcium channel blockers reduced the incidence of neonatal respiratory distress syndrome, necrotising enterocolitis and intraventricular haemorrhage yet there was no significant difference in admission to the neonatal intensive care unit compared to other tocolytics (King et al 2003).

1.3.5 Progesterone

Progesterone is a hormone produced by the corpus luteum in the ovaries and by the placenta, and is discussed in further detail in another chapter. A recent Cochrane review which evaluated the use of progesterone for prevention of preterm birth concluded that further research was necessary to find the most effective method of administering progesterone in singleton and multiple pregnancies (Dodd 2009).

1.3.5.1 A history of progesterone

In 1672 the first published work was issued describing the corpus luteum by Regner De Graff, associating it with the uterus and the fetus, however it was not until 1947 work by George Corner that it was shown that the corpus luteum produced progesterone and was responsible for maintaining early pregnancy by preparing the endometrial for implantation, and that it played a role in modifying myometrial activity (Corner 1974).

1.3.5.2 Role of progesterone in labor

Experiments with mice, rats, and rabbits have found that the progesterone produced by the corpus luteum helps to maintain pregnancy, and that there is a withdrawal prior to delivery as a result of destruction of the corpus luteum by prostaglandin $F_{2\alpha}$. Early human pregnancy follows a similar pattern, with the progesterone from the corpus luteum maintaining early pregnancy, however after the ninth week synthesis of progesterone occurs at the placenta.

Even though it has been observed in animals there is a reduction in progesterone levels prior to parturition, no such observations have been seen in humans until the delivery of the placenta occurs. In twin pregnancies progesterone plasma levels are double that found in singleton pregnancies at 26 weeks yet the progesterone-oestrogen ratio remains similar and does not decrease prior to labour. However work by Smith et al also found that there was a decrease in progesterone levels at the onset of labor and a reduction in the median of the ratio of progesterone-oestrogen from 4:1 to 1:1 at labour, finding a similar fall in preterm and twins (Smith et al 2009). Despite there being no dramatic change in the levels of progesterone it has led to various hypotheses about the direct mechanism that causes labor. One theory is that a change in the progesterone receptor ratio initiates a reduced response to progesterone, which in turn inhibits myometrial contractility (Astle et al 2003).

There are two types of progesterone receptor; progesterone A (PRA) and progesterone B (PRB). PRA is a dominant transrepressor of PRB mediated transcriptional activity in the myometrial cells, whereas PRB is the main ligand-dependent transcriptional activator of progesterone responsive genes. These isoforms originate from the chromosome 11q11-q23, a single gene that encodes for both isoforms. PRB has an extra 165 amino acids due to difference translational start sites and separate promoters. It is the ratio of the PR isoform's

messenger RNA that has been linked to progesterone withdrawal before labor, and which has been observed to increase the expression of PRA/PRB.

Transcription coupled polymerase chain reaction technology found that a functional progesterone withdrawal coincided with an activation of oestrogen promoting uterine contractility, with an increase in oestrogen receptor-α (Safakianaki 2006). However work by Condon et al failed to find PRA protein in nuclear form before or during labor, with mRNA levels low and unchanging(Condon et al 2006).They also found a truncated form of PRB in the human term myometrium, which lacks a larger N terminal segment than PRA. This other isoform of the progesterone nuclear receptor (progesterone receptor C)was found to increase prior to labor, and is absent from fundal myometrium (Zakar et al 2007).

1.3.5.3 Progesterone and co-activators in labor

When progesterone binds to an inactive receptor multi protein complex, it induces a change in the structure of the progesterone receptors within the cytoplasm, and thus stimulating the homodimerization of the PRs within the nucleus. The rate of gene transcription is dictated by regulating the number of RNA polymerase 2 complexes binding to the site, these complexes control the number of receptors that engage the co-activators. These co-activators help start the process of transcription by improving interaction between the protein complex and transcription apparatuses. The number of progesterone and steroid receptor co-activators appear to be reduced in the myometrium in labor compared to non labor along with the cAMP response element binding protein. This is another mechanism that may play a role in the initiation of labor (Sfakianaki et al 2006).

1.3.5.4 Progesterone and its anti-inflammatory effect

It is hypothesized that the anti inflammatory properties of progesterone may also play a role in labor. Progesterone inhibits nuclear factor kB (NF-kB). This transcription factor regulates the synthesis of prostaglandins by the enzyme cyclo-oxygenase 2 (COX-2), a process that results in inflammation. COX-2 is found in the amnion, the decidua of the chorion and the myometrium (Skakianaki et al 2006), with increased levels of COX-2 mRNA being upregulated in the fundus at labor (Hardy et al 2006). This inhibition of prostaglandins helps to maintain uterine quiescence. This is further supported by the use of the prostaglandin

antagonist mifiepristone in terminations of pregnancy. Prostaglandins help the uterus to expel the fetus by cervical ripening and uterine contraction (Gibb et al 1998).

1.3.5.5 Progesterone and the myometrium

Progesterone is secreted in early pregnancy by the corpus luteum allowing the embryo to implant, and ultimately produced by the placenta from 9 weeks. In later pregnancy it plays a role in uterine quiescence; reducing intracellular calcium by inhibiting voltage gated calcium channels (Jayasooriya 2009).

It is thought that progesterone causes uterine relaxation by amplifying levels of cAMP, and preventing its hydroxylation by cyclic nucleotide phosphodiesterase. However a study by Fu et al found that progesterone also increased the frequency and tone, yet decreased the amplitude of contractions, as well as increasing cAMP release in human myometrium (Fu et al 1998). However it should be noted there is still much uncertainty over the direct action of progesterone on the myometrium, and significant work needs to be done to help resolve this.

1.3.5.6 Progesterone's non genomic effect

With progesterone being found to have a rapid inhibitory action on myometrium amplitude *in vitro*, it must be working via a non genomic pathway. The exact mechanism however has not been fully elucidated, and is surrounded by much debate. It is believed this rapid action occurs via a membrane progesterone receptor rather than the nuclear receptor. Originally found in *Cynoscian nebulosus*, different isoforms of the membrane progesterone receptors α,β, and γ have now been established in the human cells with mPRα being predominantly found in the human myometrium, β in neural tissue, and γ in the kidney and gastrointestinal tract (Fernandes et al 2008). These receptors consists of seven transmembrane progesterone adiponectin Q receptors, and directly coupled to G proteins (Thomas 2008).

What is known is that the steroid can have many different effects on all species as well as different organs using the non genomic pathway. In the seatrout *Rana pipiens* progesterone initiated a temporary release of Ca^{2+} ions within the oocytes, following a decrease in the intercellular cAMP, and rise in cGMP. In spermatozoa, progesterone produced a dose

dependent rise in intracellular Ca^{2+} which was not inhibited by RU486 (Mifepristone) a nuclear progesterone inhibitor. 17α hydroxyprogesterone (a synthetic form of progesterone) has also been shown to initiate a rapid Ca^{2+} response in spermatozoa (Falkenstein et al 2000).

It has been confirmed that progesterone has no effect on potassium channels as was previously hypothesized, with potassium channels inhibitors having no effect on the progesterone inhibition of contraction (Anderson et al 2009). Patch clamp techniques have also found that progesterone had no acute effect on potassium (Knock et al 2001).
In vascular smooth muscle cells it was found that progesterone significantly reduced calcium influx through the L-type channels, when calcium levels were measured using a whole cell patch clamp technique decreasing the peak of the inward current to 65.7±4.3% (Barabagallo 2001).

In rat osteoblasts1nM progesterone increased IP_3 female and male osteoblasts and cAMP levels were not affected by 1pM-µM progesterone (Grosse et al 2000). Yet in the human myometrium using medroxyprogesterone acetate there was no effect on IP_3 and IP_3 calcium stores (Fomin et al 1999).As discussed previously application of progesterone was followed by an increase of cAMP (Fu et al 1998), it may be that this is via the mPRα receptor.

1.3.5.7 Genomic actions of progesterone

These actions occur via the nuclear progesterone receptors PRA and PRB. PRB is believed to be the principal receptor that helps maintain uterine quiescence, although PRA modulates transcription of genes, it also regulates PRB activity. PRB enhances relaxation by inhibiting oxytocin and prostaglandin receptor expression as well as reducing the expression of connexin 43 and gap junction formation (Mesiano and Walsh 2007).

1.4 Multiple pregnancies

Multiple pregnancies are when there are two or more fetuses present in the uterus. This can be confirmed by using routine ultrasound to confirm the presence of two or more heart beats, and establishing the chorionicity/amnionicity of the placenta. By using ultrasound it is also possible to detect any fetal abnormalities as well as the sex of the fetuses.

1.4.1 Classification of multiple pregnancies

Multiple pregnancies can occur as result of two individual oocytes being fertilized by two different sperm, known as dizygotic, or due to the division of a conceptus produced by one ovum and sperm, monozygotic. Triplets may be created from three separate zygotes or from two, with one of these dividing into monozygotic twins.

Dizygotic twins are non-identical and can be of either sex, and always have two different placentas and amnions therefore are known as dichorionic and diamniotic.
Whereas monozygotic twins are identical and can be dichorionic diamniotic or monochorionic diamniotic or even monochorionic monoamniotic depending on the time of the division. Figure 1.2 illustrates when division occurs and the resulting classification.

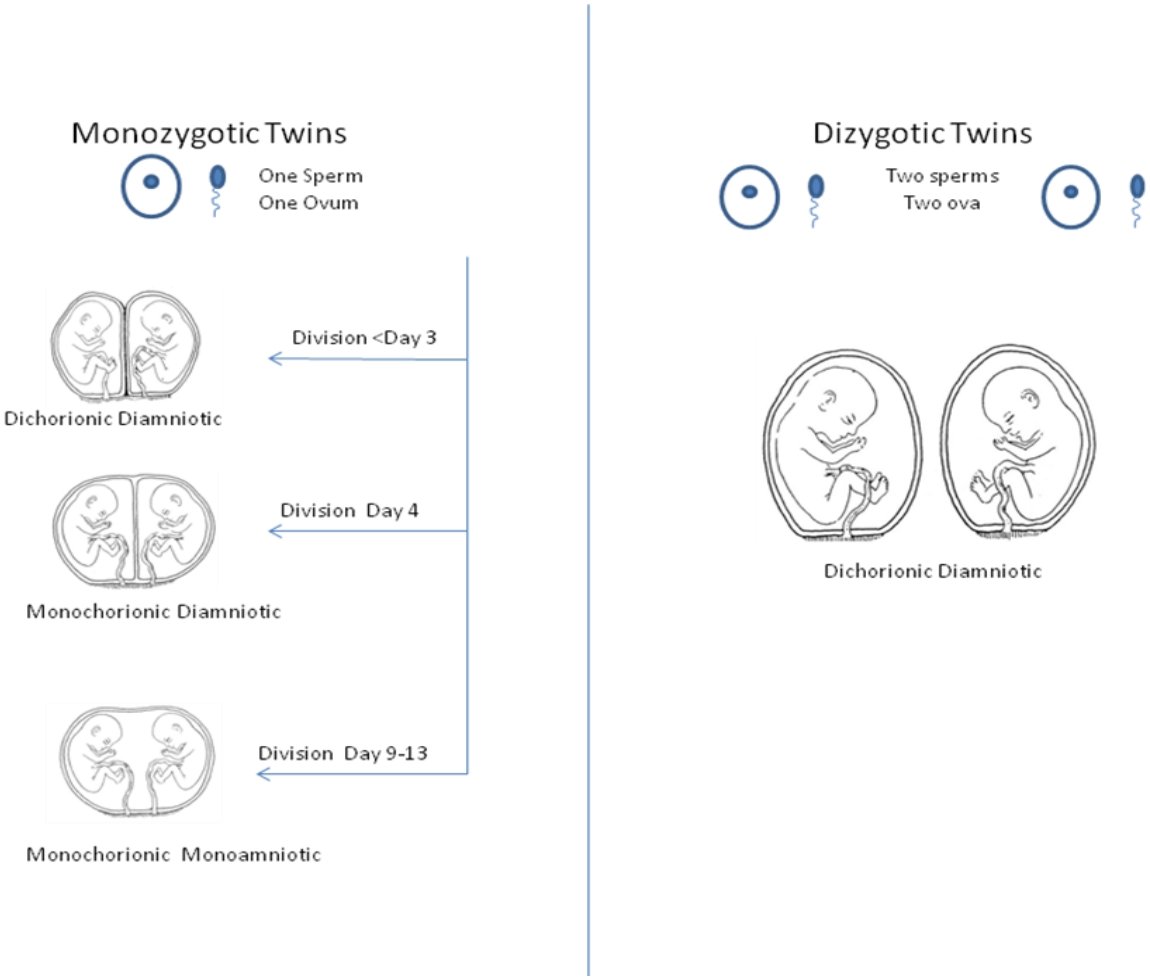

Figure1.2. Mechanisms of Twinning, Adapted from reference (Government of South Australian 2008).

It is important to establish the correct classification of the twins as there are specific complications associated with each classification that require monitoring.

To establish the chorionicity, fetal ultrasound can be performed at seven-nine weeks gestation. An observation of two placental masses and the lambda sign indicate dichorionic twins. The lambda sign is a triangular extension of the chorionic villi between the two layers of twin membrane, and was first described by Bessis and Papiernick in 1981. Monochorionic twins have a T sign where the membrane divides the amniotic space and reaches the placenta at a 90ºangle (Alhamdan et al 2009).

1.4.2. Aetiology

Dizygotic twins are associated with increased maternal age, parity, inheritance and high levels of follicle-Stimulating Hormone (FSH). Chromosome 3 has been indicated as the gene responsible, showing patterns of autosomal recessive and dominant inheritance, however the exact nature of the genetic mechanism is still yet to be fully understood. There has been links to the autosomal recessive gene being inherited along the maternal line of the family allowing the woman to produce two eggs. It has also been noted that the male needs to have high numbers of high quality sperm.

The use of In Vitro fertilisation (IVF) is associated with higher rates of dizygotic twins as a result of the implantation of two genetically different zygotes. 30-50% of twin pregnancies are associated with Assisted Reproductive Technology (ART), and over 75% of higher order pregnancies are due to ART (Verberg et al 2007).

1.4.3 Epidemiology

Over the last hundred years there has been a change in the pattern of twinning. Data from Sweden and Finland has showed a decrease from the 1930s to the 1950s and in Europe the incidence of twins reached an all time low in the 1970s: England and Wales 9.6 per 1000, France 9 per 1000. Over the next 20 years there was an increase of over 60% to 13.9 per 1000 in England and Wales, 14.4 per 1000 in France. In the USA there was an increase of 58% from 1972 to 1998. This dramatic increase is likely to be a result of the increased use of ART and lack of regulation in the USA where the number of embryos transferred to the uterus is based on an individual's situation and recommended guidelines rather than statutory law (ASRM 2009), hence the more dramatic increase in high order pregnancies (three or more) of 696%, compared to other countries such as France with 310%, and 430% in England and Wales in 1998 (Blondel et al 2002). In 2008 in the UK, there was 10,334 sets of twins born in the UK, while only 137 higher order pregnancies occurred (ONS 2008).

Multiple pregnancies have been on the rise as a result of ART. ART places two or more fertilized eggs into the uterus, assisting the chances of a zygote implanting and ultimately the birth of a fetus. Another technique is to give the mother hormones in order to cause multiple follicles to develop improving the chances of a ovum being released and increasing the

number available for removal in order to perform In Vitro Fertilisation(IVF). IVF is when conception occurs outside the uterus and an embryo is placed back in the uterus (Verberg et al 2007). In the UK 75.8% of IVF pregnancies resulted in singleton deliveries compared to 23.9% resulting in twins, but it is important to note that UK Law only allows the implantation of two embryos (Anderson et al 2008).

Despite this rise being mostly attributed to the increased use of ART, maternal age also plays a strong role, see Table 1.1 for age specific multiple birth rates for England and Wales and Table 1.2 for age specific multiple birth rates for the USA.

Year	All Ages	Under 20	20-24	25-29	30-34	35-39	40-44	45 and over
1998	14.4	6.3	9.5	12.8	17.9	21.0	19.7	56.7
2008	15.5	6.0	9.7	13.2	17.7	23.7	23.1	93.1

Table 1.1 Multiple Births per 1000 all maternities England and Wales (ONS 2008).

Year	All Ages	15-19	20-24	25-29	30-34	35-39	40-44	45-54
2006	33.7	16.5	23.2	31.5	42.6	51.9	58.8	212.1

Table 1.2: Multiple Births per 1000 all maternities USA (Martin et al 2009).

In the USA 65% of multiple births with a maternal age of 45 or over was due to ART, 36.3% for ages 40-44 and 22.3% for ages 35-39 and only 16% for 30-34. However for triplets with maternal ages 35-49 the majority were from ART. However maternal ages from 20-30 had at least 30% unexplained triplets. It is important to note that this study used natural conception rates from 1972 to indirectly apply age specific multiple birth rates for each cohort, in the assumption these figures were accurate for spontaneous conception as it was before the use of ART (Reynolds et al 2003). 30% of twin pregnancies are monozygotic, 25% of monozygotic twins are dichorionic (Alhamden et al 2009). 5% of all preterm birth are dichorionic, and 10% are monochorionic (Siddiqui, Fekrat 2005).

1.4.5 Maternal Complications

Twin pregnancies are associated with higher rates of nausea and vomiting affecting over half of all women with multiple pregnancies. Women with multiple pregnancies are also more likely to suffer 'minor ailments' of pregnancy such as heartburn, fatigue, constipation and a higher frequency of micturition due to compression on the bladder from the uterus, and a raised abdominal pressure adding to the discomfort of an already demanding pregnancy.

Gestational hypertension has a relative risk of 2.04 (95% CI 1.6-2.6) in twin pregnancies compared to singleton pregnancies. A similar pattern is seen in pre-eclampsia with a relative risk of 2.62 (95% CI 2.0-3.4). Risk of acute fatty liver also increases-a rare disease but often lethal disease that affects the mother in the third trimester and which can progress to renal failure and hypoglycaemia, requiring immediate delivery (Rao et al 2004). Placenta praevia is more common in twins: 3.9 per 1000 compared to 2.8 per 1000 singletons (Siddiqui and Fekrat 2005).

1.4.6 Fetal complications

Twins are associated with higher rates of miscarriage, fetal loss, intra uterine growth restriction and preterm delivery. In general all types of twins have a tenfold risk of being small for gestational age, and the average length of gestation is 35 weeks compared to that of singletons whose average length is 39 weeks gestation (Rao et al 2004).

1.4.6.1 Fetal mortality

Multiple births are associated with higher rates of perinatal mortality and stillbirth rates. In 2000 the stillbirth (a fetus born with no signs of life after 24 weeks) rate was 17.7 per 1000 for twins and 28.4 per 1000 for high order births, compared to the singleton rates of 5 per 1000, which in 2003 rose to 5.3 for singletons, 19.6 for twins and 60.9 for high order births declining to 4.9, 12.2 and 20.5 in 2006 (CEMACH 2006).

1.4.6.2 Prematurity in twins

Twin pregnancies are associated with higher rates of preterm delivery with rates in Canada of 50.2%, USA 53.7 % and France 43.7 % before 37 weeks from 1995-1997, compared to singletons who had a preterm rate of 5.9%, 9.8% and 4.6% respectively (Blondel 2002), with another study finding 8.4% of twins were small for their gestational age (Joseph et al 2001).

Despite advances in treatment for preterm births such as the introduction of steroids, surfactant, and the use of incubators, there are still very severe complications associated with preterm birth. For instance there is an increased risk of intraventricular haemorrhage, periventricular haemorrhagic infarction and periventricular leucomalacia with the risk increasing the earlier the gestational age, and these can lead to neurodevelopmental problems including cerebral palsy (Lissauer et al 2008).

Twins with a birth weight over 2500g have a greater incidence of cerebral palsy than in singletons, 3.9% compared to 1.9%, however no significant difference was found when the birthweight was under 2500g in singleton and twins. However the incidence of cerebral palsy was over 69% for both groups (Pharoah 2002).

Due to the inadequate development of the lung, preterm neonates can suffer from severe respiratory problems such as respiratory distress syndrome where there is a deficiency in the production of surfactant, and infections that can lead to long term respiratory complications in childhood and early adolescence.

Another concern for preterm neonates is the development of necrotising enterocolitis where bacterial invasion of the superficial mucosa of the gastrointestinal lining cause intramural gas, leading to small bowel syndrome. The incidence of necrotising enterocolitis increases as the birth weight and gestational age decrease, with a rate of 1 in 100 in preterm infants.

Despite the advances in treatment for preterm infants the most effective environment is the uterus and ideally the neonate delivered at term, current guidelines from the Royal College of Obstetricians and Gynaecologists recommended the use of Atosiban or Nifedipine however it is not recommended to use as maintenance of pregnancy rather than a 24 hour delay in order to allow the completion of action by corticosteroids to aid lung maturation as preterm infants

have immature alveoli and resultant low levels of surfactant. Surfactant prevents alveoli collapse and corticosteroids reduced the risk of intraventricular haemorrhage by the vasoconstriction of cerebral blood flow. However if there is hypercapnea vasodilation will occur (Roberts, Dalziel 2006).

The lack of guidelines for preterm labor stems from the lack of knowledge about what mechanism directly causes the initiation of labor. However more recent trials have found progesterone to be effective in reducing the reoccurrence of preterm delivery and morbidity in high risk singletons with a history of preterm delivery (Meis et al 2003). Trials in twins and triplets have since been carried out, however progesterone has been found to be ineffective at reducing preterm birth (Norman et al 2009, Briery et al 2009, Caritis et al 2009).

1.4.6.3 Fetal growth discordancy

Growth discordance is when there is a difference between the birth weight of the two fetuses. This can be classified as mild <15%, moderate 15-30%, or severe >30%. This can occur due to a variety of reasons such as a small placenta, placental dysfunction, fetal sex differences, and chromosomal abnormalities (Siddiqui, Fekrat 2005).

1.4.6.4.Twin specific complications

Monochorionic twin pregnancies have unique complications. Twin-to-twin Transfusion Syndrome (TTTS) occurs as result of an imbalance of placental blood flow from one twin (donor) to the other (recipient). This imbalance occurs as there are various types of anatomises on the placenta, some that allow the blood to flow in both directions, and others that restrict the blood flow to only one direction. For example arterio-venous (AV) and veno-arterial anatomises only allow blood to flow in one direction, so if there is higher number of AV anatomises TTTS occurs. This usually occurs between 15 and 26 weeks gestation, and is made up of 5 stages, which is detailed in table 1.3 (El Kateb, Ville 2008).

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Stage I	The bladder of the donor is still visible
Stage II	Bladder not visualised for 1 hour, Normal Doppler
Stage III	Doppler abnormalities in either fetus
Stage IV	Hydrops
Stage V	Demise in one or both twins

Table 1.3. Stages of twin to twin transfusion syndrome (El Kateb, Ville 2008)

TTTS is diagnosed on ultrasound by the presence of polyhydramnios in one sac with a deepest vertical pool of amniotic fluid (DVPAF) of at least 8 cm after 20 weeks, with oligohydramnios present in the other 'donor' twin's sac with a 2 cm DVPAF score. It is usually treated with laser therapy ablation.

Monozygotic twins are also at risk of Twin Reversed Arterial Perfusion sequence (TRAP) where one twin fails to develop normally and has an absent head, and upper limb structures as well as being acardiac. This occurs in 1% of pregnancies and is treated with diathermy to prevent blood going to the acardiac twin (Duncan 2004).

1.5 The effect of stretch on the uterus and protein expression

The uterus increases in size and weight as result of hypertrophy and hyperplasia due to the mechanical stretch applied by the growing fetus. This enhances the expression of the gap junction protein connexin 43, as well increasing the production of prostaglandins before labour (Salameh et al 2010). It was also found that COX-2 activity was increased when exposed to mechanical stress, and had greater activity in myometrium taken from labouring samples (Sooranna 2004).

Stretch and inflammation are both potential triggers for labour through IL-8, which is associated with infection-induced labor. Stretch indirectly affects IL-8 through the MAPK pathway, with mechanical stretch activating all three types of MAPK (Sooranna 2005).

1.6 Aims

Multiple pregnancies are associated with higher rates of morbidity and mortality compared with singletons, and a significant contributor to this is preterm labour and delivery. Currently there is no gold standard treatment for the prevention of preterm delivery and the pathophysiology is still not fully understood.

The first aim was to examine the difference in myometrial properties of multiple and singleton pregnancies, building on previous work in the laboratory.

The second aim was to explore the effects of progesterone and to examine if there is a dose response relationship, and to test the hypothesis that progesterone differs in its effect in multiple and singleton pregnancy myometrium. As progesterone plays a role in uterine quiescence, it has been used in various clinical trials for the prevention of preterm delivery (before 37 weeks) to be effective in high risk singleton pregnancies (Meiss et al 2003), but not in multiple pregnancies (Norman et al 2009, Briery et al 2009, Caritis et al 2009).

The third aim of this study was to examine the effect of stretch on progesterone response. Increased stretch could be the underlying reason why there is a reduced response to progesterone in twin pregnancies.

The final aim of this study is to review current dietary advice for multiple pregnancies as there is much debate and some advocate specific calorific intake and diet components in an attempt to reduce complications including preterm delivery. Currently there are no guidelines on nutrition for multiple pregnancies with some advocating a diet of over 3000 calories a day (Luke et al 2003) to a more conservative approach, and it is important to take into consideration the need of the mother as well as the fetus as obesity is associated with long term health complications.

Chapter 2: Methods

2.1 Abstract

This chapter will discuss the general methods and solutions used. It will describe how consent was gained, the dissection process used and how the experiment was performed. It will also explain what computer software was used and what was done to reduce bias.

2.2 Introduction

All experiments used human uterine tissue obtained from consenting women, having previously had the ethics approved by the national ethics committee. The anonymity of the patient was maintained by samples being collected and identified by a reference number only. Samples were allocated a unique reference number as part of the Myometrial Research Tissue Bank e.g. M00100. The Myometrial Research Tissue Bank is maintained and regulated at the Liverpool Women's Hospital, and all consent forms and proformas are securely kept there. Singleton samples were kindly consented for by the Research Midwives at the Liverpool Women's Hospital in pre-operative clinic. Multiple births samples were consented by the author or Doctors in the Multiple Birth Clinic at the Liverpool Women's Hospital.

2.3 Consent

When women were asked for consent they were given the following information:

- The Myometrial Research Tissue Bank collects and stores tissue taken at caesarean section.
- The surgeon will removed a small piece of tissue approx 2 x4cm after removing their baby(s)
- They will not have long term effects as a result of the biopsy, and previous experience has found that it will not delay healing or increase the risk of bleeding.
- Some pieces are used to study muscle contraction, or frozen or used to investigate other factors including ribonucleic acid (RNA).
- All women are allowed to change their mind at any time even after the sample has been taken and stored.
- There will be no financial rewards for the patient or the Research Tissue Bank
- Medical records that relate to the pregnancy/labour will be periodically examined
- Any information used will be treated securely and confidentially

If they were happy to consent then a carbon paper form was signed and dated. One copy was given to the patient, one to the research tissue back and one copy would be attached to the patient's hospital notes.

The majority of women were consented for an elective caesarean section as a result of breech presentation in twin one or in singleton pregnancy, medical advice if they had had

a previous caesaraen section. Samples obtained from Emergency Caesarean Section were due to fetal distress . Figure 2.1 shows the method of sample collection and what path samples followed e.g collection or loss of samples due to unexpected early emergency caesarean sections.
When consent was obtained a proforma was used to collect the patient details that were stored at the research bank at the Liverpool Women's Hospital.

2.2.2 Collection of tissue biopsy

The myometrial biopsies were removed post delivery of infant(s). When a sample was collected it was placed into Hank's Buffered solution in a 130ml sample pot, labelled with the unique identification sample number and the date is was taken.
It was then placed in a fridge at $5^{\circ}C$ or $4^{\circ}C$ and then transferred to the Physiological Laboratory. This occurred within 0 – 8 hours in 80% of the samples.
Once transferred to the laboratory the tissue was finely dissected using a light microscope (Nikon, UK). Each strip was dissected into 5 mm x 5 mm x 3mm. Once dissected metal foil clips would be attached to each end, and one end attached to a fixed point and the other to a force transducer. The force transducer was connected to a computer via an amplification box and recorded using the programme Axoscope version 8.0. See Figure 2.2 for an overview of biopsy method. The force was measured isometrically, by having resting tension on the strip so that when it spontaneously contracted it would 'pull' on the transducers.

The solution used to superfuse the tissue in a perfusion bath, was a physiological saline solution and the temperature was $37^{\circ}C$, and pH 7.4.

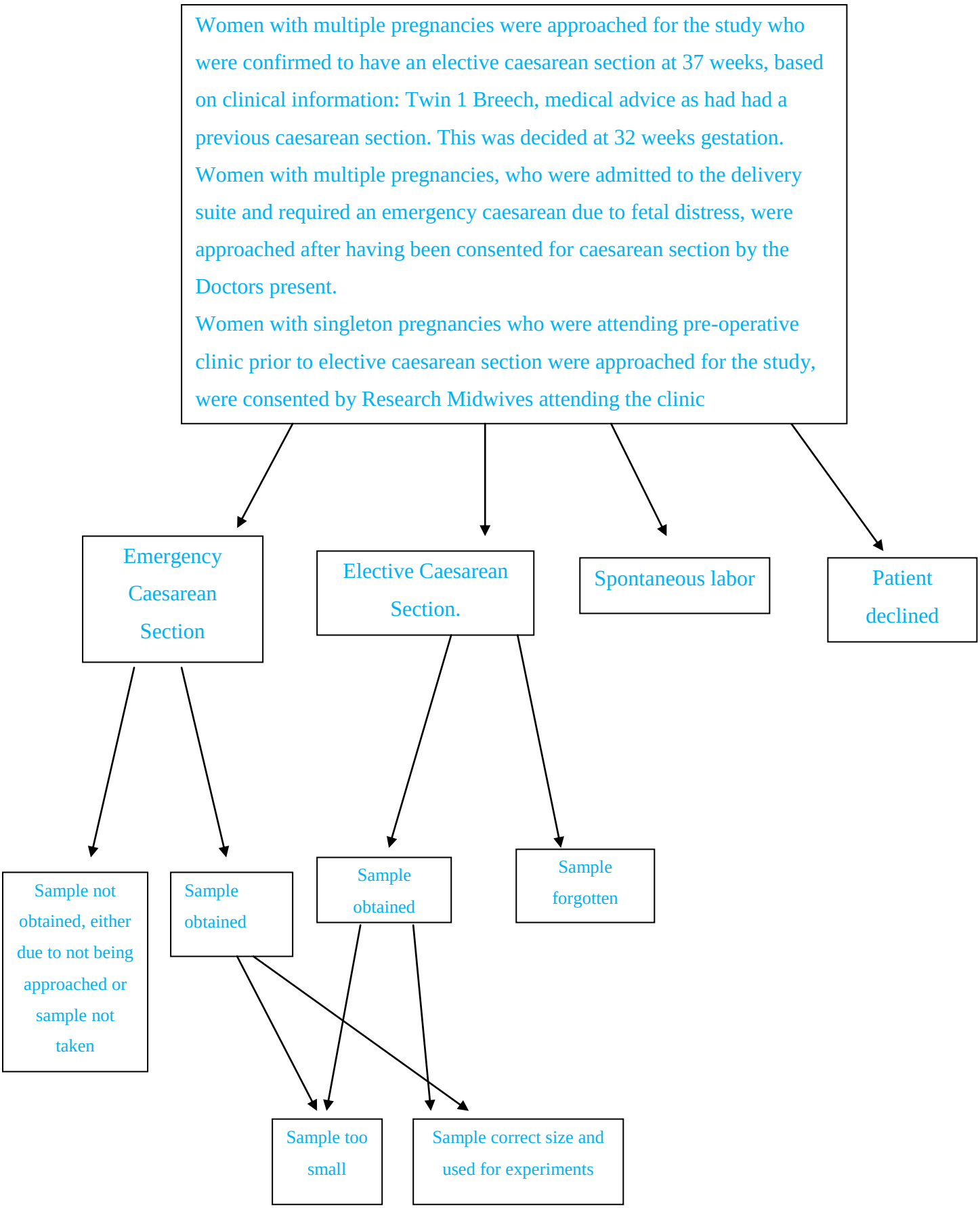


Figure 2.1. A flow chart showing how patients were recruited and potential outcomes

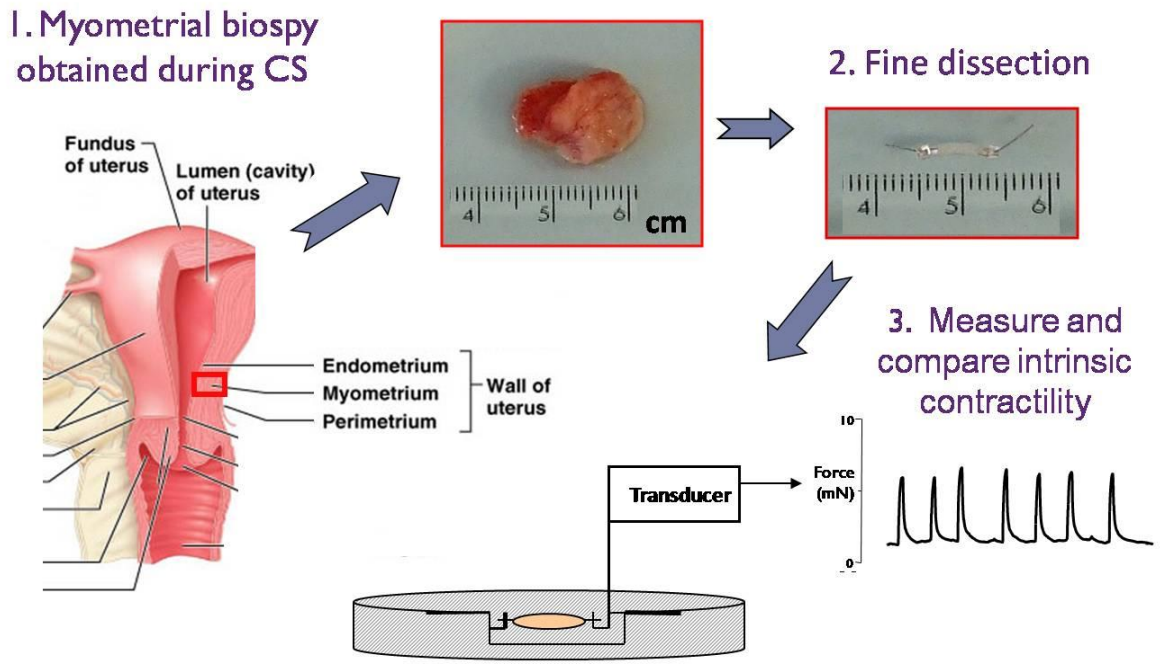


Figure 2.2 Overview of biopsy method (Kindly donated by S Arrowsmith.)

2.3 Solutions

All chemical were from Sigma (Dorset, UK), and were handled using the required safety precautions in the laboratory.

2.3.1 Physiological saline solution

Physiological saline solution was used to superfuse the sample, and the pH was adjusted to 7.4 using alkali or acid e.g. Na OH, HCL. The solutions were buffered with 4-(2-Hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES), and made up in double distilled water. See Table 2.1 for composition of the physiological saline solution.
All solutions were mixed using a magnetic stirrer.

Substance		Amount measured to make 1l(g)	Molarity(mmol)
Sodium chloride	154	8.99	153.90
Potassium chloride	5.4	0.40	5.36
Magnesium sulphate	1.2	0.29	2.40
HEPES	11.0	2.62	10.99
Glucose	8.0	1.44	7.90
Calcium Chloride	110.98	2.00(ml)	2.00

Table 2.1 Physiological saline solution

The calcium chloride was added prior to adjustment of pH.

A high K^+ ion solution of 40 mM potassium chloride was used to produce a maximal force contraction. This was made by adjusting the sodium chloride and potassium chloride quantities in a 1L solution to 6.99 g Sodium Chloride, and 2.98g potassium chloride. This was added for 2 minutes in order to elicit maximal force from the preparation, to enable standardization of results between samples.

Progesterone

A stock solution of progesterone was made using 70% ethanol to make a 10 mM progesterone. This was used to make the three concentrations of progesterone used: 1μM, 10μM, and 100μM. 100ml of 100μM progesterone was made using 100μl of the stock solution into 100ml of physiological saline solution. In order to minimise the amount of ethanol used the 100ml solution of 100μM progesterone was used to make the 10μM by adding 10ml of this solution to 90ml of Krebs. The 1μM progesterone was made adding 10ml of the 10μM progesterone solution to 90ml of Krebs solution. The stock solution was made by adding 0.3g of progesterone to 7ml of ethanol and once this had dissolved adding 3ml of diluted water.

2.3.3 Vehicle control solution

In order to confirm that any effect found was due to the progesterone rather than the 70% ethanol, a control solution was made using the strongest concentration of ethanol used. As the highest concentration of progesterone was 100µM, the dilution of from this concentration was tested. A solution of 1 in 1000 dilution of 70% ethanol was made adding 100µl of 70% ethanol to 100ml of physiological salt solution.

2.4 Force measurements

The contractions of the muscle strips were recorded by a tension transducer that was calibrated so that 1mN equalled a 1mV deflection, as shown by the Axoscope software. This was done by placing a 1mN weight on the transducer and by adjusting the amplification box appropriately.

Before the strip was clipped on using the foil metal clips, physiological saline solution was run continuously through for ten minutes in order to allow the heat resistor to have reached the appropriate temperature for the strip. Once the strip was attached it would be then stretched by 1.5mN which equated to 1.5mV by using the baseline on the axoscope programme.

The strip was then left until spontaneous contractions occurred. Once contractions had started no solutions or stretch were added until there was a regular control period. The next chapters will give more details about the specific experiments.

Chapter 3: The contractile properties of multiple pregnancy and singleton myometrium.

3.1 Abstract

Multiple pregnancies are associated with higher rates of preterm delivery than singleton pregnancies, however the direct cause for is unknown. Studying the difference between the properties of multiple birth and singleton myometrium will enable us to further our understanding as to why over 50% of twins deliver preterm.

Previous work has shown that twins have a higher mean weekly frequency uterine contractions using home monitoring systems (Hernandez et al 2008). Work in vitro has also shown that there was a significant longer duration (Turton et al 2008).

Biopsies were obtained for triplet, twin and singleton myometrium, and were then dissected. The biopsies were placed in a physiological salt solution perfusion bath, and were left until spontaneous contractions occurred.

There were no significant differences between the triplets, twins, and singletons for force, intercontraction interval, duration, and force integral.

3.2 Introduction

Multiple pregnancies are associated with higher rates of preterm delivery and perinatal mortality than singletons, as a consequence of the uterus containing two or more fetuses. However the direct link as to why multiple fetuses cause preterm delivery is unknown. In order to obtain a greater understanding, it is necessary to take into consideration how the multiple pregnancy uterus differs in its properties to a singleton uterus.

Work by Hernandez et al using daily uterine monitoring found that preterm twin pregnancies had a higher level of uterine activity in the third trimester compared to twin pregnancies that reached term. (Hernandez et al 2008) Uterine contraction frequency in singletons is also increased prior to preterm labor, but not in triplets (Newman et al1989).

In the twin uterus it has also been found to have a significantly higher mean weekly frequency of uterine contractions than singleton pregnancies, increasing each week prior to labor (Newman et al 1986).

There has only been one study of the *in vitro* characteristics of twin and singleton myometrium. This also reported differences. The amplitudes of force were similar between groups but it was also observed that there was a higher frequency of contractions in twins, and contractions were significantly longer in duration (Turton et al 2008).

This chapter will continue to investigate the different myometrial properties measuring the force amplitude, average intercontraction interval, average duration, and the force integral (area under the curve).

3.3 Methods

All patient samples were collected as stated in chapter two, and were consented before caesarean section took place. The sample was taken after delivery of the fetus(s). Once the sample was taken it was anonymized, and given a unique reference number.
Samples were collected from the Liverpool Women's Hospital, and placed in Hank's Solution. During dissection they were placed in a physiological Krebs salt solution with pH 7.4, which was used throughout the control experiments.

3.3.1 Contractile Experiments

Once samples were obtained and dissected into small strips of 10mm x 2 mm x2mm, foil metal clips were placed on both ends. The metal clips were placed on a force transducer, submerged in a water bath of Krebs at temperature 37ºC, with 1.5mN of stretch applied. The strips were left until spontaneous contractions occurred. A minimum of 4 contractions that were regular in force and intercontractile interval were used for analysis. This was recorded using Axoscope, and analysed using Origin 8 (supplied by the University of Liverpool).

3.3.2 Measurements

Using Origin pro 8 the force, intercontraction interval, duration and area under the curve (AUC) was calculated for each sample.
Force was measured by calculating the amplitude of the contraction, then using the average contraction in this control period. The amplitude was calculated by subtracting the y value at the base of the contraction from the y value at the peak of the contraction. See figure 3.1
Intercontractile interval was measured using the x value from the base of each contraction then subtracting it from the previous x value see figure 3.2 Due to the variability of samples., it was decided to record the time taken for each contraction rather than a selected time period.

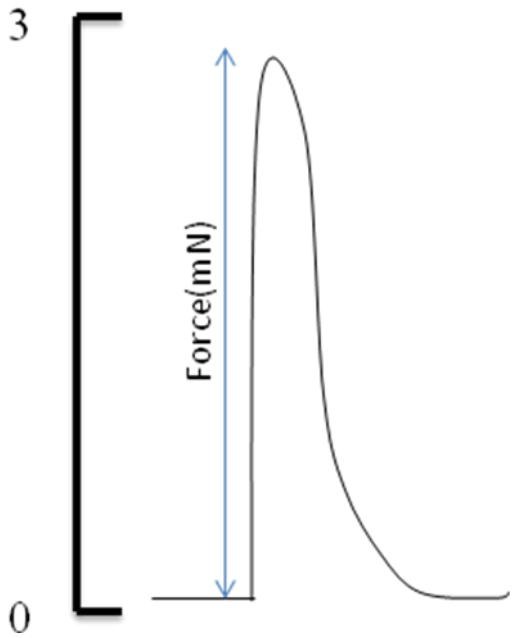


Figure 3.1 Measuring Force

The force amplitude was calculated subtracting the base y value from the peak y value.

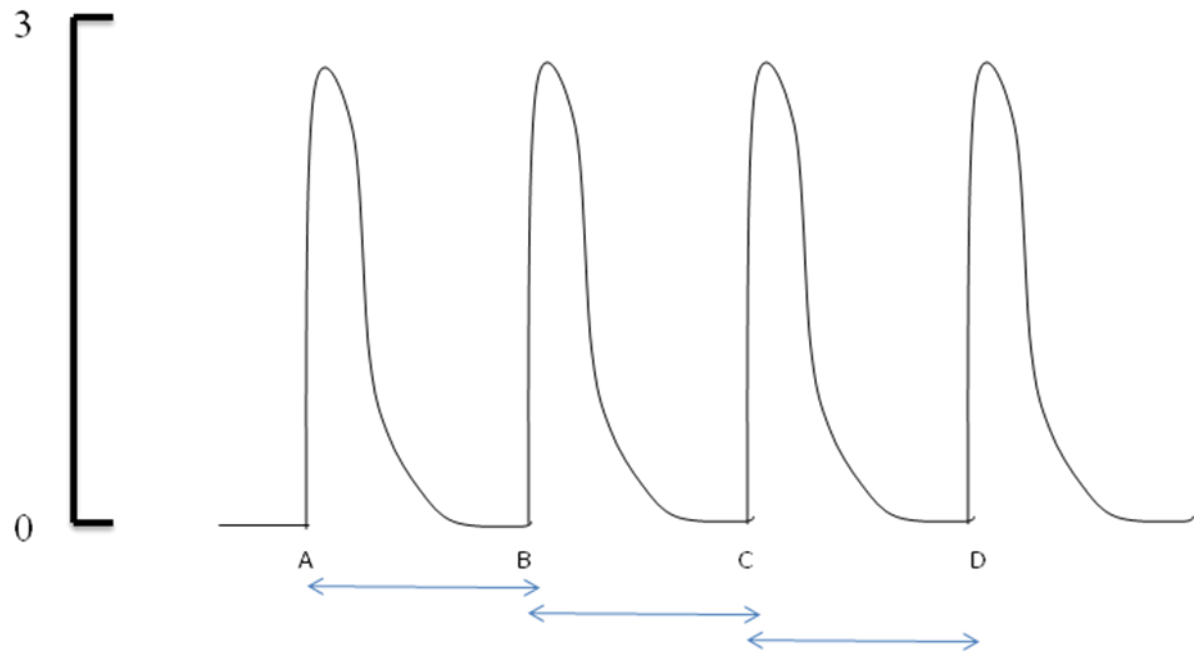


Figure 3.2 Measuring intercontraction interval

ABCD are the x values at the base of each contraction. The intercontraction interval was calculated by subtracting C from D, C-B, B-A. The average intercontraction interval was then calculated by adding the sum of these values then dividing by three.

The duration of the contraction was calculated at the midpoint of the force amplitude i.e. at half height. From this value the x value was noted for the period of contraction and relaxation, see figure 3.3.

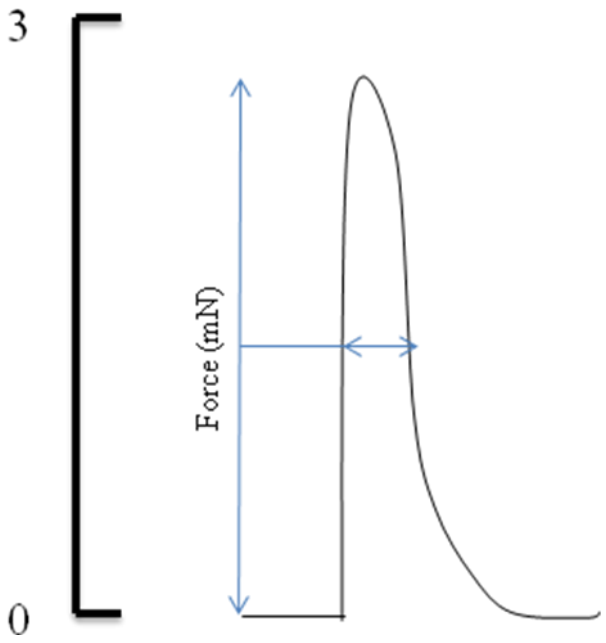


Figure 3.3 Measuring Duration

The midpoint of the force is used to calculate the duration by using the two x values that cross this point, and then calculating the difference. The average was then calculated from three contractions.

Area under the curve is the measurement of the mean force integral. This was calculated using the last twenty minutes of the control period, and using origin to give the area under the curve value. See Figure 3.4

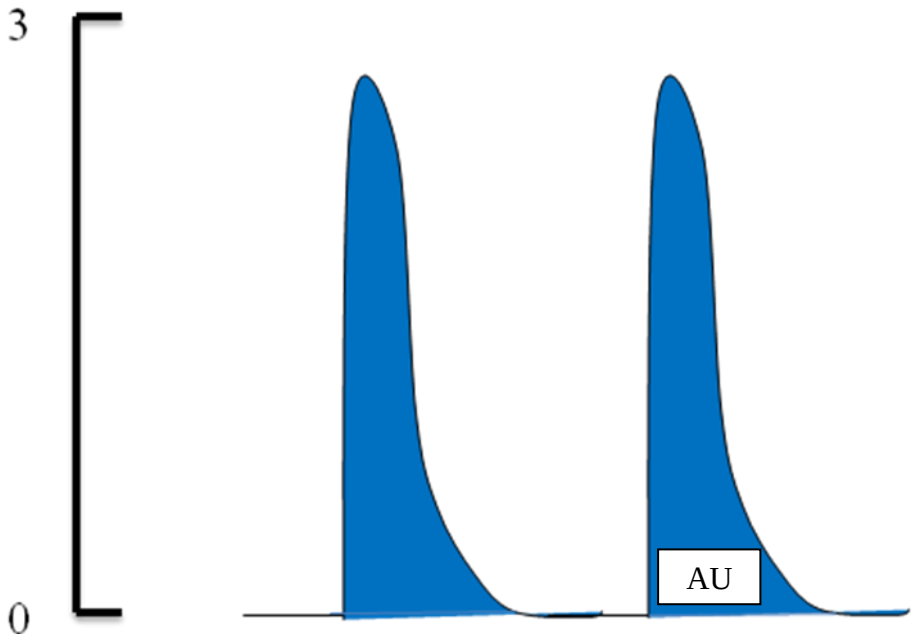


Figure 3.4. Calculating area under the curve

Using Origin 8.0 the area under the curve (as indicated by the blue above) was calculated for a ten minute period.

3.4 Results

3.4.1 Baseline characteristics

19 singleton, 10 twin and 2 triplet samples were obtained. 2 singleton samples were 31 and 33 weeks gestation and were a twin sample with a gestation of 27 weeks were also excluded. Due to the small n number of triplets they were also excluded from analysis. Table 3.1 shows the general characteristics of the two different groups. See Appendix A for full details of patient information.

Table 3.1 Characteristics of the groups

	Twin (n=9)	Singleton (n=17)
Median Gestation	37(37-37+4))	39+1(39+1-39+1)
Mean Maternal Age	33±1	30±2
Median Parity	1(0-1)	1(1-2)
Median Gravida	2(1-3)	2(2-3)
Mean BMI	23.2±1.5	25.4±1.4

*P=<0.05 Mann-Whitney Test

Using the Shapiro-Wilk W test to establish normal distribution it was found that the gestation, parity, and gravid were not normally distributed, and therefore the Mann Whitney Test was used to test for significance.

The gestation of each group was significantly different however all other factors were not significant. All data was obtained from elective caesarean sections due to a breech

presentation (Twin 1 breech) or a previous history of caesarean section. The emergency sections were the preterm samples that were not included in analysis.

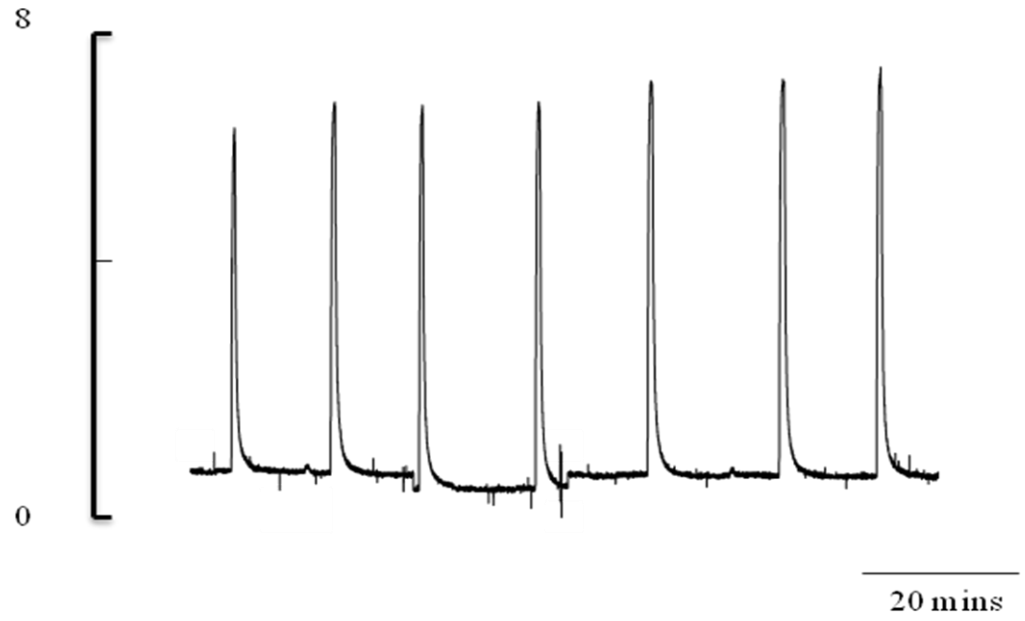


Figure 3.5 Singleton myometrium contractile trace in a physiological salt solution

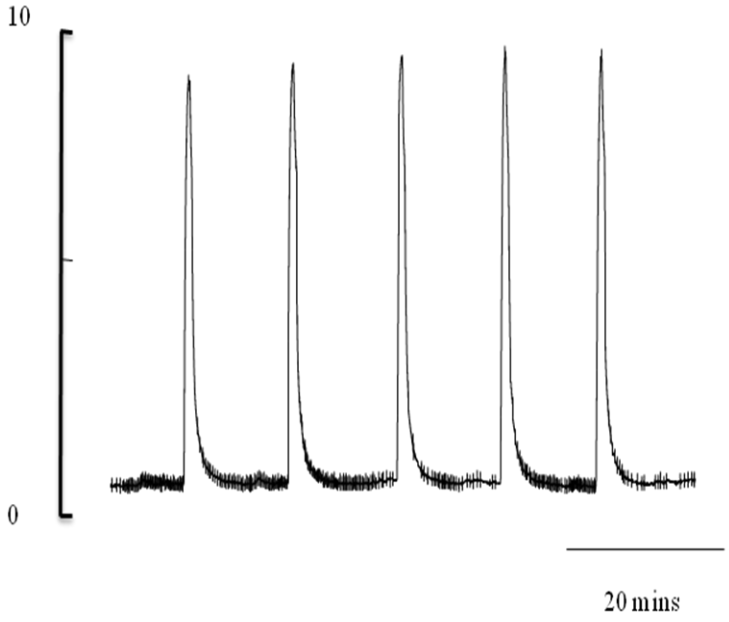


Figure 3.6 Twin myometrium contractile trace in a physiological salt solution

3.4.2 Force Amplitude

There was no significant difference between force amplitudes for each group; Twins (5.3±2.4), Singletons (4.9 ±1.2). See figure 3.7.

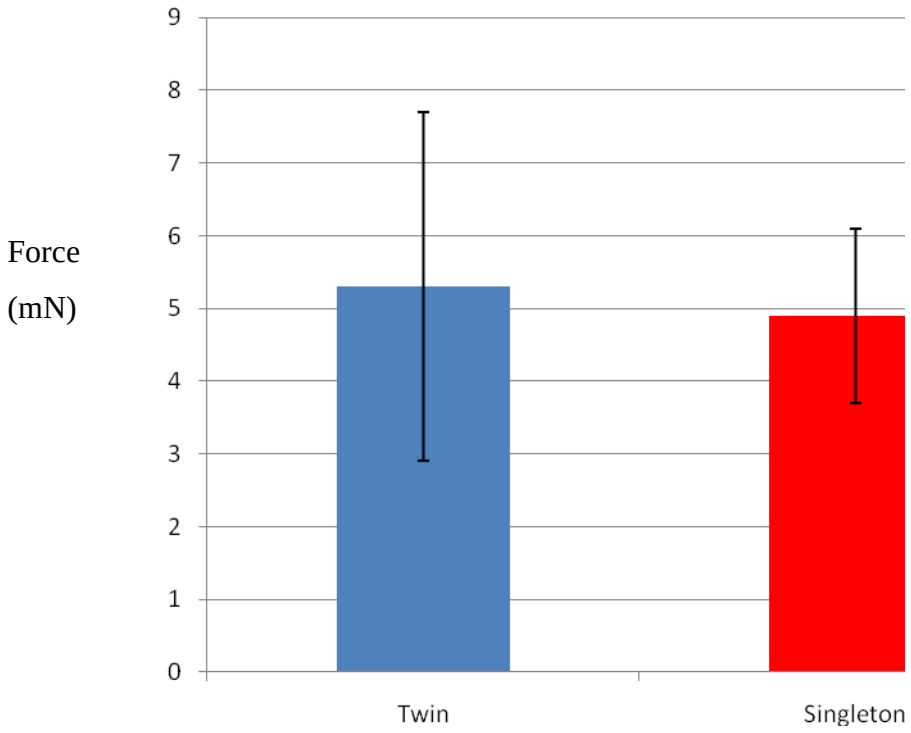


Figure 3.7. Mean force amplitude of twins and singletons.

3.4.3 Intercontraction interval

The mean intercontraction interval for twins, and singletons was not significant when $p \leq 0.05$, Twins 7.7±1.1mins, Singletons 8.7±3.6mins. See figure 3.8.

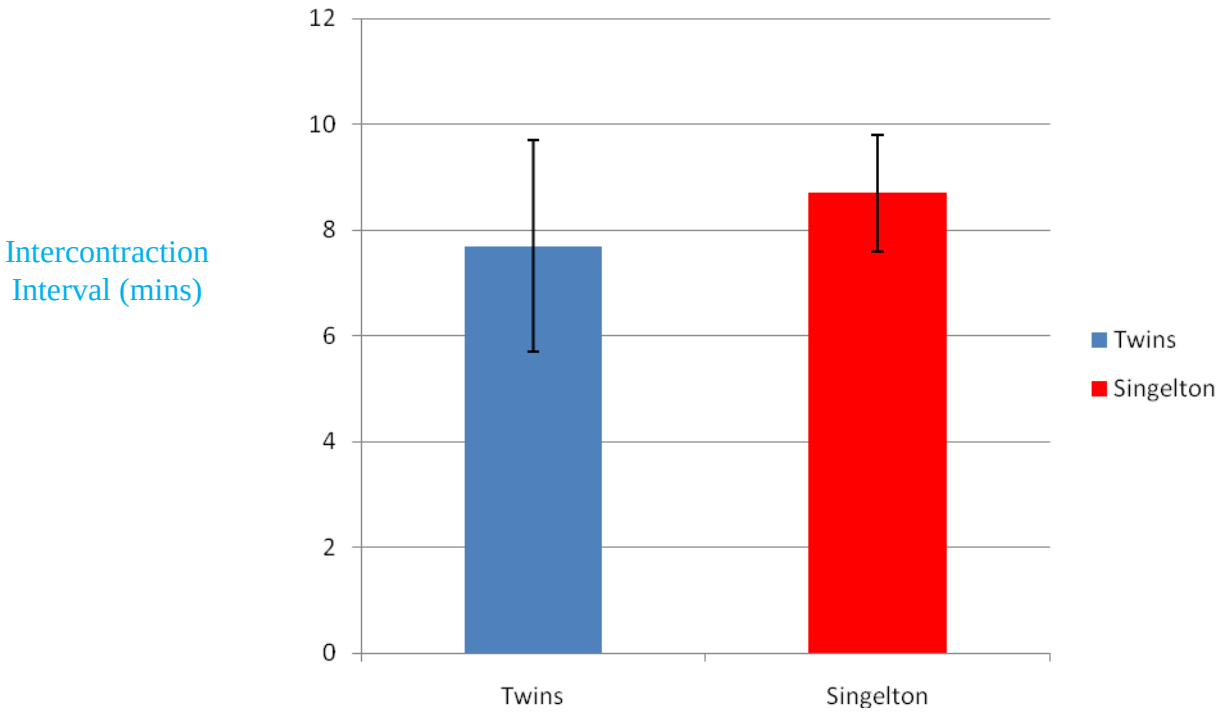


Figure 3.8. Intercontraction interval.

Although the singleton intercontraction interval was higher, it was not significantly different.

3.4.4 Duration

The average mean duration of contractions twins and singletons was 1.12±0.36, 0.73±0.05mins respectively. The twin samples had a longer duration out of all groups however this was not significant.

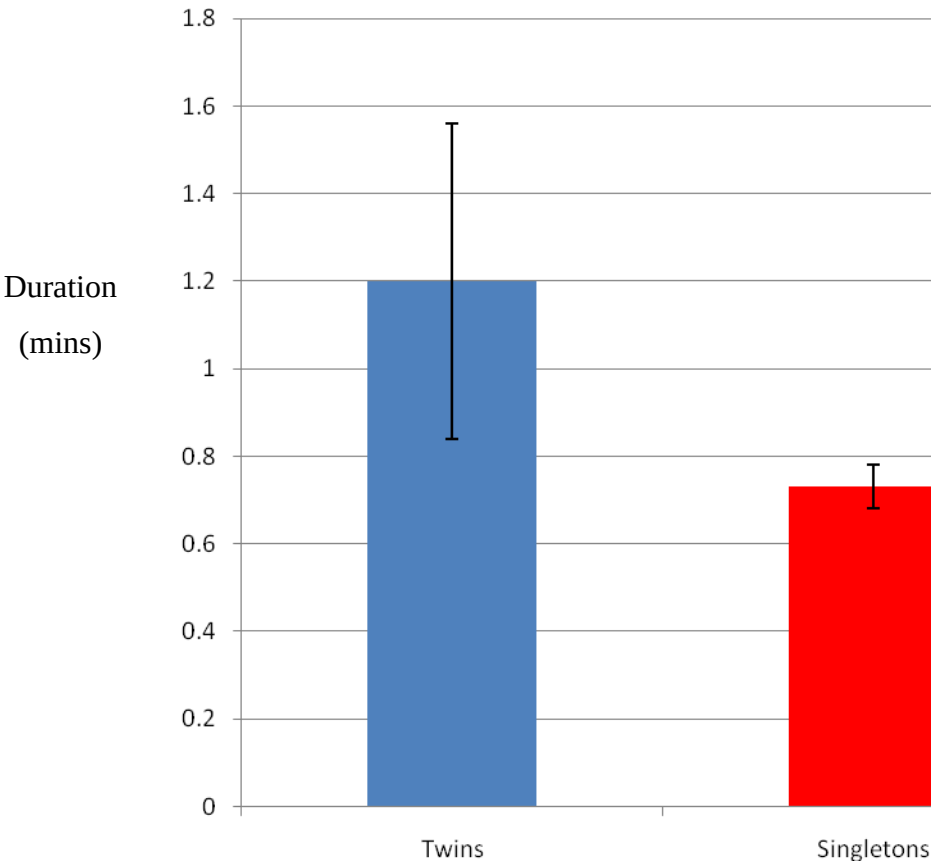


Figure 3.9 Mean duration.
The mean duration was longer in the twin duration than the singletons, however no significance was found.

3.4.5 Force Integral (Area under the Curve)

The average force integral was higher in both twins and singletons 17.6±8, and 18.8 ±3.5. See figure 3.10.

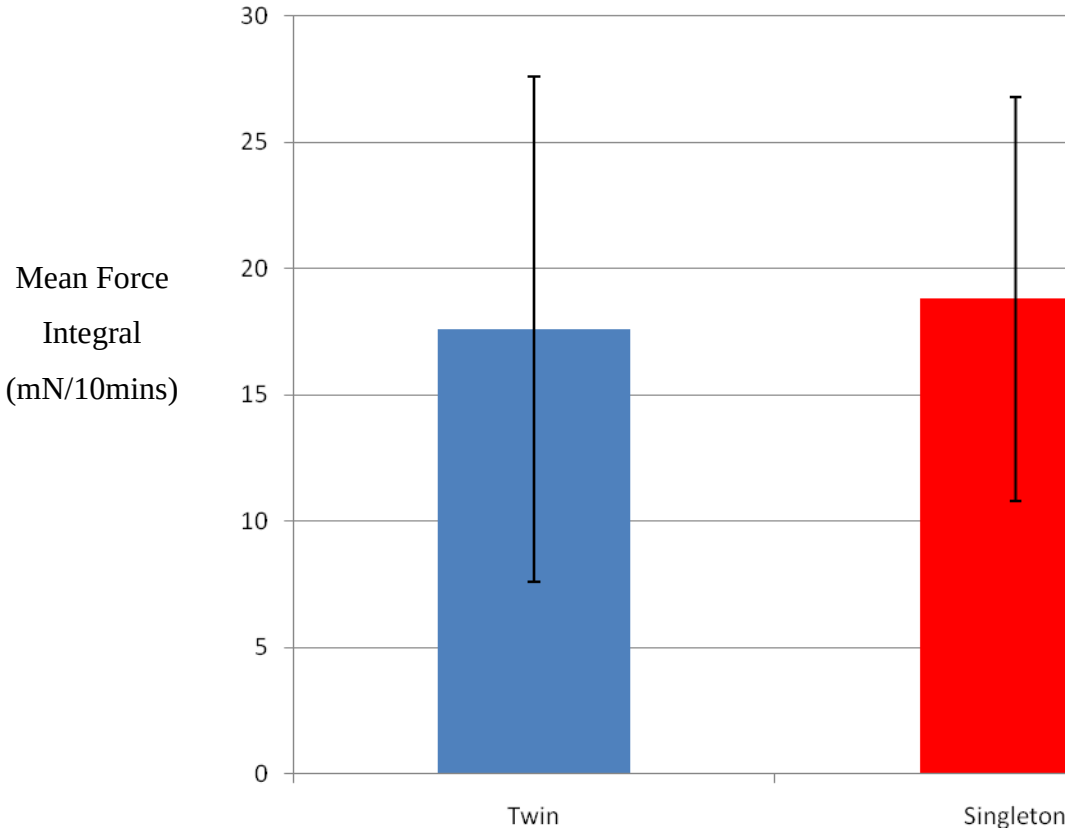


Figure 3.10 Mean force integral (mN/10mins).

The force integral was higher in the twin myometrium, however this was not significant.

3.4.6 Triplets

With the two triplet samples, the mean maternal age was 33, average gestation was 32 weeks, G2, P3 and mean BMI was 24.5. However at 32 weeks triplets pregnancies have a caesarean section so this is not a true gestational age.
Mean Force was 5.39±4.8, Intercontractile interval 7.6±0.5 mins, duration 0.76±0.02, and area under the curve 11.6±9.

3.4.7 Preterm singletons

Preterm singletons had a mean maternal age of 20, gestation 31 weeks, G2, P1, and BMI 31. Mean force was 10.3±0.9, intercontractile interval 3.86±2.7 mins, mean duration 0.42±0.1, and mean area under the curve 28±3.3. In comparison to the term singletons, the force is twice as high with contractions twice as common, and of shorter duration, and larger area under the curve. However as n=2 it is difficult to confirm significance.

3.4.8 Preterm twin

Maternal age was 29, gestation 27 weeks, G1, P0, and BMI was 26.2. Force was 0.88, intercontractile interval was 8.7, duration 0.65 and area under the curve was 4.44. Preterm twins had a lower force, higher intercontractile interval, shorter duration, and smaller area under the curve. It should be noted that only one preterm sample was collected.

3.5 Discussion

Earlier work has demonstrated that twin pregnancies have a higher contraction frequency than high risk singletons at term. *In vitro* evidence showed that duration was significantly longer in twins, as well as observing a higher frequency (Turton et al 2008).

A comparison of the three groups showed no significant differences in force amplitude, and force integral.
Twin pregnancies showed a longer duration than triplets and singletons however no significance was shown, contrasting with previous work in vivo that had larger sample numbers. On comparison between the preterm samples and the term samples the preterm singleton samples have a much higher force and Intercontractile interval, a shorter duration, and a much higher force integral, nearly 10 mN/10mins higher. Yet it should be noted there is a much smaller n number for this group of preterm singletons, and it was not significant.

The comparison of the preterm twin and term twins showed that the intercontractile interval was similar, but there was a greater force, and longer duration in the term twin. It should be noted that there was only one preterm twin, as there are many limitations obtaining a preterm twin, as will be discussed in the limitations and future work.

As the myometrial properties of all groups are not statistically significant, it may be likely that other factor plays a greater role in stimulating preterm delivery in multiple births, rather than an inherent property of the myometrium. It may be either hormonal factors triggering the myometrium or an inflammatory process. However as noted below, my twin numbers were not large and this, coupled with inherent variation between women, may have made it difficult to reveal differences in the parameters of force, if they are not large. Previous *in vitro* work that used the same methods as noted in this chapter did find significant longer durations in twins and observed a higher frequency. However they did have a greater sample size of eighteen compared to a sample size of nine (Turton et al 2008).

3.6 Limitations and Future Work

One of the main problems was obtaining the samples, despite twins and singletons mothers being consented. There were issues with remembering to take the sample, as theatres are busy places, and the care of the women is the priority of the doctor. For example if twin one was breech then the mother would be booked for a caesarean at thirty seven weeks, however that did not prevent the twins from delivering early, out of hours or prevent complications. As some of the twins did require emergency caesarean sections out of hours, it was understandable that the sample was forgotten. This reduced the total number of twins available. This is was also a problem with obtaining preterm twins as the majority would be via a vaginal birth and would have no need for caesarean section.

Another limitation is the dissection process itself, although the dissection skills improved over the year however it was a new skill that needed to be learnt, and this would limit the quality of the traces. There were also inherent variations between biopsies.
For future work it would be important to increase the total number of triplets as this was also a limiting factor. And to find a way to encourage doctors to remember to take a sample, as through the year this did improve yet it was still an issue.

3.7 Conclusion

Twins had a longer duration than both groups, however this was not significant. There were no significant differences between the force, intercontractile interval, duration and force integral.

Chapter 4: The effect of progesterone on twin and singleton myometrial contractility

4.1 Abstract

Early in pregnancy progesterone plays a role in implantation, and later is responsible for uterine quiescence. Previous work *in vitro* has found it to be effective in inhibiting uterine contractions (Anderson et al 2008). In high risk singleton pregnancy studies, it was found to be it effective in preventing preterm birth in women who had a history of preterm labor (Meis et al 2003). However similar results were not seen in multiple births; it was found to be ineffective in preventing preterm delivery (Norman et al 2009, Briery et al 2009, Caritis et al 2009).

One of the reasons for this difference could be due to a higher dose being required in twin pregnancies. The efficacy of progesterone in twin myometrium studied in vitro is unknown. To test these myometrial biopsies were obtained from ten twins and sixteen singletons pregnancies during caesarean section, and dissected. A control period was established, and then 1, 10,100 am progesterone solutions were added, and the force, intercontractile interval, duration and force integral were measured.

Progesterone displayed a dose response relationship in singleton and twin myometrium inhibiting uterine contractions. At 10 and 100μM progesterone there was a significant difference in response to progesterone, showing that progesterone was more effective in inhibiting singleton myometrium than twin myometrium.

4.2 Introduction

Progesterone has long been established to play a role in maintaining uterine quiescence. In small mammals it has been found that labor is preceded by a fall in progesterone, however in humans progesterone levels keep rising (Astle et al 2003). It has been hypothesised that this may be due to a change in genomic progesterone receptors (PR), which has two isoforms PRA and PRB, and a change in the ratio of PRA/PRB is responsible for the more active uterus (Zakar 2007). However more information is coming to light that shows progesterone also works non genomically, via plasma membrane receptors that are similar in structure to G-protein coupled receptors (Fernandez et al 2008). When this receptor is activated it causes an increase in cyclic adenosine monophosphate (cAMP) by inhibiting the enzyme responsible for its hydrolysis, as discussed in greater detail in Chapter one (Gellersen et al 2009).

Much in vitro work has found that progesterone is able to inhibit uterine contractility (Anderson et al 2009) although its synthetic form 17-alpha hydroxy-progesterone may be ineffective (Ruddock et al 2008).
In several studies it was found to be effective in the use of preventing preterm delivery in high risk singleton pregnancies, where there was a history of preterm delivery (Meis et al 2003). Despite its success with high risk singletons however studies that used twins and triplets were not as successful, finding that it did not prevent preterm delivery (Norman et al 2009, Caritis et al 2009, Briery et al 2009).

In vitro progesterone is effective in inhibiting spontaneous human uterine contractions in a dose dependent manner (Ruddock et al 2008). This led to the hypothesis that progesterone differs in effect in twin and singleton myometrium, specifically it is less effective in twins and its dose response curve is shifted to the right.

4.3 Method

Nine twins, sixteen singletons, two triplets, two preterm singletons were used for analysis. Patient recruitment and consent was obtained as discussed in chapter two at the Liverpool Women's Hospital. Once samples were obtained they were placed in a Hanks salt solution, and dissected into small strips of 2mm x 2mm x 10mm and placed onto a force transducer with metal foil clips.

4.3.1 Progesterone Protocol

Once a suitable control period was established with a minimum of four contractions with similar force and intercontractile interval, 1μM progesterone was added for the next four contractions, then 10μM progesterone for four contractions, followed by 100μM progesterone for four contractions. Chapter two contains information about how these solutions were made using ethanol as the vehicle for progesterone.

Each period of different concentrations was used for analysis with Origin8. For each period the force, intercontractile interval, duration and force integral were measured as discussed in Chapter three to compare the effect of progesterone between multiple pregnancies and singleton myometrium, the percentage of the original control force was calculated for 1μM, 10μM and 100μM by dividing the progesterone force amplitude by the control force, and then multiplied by one hundred.

Control strips were also placed in vehicle only solutions to ascertain if any effect was due to the ethanol rather than the progesterone. Experiments were also made using only 1μM or 10μM progesterone, to see if the response made was due to an accumulation of progesterone rather than due to a higher concentration of solution.

4.4 Results

Progesterone had a dose dependent response in both twins and singletons, however this response was reduced in twins, see figure 4.1 and 4.2 illustrating progesterone's effect on myometrial contractility in twins and singletons.

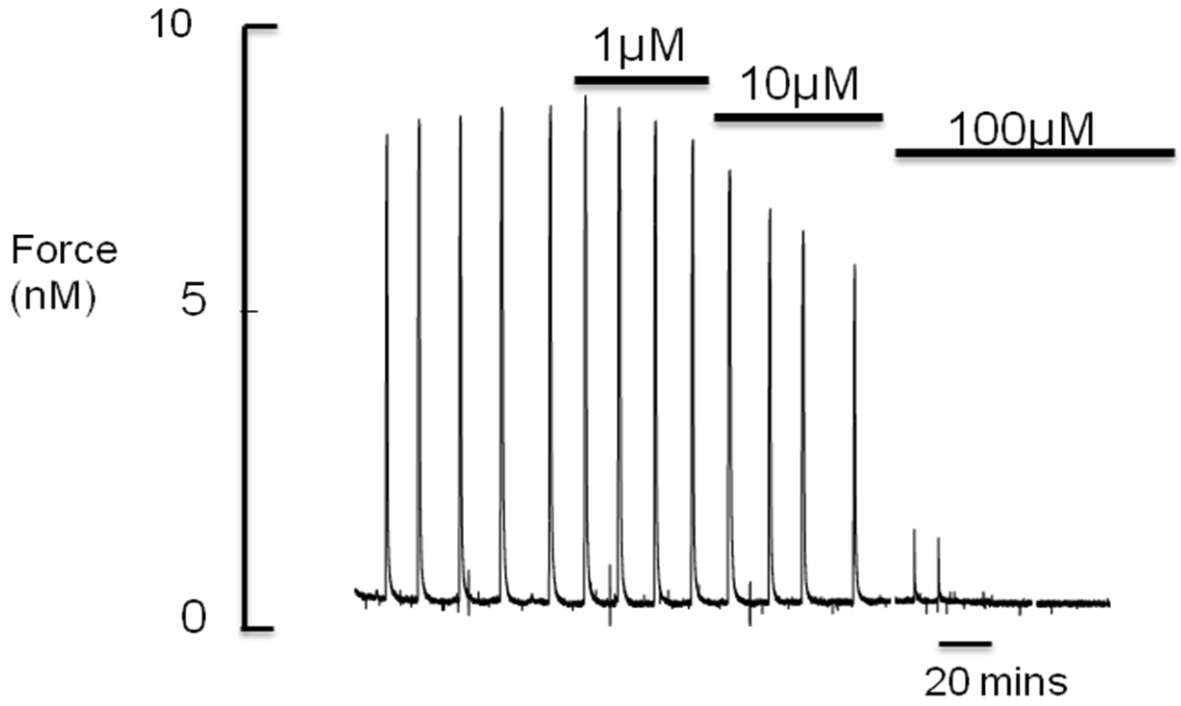


Figure 4.1 the effect of progesterone in singleton myometrium

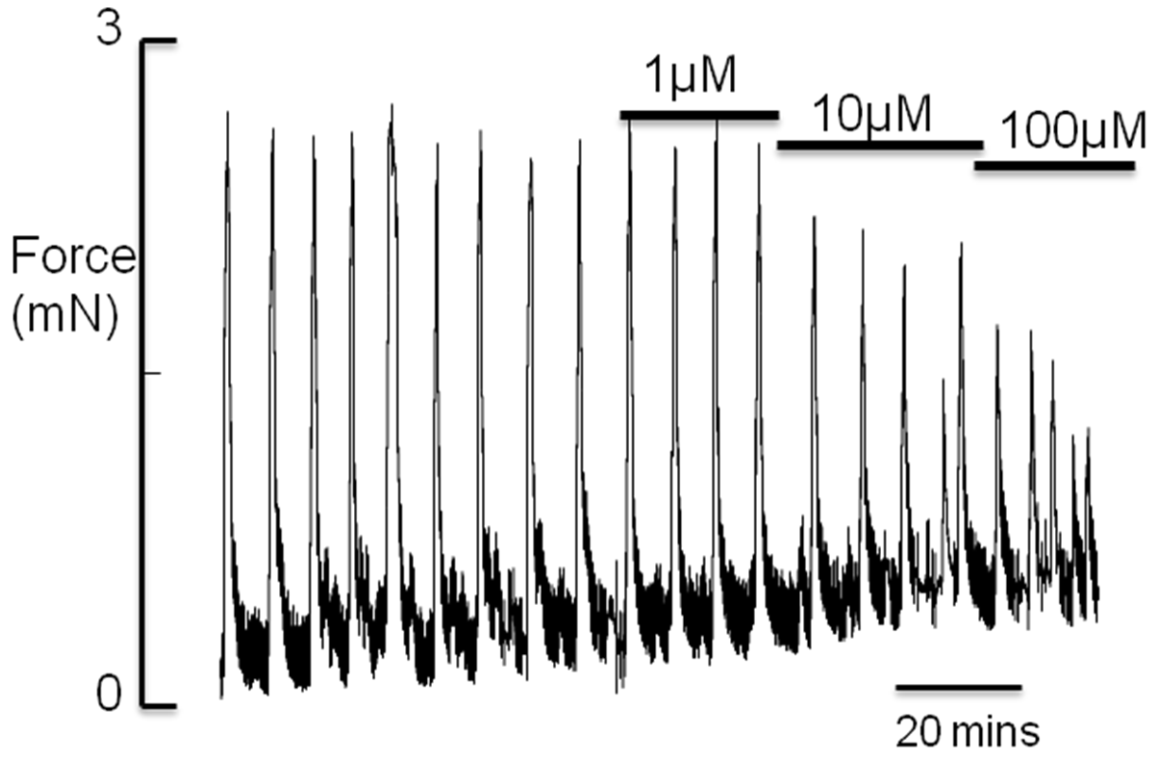


Figure 4.2 The effect of progesterone in twin myometrium

4.4.1. Force Amplitude

At 1μM, progesterone reduced the force amplitude in both twin and singleton groups, having a greater effect in the singleton myometrium however this was not significant. When the percentage of force amplitude was calculated, progesterone reduced the force of the contraction in twins to 87±3% and 72±5% in singletons. However at this concentration little or no effect was seen in preterm singletons (98±0.4%) and triplets (100 %).

A difference in response between multiples and singletons starts to be seen at 10μM progesterone as the force amplitude is reduced with incremental doses of progesterone, and the response in singleton myometrium was significantly greater than in twin myometrium, reducing the percentage control amplitude to 32±7% while only reducing the percentage of control amplitude in twins to 68±4%, p=<0.02. In the triplets the average control force was 100%, yet in the preterm singletons it was reduced to 72±1%.

When the concentration of progesterone was increased to 100μM progesterone force was almost completely abolished in the singleton, reducing the mean force to 0.5±0.2mN and mean percentage control force to 7±1.7%. A significant difference was seen in twins with a mean percentage of control force of 31±7%, p=<0.001. Figure 4.3 shows the dose response relationship of progesterone in singletons and multiples.

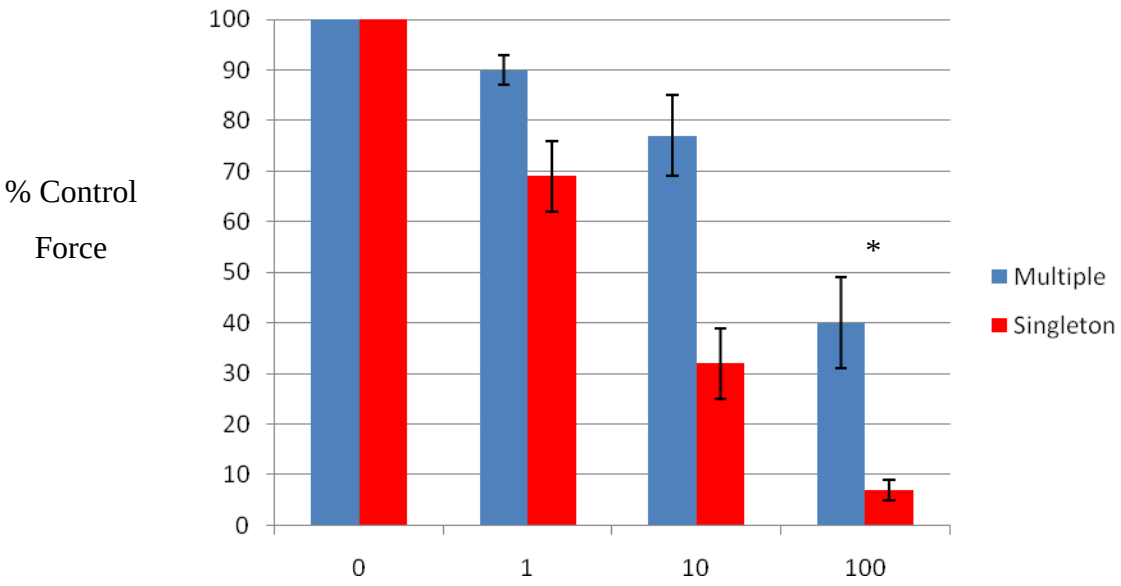


Figure 4.3 The effect of progesterone in multiples vs singletons * P≤0.05

* P≤0.05

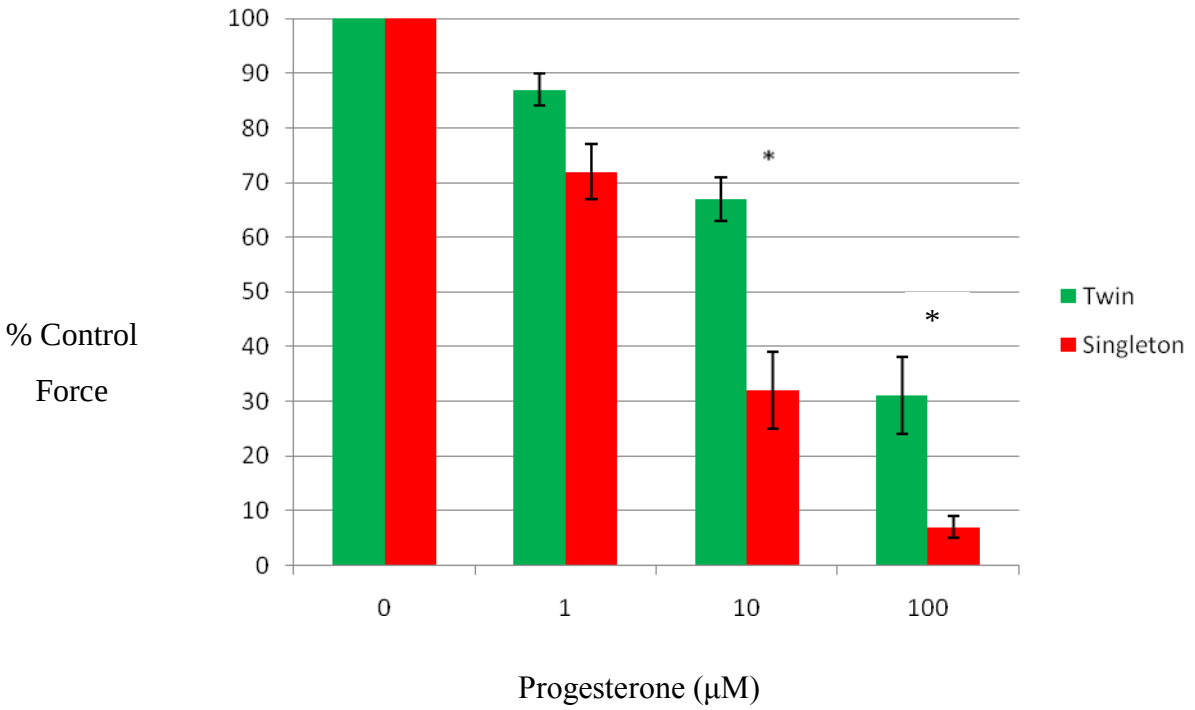


Figure 4.4 The effect of progesterone on percentage of control force in twin vs singleton myometrium

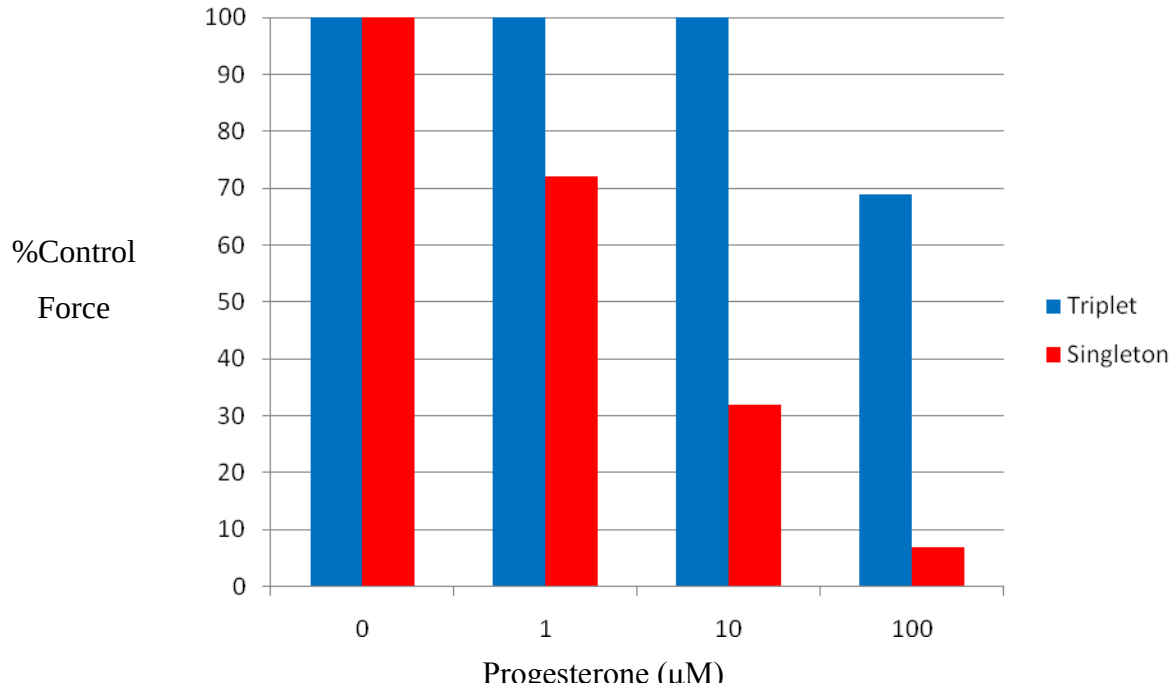


Figure 4.5 The effect of progesterone on percentage of control force in triplet and singleton myometrium.

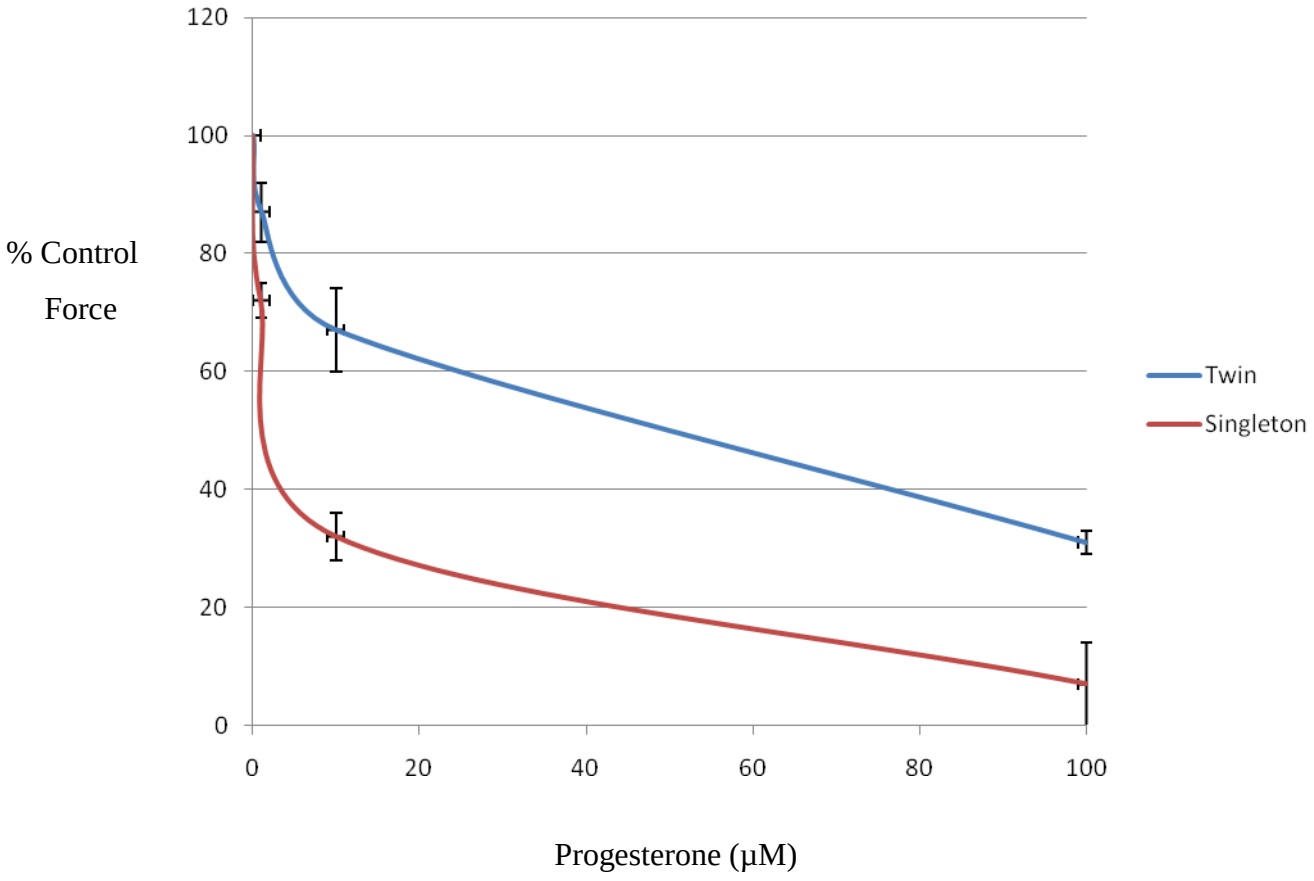
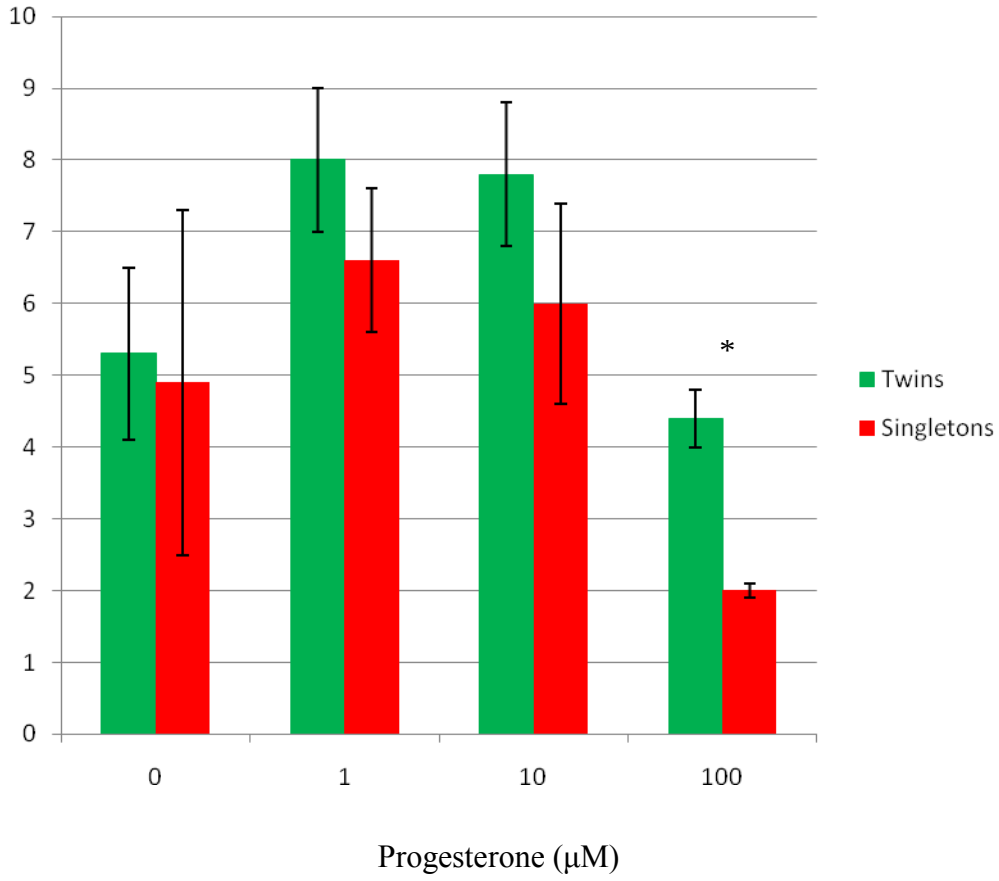


Figure 4.6 A dose response curve of progesterone illustrating that a higher dose of progesterone is required to reduced the control force to the same degree as in the singleton myometrium,

4.4.2 Intercontractile interval

At 1μM progesterone there was a decrease in the mean intercontractile interval of contractions in twins and singletons from 5.3±1.2 mins, 4.9±2.4 mins to 8±1 and 6.6 ±1, showing that progesterone increased the time taken for a contraction to occur.

When the concentration of progesterone was increased to 10μM, the mean intercontractile interval decreased to 7.8± 1 mins for twins and 6±1.4 mins for singletons. A similar effect was also seen in the preterm singletons and triplets (7±0.3 to 6.2±0.1 mins).
There was a significant difference between the singletons and twins at a 100m with a greater increase in the singleton myometrium to 2±0.1 where as the twin mean intercontractile interval was 4.4±0.4. The triplet intercontractile interval was also lower than singleton however this was not significant, 4±1.



* P≤0.01

Figure 4.7 The effect of progesterone on intercontractile interval

4.4.3 Duration

At 1μM progesterone there was no change in the duration of contraction in singletons and triplets however the mean duration changed to 0.7±0.1mins in twins, yet this was not significant.

When the dose of progesterone increased to 10μM the only change in duration was term singleton group to 0.6±0.07mins. However when the dose was increased to 100μM the singleton, twin group and the preterm singletons mean durations were decreased to 0.3±0.07 mins, 0.6±0.1mins, 0.3±0.1mins respectively however the p value >0.05.

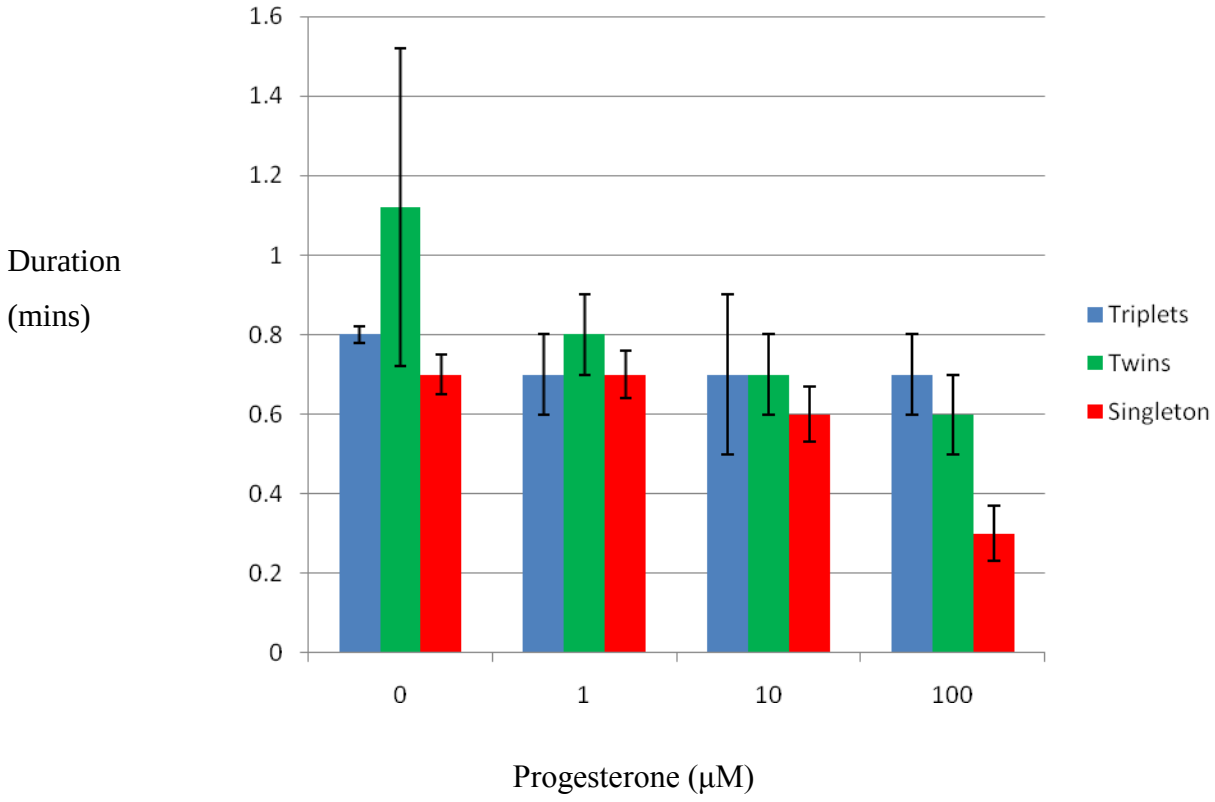


Figure 4.8 The effect of progesterone on duration

4.3.4 Force integral (area under the curve)

In general there was a decline in the force integral as the concentration of progesterone increases, however there were no significant differences between the groups.

The greatest decreases occurred at 1μM and 100μM progesterone as can be seen in table 4.1. At the 100μM progesterone in triplets and preterm singleton the force integral increases.

	Progesterone (μM)			
AUC(mN/10mins)	0	1	10	100
Triplets	11.6±10	9.6±5.9	8.8±6.1	10.9±5.8
Twins	17.6±8	15.0±6.6	15.2±7.3	8.0±3.6
Term Singletons	18.8±3.5	14.3±3.3	15.7±1.6	4.1±1.3
Preterm Singletons	28±3.3	23.1±7.0	22.6±7.7	23.4±13.5

Table 4.1 The effect of progesterone on the area under the curve.
This table shows that in triplets there is no significant difference between control and progesterone groups. However despite the reduction in force integral for singletons and twins, there were no significant differences between the groups.

4.4.5 Control Experiments

In order to confirm that the change in uterine contractility was a result of an increase in progesterone rather than an accumulation of the vehicle (70% ethanol), control strips were used with 1 in 1000 dilution, as this was the strongest concentration of ethanol used. This was maintained for two hours, and showed that the ethanol had no effect. See Figure 4.9

Experiments using only 1μM, 10Mm, or 100μM were used, in order to ascertain if there was a dose response relationship or if it was an accumulation of the lower doses. These strips showed that the initial reduction of force was maintained and that the decrease in force was due to dose response relationship.

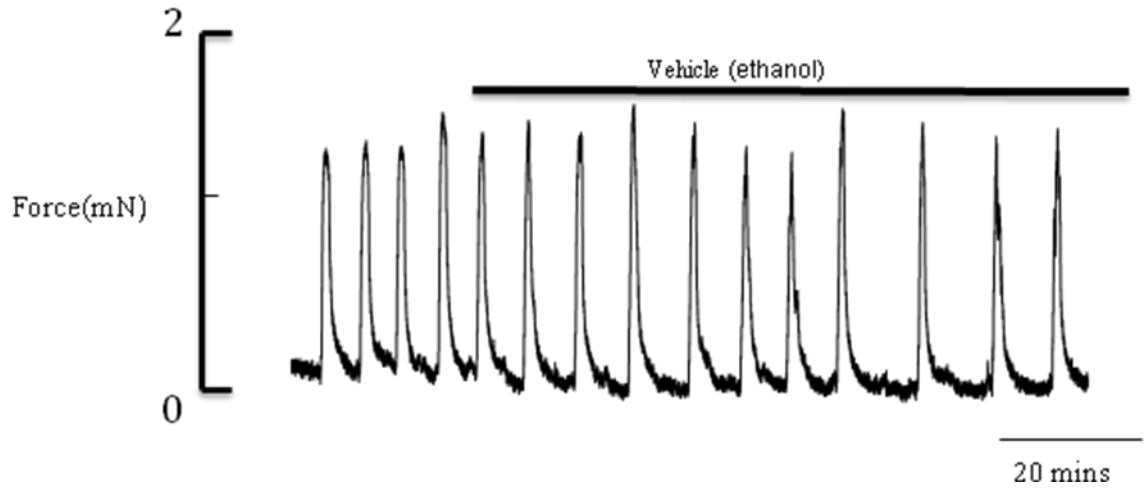


Figure 4.9 The effect of vehicel on myometrial contractility
This figure shows that when the vehicle was added, it did not cause a reduction in the force of amplitude, and therefore the reduction in force shown in the progesterone experiments was due to progesterone rather than the ethanol.

4.5 Discussion

Progesterone had a dose-dependent effect on contractions over the range examined, in both singleton and twin pregnancy. Significant differences in the dose effects results were found at 10μM and 100μM progesterone. At lower doses of progesterone there was a reduction in the force amplitude, increase in intercontractile interval, shorter duration and decrease in force integral however there were no significant difference between the groups. At higher doses 10μM and 100μM progesterone, there was a significant difference in reduction of the force amplitude that supports the hypothesis that the twin myometrium has a greater resistance to progesterone.

This difference could occur as a result of the increased stretch placed on the twin myometrium earlier on in the pregnancy, and reduce the expression of the progesterone receptor. As progesterone acts rapidly within minutes, it is likely that *in vitro* it is working via the progesterone membrane receptors, which recent work has shown increases the cAMP levels. The raised cAMP enhances uterine relaxation (Fu et al 1998).

The intercontractile interval of contraction generally increased as the dose increased. However this occurred as the result of the force of the contractions being reduced, and therefore the recovery period was shorter allowing a new contraction to occur sooner. This was also accompanied by a shorter duration supporting this action rather than the progesterone stimulating calcium channels to open. At 100μM there was a significant difference between the twin and singleton myometrium, the singleton had a higher intercontractile interval than the twin myometrium.

Clinically with progesterone showing greater efficacy in singleton pregnancies, it would be useful to consider a higher dose of progesterone for twin pregnancies. Current doses used for singleton studies have explored using vaginal pessaries from 90-400mg for singletons. With giving a higher dose it may be beneficial to give it vaginally as it has been shown to avoid hepatic first pass side effects, which would increase with a higher dose given orally (Tita et al 2009).

Mean serum progesterone in singletons at 16 weeks was 140.2±4.9nmol/L, and rises to 265.7±9nmol/L at 27 weeks (Lagiou et al 2003). However there is little literature of exact levels in twin pregnancies, however it has been reported that as a result of a larger placenta, there are increased levels of progesterone in twin pregnancies, with levels at 26 weeks having a median level of 688(349-1105) nmol/l in twin pregnancies (Johnson et al 1994).
With the number of fetuses increasing hormonal levels it is difficult to estimate what appropriate dose would be worthwhile, with high circulating plasma levels.
It is important to question whether or not a higher dose of progesterone for twin pregnancies would work.

A progesterone metabolite (5β-dihydroprogesterone) has also been previously found to reduce the force, frequency and force integral of singleton uterine contractions, and this could also have a role in uterine relaxation (Thornton et al 1999).

4.6 Limitations and further work

The effects of progesterone were so different between twins and singletons that even with relatively small n numbers, significant differences were found. Future work could concentrate on the difference between term and preterm twins, and preterm singletons, however there is difficulty obtaining these samples as these would likely be obtained from emergency sections. Also other progesterone preparations could be examined such as 17-alpha hydroxyl progesterone to explore the in vitro relationship in twins. Future work could also explore the expression of the membrane receptors in multiple births and singletons.

4.6 Conclusion

Progesterone displays a dose response relationship in singletons and twin myometrium. There was a significant difference at 10 & 100μM progesterone in the reduction of control force amplitude, supporting the hypothesis that progesterone has less of an effect in twins.

Chapter 5: The impact of stretch on the effect of progesterone

5.1 Abstract

Multiple pregnancies increase the amount of hyperplasia and hypertrophy in the uterus, in order to accommodate for two or more growing fetuses.
It is hypothesized that this higher degree of stretch is responsible for the higher of preterm deliveries in this particular group.

In the previous chapter it was found that progesterone is less effective in reducing force amplitude in twin myometrium than singleton, and that the reason for this could be as a result of stretch influencing the progesterone membrane receptor expression.

Therefore in order to explore this relationship paired experiments were performed. A comparison of the effect of progesterone with and without 'stretch' was made. Although it appeared that stretch reduced the effect of progesterone, this was not significant, probably due to low sample numbers (n=5).
More work is required to explore the effect of stretch on progesterone, and its effect on human myometrium.

5.2 Introduction

It has long been established that multiple pregnancies are associated with higher rates of preterm delivery and perinatal mortality than singleton pregnancies (ONS 2009). The direct cause of the reason why twins deliver preterm is unknown; however stretch may play a part.

Throughout pregnancy the myometrium undergoes hyperplasia and hypertrophy, and stretch increases levels of contraction associated proteins for example connexin 43, cyclo-oxygenase-2, and oxytocin receptors, as well as interleukin eight mRNA expression (Ou et al 1997, Salameh et al 2010, Sooranna et al 2004, Sooranna et al 2005, Loudon et al 2004). In multiple births the uterus undergoes greater quantities of stretch in order to accommodate the demands of two or more growing fetuses.

All these changes maybe speculated to be increasing or occurring earlier in gestation in multiple pregnancies, leading to an increased contractile drive. This may therefore be a cause of the increased prematurity in multiple pregnancies in comparison to singleton pregnancies. In chapter four it was found that twins had a greater resistance to the tocolytic effects of progesterone than singletons. This could be as a result of stretch reducing the expression of progesterone receptors; or switching the PR isoforms at an earlier stage in gestation, to the forms that are less receptive to progesterone. There is however no literature on the relationship between progesterone and stretch. In order to gather further information, I undertook preliminary experiments to test the hypothesis that stretch reduces the effectiveness of progesterone.

5.3Methods

Patient recruitment was performed as described in chapter two, and the myometrium was then dissected into strips of 10mm x 2mm x 2mm, and placed on the force transducer.

5.3.1 Stretch protocol

Two strips from each sample were used as paired data; one with 100µM progesterone, and another strip with 100µM progesterone and stretch. 5 term samples were used.
The 'stretch strip' was initially set to the same degree of resting tension. Both strips were allowed to start spontaneous contractions, and once control activity was established, a 40mmol potassium solution (High K+) was added which causes the muscle to depolarize and contract at its maximum amplitude. Once the tissue had recovered, it would then be stretched to 50% of its maximum amplitude see figure 5.1.

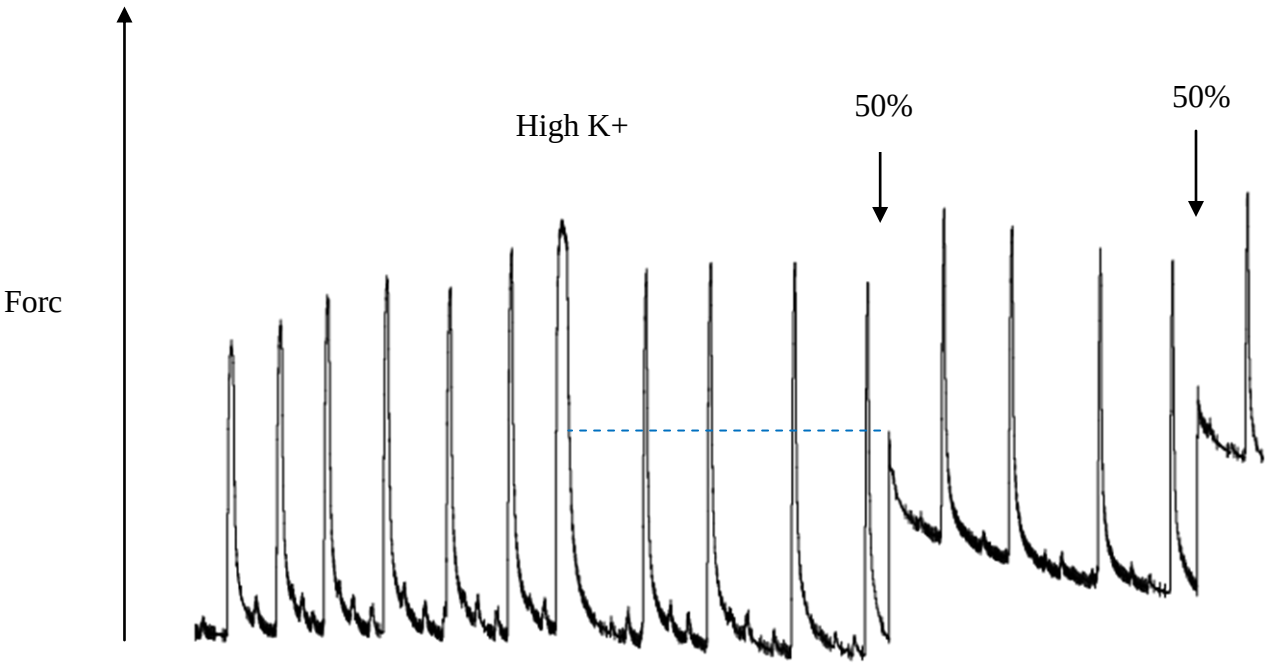


Figure 5.1 A trace showing how stretch was applied.

The spontaneous and high K+ contractions in term singleton myometrium were superfused with physiological saline at 37 C and pH7.4. The arrows indicate increases in passive stretch in the tissue.

100μM Progesterone was also added when the 50% of stretch was applied, and after every 4 contractions another 50% of the maximum amplitude was added.

The force, the percentage of the control force, duration and intercontractile interval was calculated for each sample

The solutions protocols for the 100M progesterone and the physiological salt solution were the same as discussed in previous chapters.

5.4 Results

Stretch appeared to reduce the effect of progesterone showing a lesser reduction in control force amplitude however this was not statistically significant when $p \leq 0.05$. See figure 5.2

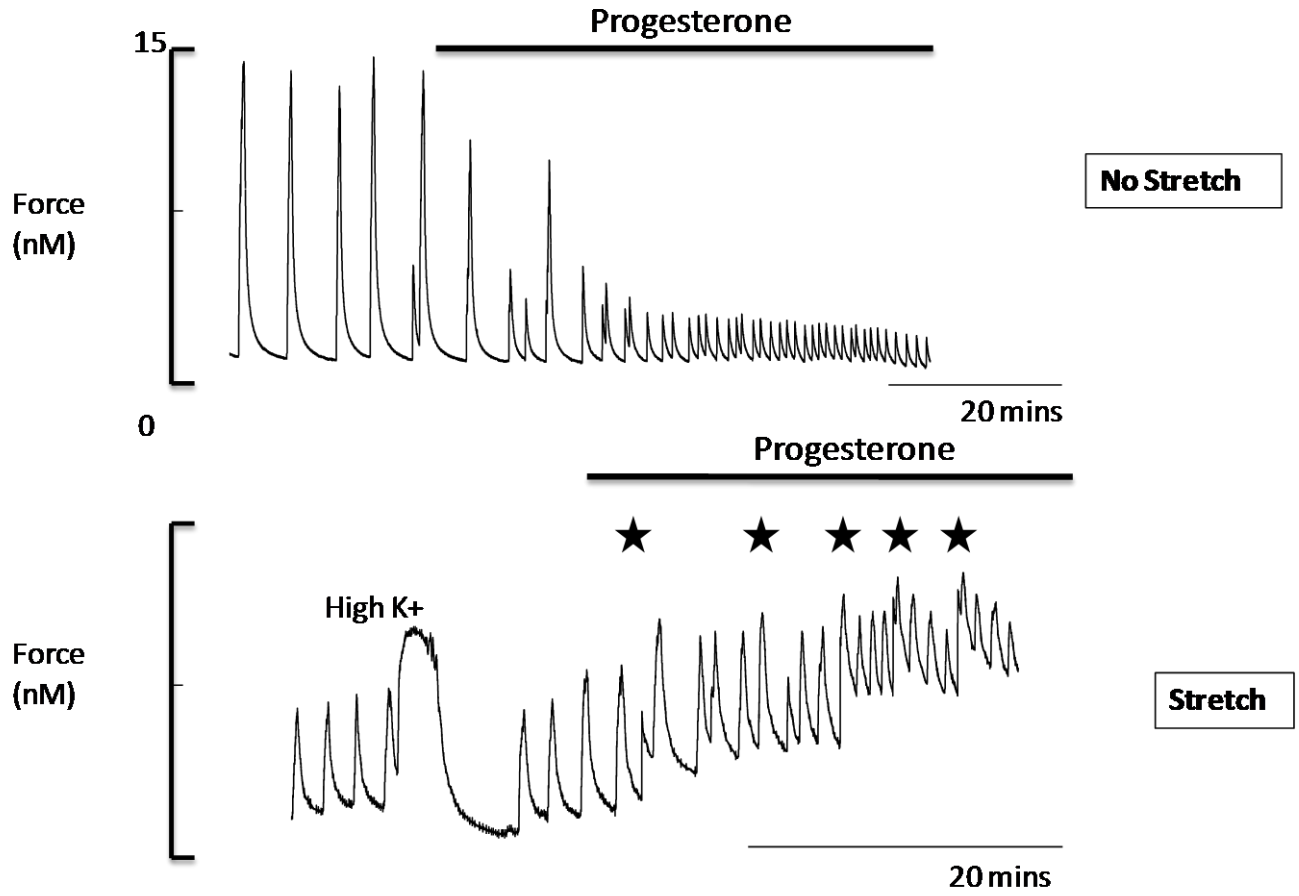


Figure 5.2 The effect of stretch on progesterone response
The no stretch strip shows the effect of progesterone reducing the force of contractions. However when stretch was added in addition to progesterone the effect is reduced.

After application of progesterone the force amplitude and the percentage of control force were reduced in both groups however when stretch was combined with progesterone this effect was greatly reduced, with some values being three times the percentage in the no stretch group. The mean percentage of the original control for the stretch group was 52±18%, while the non stretch group was half this at 19±11.5% however this was not statistically significant p= 0.14 using paired student t test. See Figure 5.3

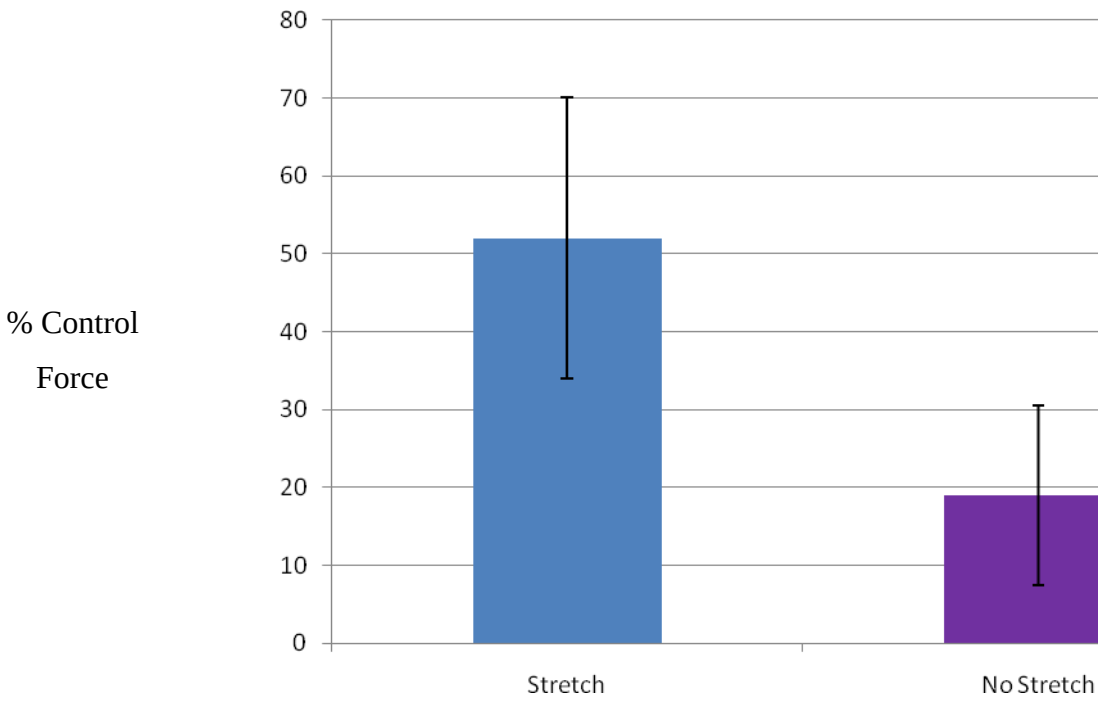


Figure 5.3 A comparison of control percentage of control force where stretch was applied with progesterone or no stretch was applied with progesterone. (n=5)
This figure shows that when stretch was applied with progesterone the reduction in the percentage of control force was not as great, and the effect of progesterone was reduced however this was not significant.

The intercontraction interval was also slower in the stretch group, with the mean intercontraction interval of one contraction every 2± 0.4 minutes in the no stretch group, and 4±1.4 minutes in the stretch group however this was not significant. The intercontraction interval was measured after the progesterone was added, either with stretch or without using the first three contractions. See figure 5.4

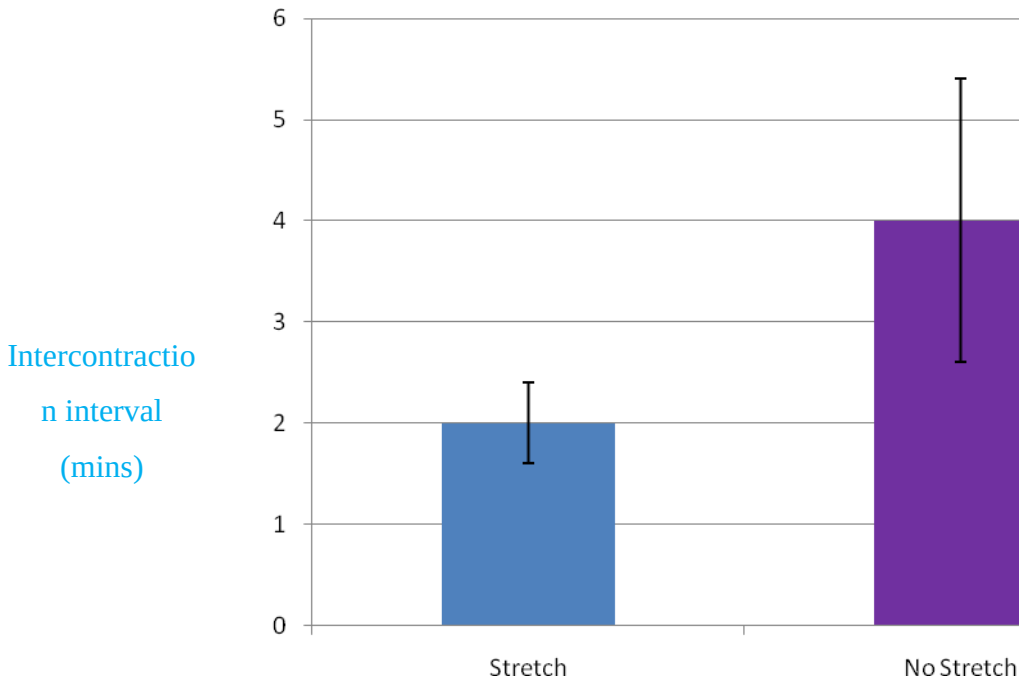


Figure 5.4 The effect of stretch on intercontractile interval (n=5)

The duration of the first three contractions was shorter in the stretch group 0.44±0.04mins and 0.6±0.14mins in the no stretch group. See figure 5.5 The effect of stretch on the duration.

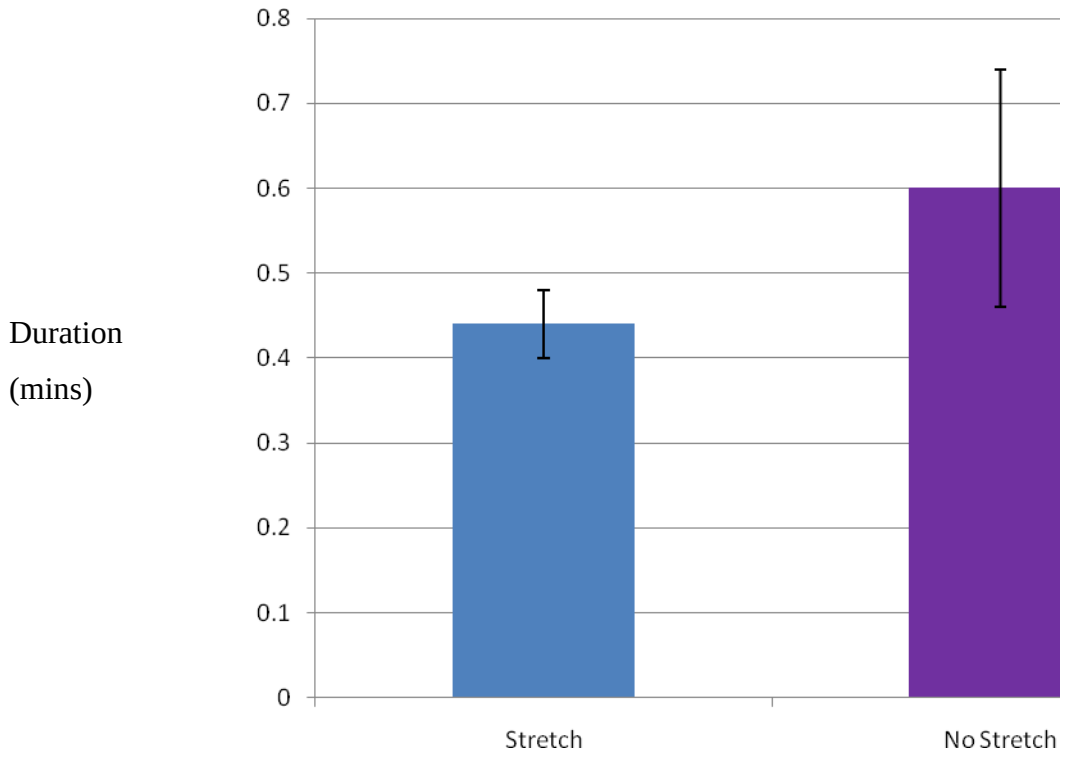


Figure 5.5 The effect of stretch on duration (n=5)

The duration of the contraction was on average longer in the first three contractions in the group where no stretch was applied, however this was not significant.

5.5 Discussion

One of the main differences between multiple pregnancies and singletons is the amount of stretch placed on the uterus with the multiple pregnancies having more fetuses to accommodate. This has led to the proposal that due to the higher levels of stretch earlier in pregnancy, contractile pathways are stimulated earlier leading to higher rates of preterm delivery. This theory was explored as a possible reason why progesterone was less effective in the twin myometrium as discussed in chapter four.

As 100µM progesterone had the greatest effect in the progesterone samples compare to 1µM progesterone, this was used for the stretch protocol as this response had the greatest significant effect. The myometrial strips that had stretch as well as progesterone applied showed a higher percentage of original control force, the less the uterus was stretched. However my data did not achieve significance. This maybe due to a small sample size or due to the response of the myometrium to the progesterone, with some samples having a greater response to progesterone. The stretch applied *in vivo* is difficult to measure; although birth weight can be used, it does not include how much amniotic fluid was also in the uterine cavity or the size of the placenta. If the mother's fundal height is used then adipose tissue is also included. Therefore it is difficult to interpret how much stretch applied *in vitro* can also apply *in vivo.* However ultrasound has been used to measure the thickness of the uterus in twins, as uterine wall thickness is inversely proportional to uterine stress (Deyer et al 2000). This study found that preterm uterine thickness had a much thinner lower uterine segment than term twins, and that twins also had a thinner lower uterine segment compared to singletons (Sfakianaki et al 2008).

Stretch has been shown to increase the expression of contractile associated proteins such as connexin 43 (Salameh et al 2010), and does appear to play strong part in the development of these pathways, whether or not it remains the trigger or is part of a more complex mechanism for preterm delivery remains to be seen.

5.6 Limitations and further work

One of main difficulties was the requirement of both strips to behave in a similar manner for the limited time available to undertake this study and thus this data should be considered preliminary.

Future work needs to explore how stretch can effectively be measured *in vivo* in order to help fully understand the impact stretch has on the uterus, as otherwise the stretch theory will never be answered and its usefulness unknown. Although the use of animals at different gestational stages could help highlight the impact different amounts of stretch has on the contractile properties of the myometrium. Also the effect of stretch on PR isoform expression could be further explored or in twin and singleton myometrium.

In vitro work could include similar experiments but with larger sample numbers, and explore the effect stretch has on other hormonal responses.

5.7 Conclusion

My preliminary data supports the hypothesis that stretch inhibits the effect of progesterone on uterine contractility. However further work is required to explore the true relationship of stretch and progesterone.

Chapter 6: Dietary advice for women with multiple pregnancies

6.1 Abstract

Maternal nutrient insufficiency may be a possible reason for higher rates of preterm delivery, and an appropriate diet providing the mother and the fetus with adequate nutrition could decrease prematurity rates. Therefore a literature search was performed to evaluate what diet is appropriate for the mother to achieve optimal fetal outcomes. This search revealed that substantial information is still required. Higher weight gain in the first 20 weeks was associated with better fetal outcomes, and recommendations about weight gain should be determined by maternal pre-gravid BMI. However, there were differences in recommendations for quantities of weight gain. Dietary support and intervention may provide substantial benefits. However, still more research is required to determine the success of a high maternal weight gain against the potential harmful effects it could have on the mother.

6.2 Introduction

1 in 15 pregnancies in the United Kingdom is a multiple pregnancy (ONS 2008), over 50% of which will deliver before 37 weeks. The perinatal mortality rate of multiple pregnancies is 27.5 [95% CI 25.3,29.8] per 1000 total births, and the neonatal mortality rate is 20 [18.1,22.0] per 1000 live births compared to the singleton perinatal mortality rate of 6.9[6.7,7.1] per 1000 total births, and neonatal mortality rate 2.6[2.5,2.7] per 1000 live births. See table 6.1 for details of differences between triplet and higher order, twins and singletons (CEMACH 2008).

	Triplets and higher order	Twins	Singletons
Perinatal Mortality rate (per 1000 total births) [95%CI]	81.8[59.0,113.4]	27.2[25.0,29.5]	6.9[6.7,7.1]
Neonatal Mortality Rate (per 1000 live births) [95%CI]	78.9[56.4,113.4]	19.3[17.5,21.4]	2.6[2.5,2.7]

Table 6.1 Perinatal and neonatal mortality rates for twin, triplet and singletons (CEMACH 2008)

One of the main factors for the high mortality rates in multiple pregnancies is the higher rates of prematurity and its associated complications. Other factors include the increased incidence of intrauterine growth restriction and small for gestational age fetuses: 35.6% of twins and 36.6% of triplets were small for gestational age compared to 9.4% of singleton deliveries (Alexander et al 1998). Small for gestational age neonates with low birthweights are more likely to develop visual, behaviour and growth problems throughout childhood (Monset-Couchard et al 2004).

When a neonate is small for gestational age possible causes include a pathological process, such as infection, pre-eclampsia or diet insufficiency. This can also be influenced by the chorionicity of the twins: monozygotic monochorionic twins can be complicated by twin-

twin transfusion syndrome leading to severe growth discordance and ultimately 80% of fetuses will demise if left untreated (Duncan 2004).

6.2.1 Maternal physiology and multiple pregnancies.

Basal metabolic rate is greater in multiple pregnancies: 10% higher in twins than singletons, increasing with each trimester. There is also an increase in maternal plasma volume, fat soluble vitamins, triglycerides, cholesterol and free fatty acids.

The placenta also secretes higher amounts of hormones and steroids which influence maternal carbohydrate metabolism, resulting in a more rapid uptake of glucose in the fetus causing accelerated depletion in the mother's glycogen stores (Goodnight et al 2009).

Fat is metabolised due to the resulting shortage of glucose, to provide energy for growth and this results in ketonuria which is linked to preterm labor and delivery (Luke 2005).

In early pregnancy, increased nausea and vomiting in twin pregnancies could hinder nutritional intake (Louik et al 2006).

With the increased insulin resistance there is an increased the risk of gestational diabetes, which is associated with poor maternal outcomes and fetal macrosomia (Rosello-Soberon 2005).

6.2.2 Current clinical guidelines

Currently the only clinical guidelines available are from the Institute of Medicine (IOM) in the USA. These guidelines are based on the mother's prenatal body mass index (BMI), calculated by weight(kg)/height(m^2), and a recommended total pregnancy weight gain. See table 6.2 for IOM guidelines.

	Recommended Total Weight Gain	
Prepregnancy BMI	**Range in kg**	**Range in lbs**
Normal weight (18.5–24.9 kg/m2)	17-25	37-54
Overweight (25.0–29.9 kg/m2)	14-23	31-50
Obese (≥ 30.0 kg/m2)	11-19	25-42

Table 6.2 IOM weight gain guidelines (Rasmussen 2009).

However this guideline does not include underweight women, who are potentially at the most risk of inadequate nutrition. Another limitation of advising weight gain is the interpretation of this by the patient: the patient may opt for a diet high in saturated fats, which is associated with an increased risk of coronary artery disease and type 2 diabetes mellitus (Mann 2002). The IOM also states that there is insufficient knowledge to give full guidance for multiple births and more work is needed to help resolve this issue: when advocating a diet for multiple pregnancies it is necessary to consider the needs of the fetus as well as the implications of a high calorie diet on the mother's future health.

Therefore the aim of this chapter is to review current literature on dietary advice for multiple pregnancies.

6.3 Methods

A literature search was carried out using OVID Medline. The inclusion criteria were: randomised controlled trials, 'quasi-random' studies, case-control, cohort, cross-sectional studies with multiple pregnancies (2 or more fetuses) either nulliparous or multiparous. The studies were selected if they focused on specialised diets or specific dietary advice for multiple pregnancies. The search strategy is included in the appendices. This produced 20 reports using the above inclusion criteria, which was subdivided into twins or triplets, to take into account the different nutritional demands of triplet and twin pregnancies.

6.4 Results

Table 6.3 summarises the method, and results of all the appropriate studies found.
All studies found were cohort studies focusing on maternal weight gain/BMI and the fetal outcomes were small for gestational age, birthweight or preterm delivery.

Table 6.3 Results of twin search

Author	Method	Results
B Luke et al 2003	Cohort Study of 2324 twin pregnancies. Inclusion criteria were; 1. Two twins born alive 2. ≤28 weeks first ultrasonography or best clinical estimate 3. Documented birthweights and sexes 4. At least two ultrasonographic weight estimates first before 28 weeks 5. Absence of major congenital abnormalities 6. BWT discordancy<20% 7. Documentated maternal height, and at least 2 maternal weights, one prenatal , one 1 week after delivery. Regression curves created for maternal and fetal weight gain.	Optimal Weight gain that caused optimal fetal growth 105-110g/wk 20-28 weeks, 28 until delivery 155-161g/wk and optimal birthweights 36-38week gestation 2850-2928g: Underweight women (BMI<19.8) 0-20wks 1.25-1.75lb(0.57-0.79kg/wk 20-28wks 1.5-1.75lb/wk(0.68-0.79kg/wk) 29-delivery 1.25lb/wk (0.57kg/wk) Normal Weight(BMI 19.8-26) 0-20wks 1-1.5lb/wk(0.45-0.68kg/wk) 20-28wks 1.25-1.75lb/wk(0.57-0.79kg/wk 29-delivery 1lb/wk (0.45kg/wk) Overweight (26.1-29.0) 0-20wks 1-1.25lb/wk(0.45-0.57kg/wk) 20-28wks 1-1.5lb/wk (0.45-0.68kg/wk) 29-delivery 1lb/wk(0.45kg/wk) Obese (BMI>29.0) 0-20wks 0.75-1lb/wk (0.34-0.45kg/wk) 20-28wks 0.75-1.25lb/wk(0.34-0.57kg/wk) 29-delivery 0.75lb/wk(0.34kg/wk)

Luke et al 1997	Cohort Study of 646 twin pregnancies >28 wks Inclusion Criteria: 1. Both twins born alive 2. >28 weeks' gestation by last menstrual period, first-trimester ultrasonography, or best obstetric estimate (a combination of clinical and ultrasonographic estimates) 3. Documented genders and birthweights 4. Absence of major congenital anomalies as documented by normal findings, 5. Documented maternal height and weight within 1 week of delivery.	Birth weight was significantly associated with weight gain before 20 weeks in underweight women, before 20 weeks and after 28 weeks in overweight women, and during all three gestational periods in normal-weight women. Weight gain before 20 weeks had the largest effect on infants of underweight women, less of an effect on infants of normal-weight women, and half as much effect on infants of overweight women. Weight gain after 28 weeks significantly affected the infant birth weights of normal-weight and overweight women, but the effect was half as great among infants of the latter group.

Lantz et al 1999	Cohort of 189 twin pregnancies Inclusion Criteria: 1. Maternal recall of pregravid weight and height, at least 3 prenatal visits 2. Delivery after 28 weeks gestation 3. Birthweights for both fetuses 4. A prenatal weight measurement within two weeks of delivery Exclusion Criteria: 1. Diabetes Mellitus, Hypertension 2. Gestational Diabetes, pre-eclampsia, fetal death 3. Congenital anomalies Outcome variable both twins at least 2500g at delivery. Multiple logistic regression performed.	Rates of Weight gain, that were associated with fetal birth weight over 2500g. BMI<19.8 0-20wks 1.13±0.24lb/wk, 20+wks 1.92±0.62lb/wk BMI19.8-26.0 0-20wks 0.61±0.12lb/wk, 20+wks 1.63±.15lb/wk BMI>26.0 0-20wks 0.46±0.14lb/wk, 20+wks 1.85±0.27lb/wk

Luke et al 2003	Prospective interventional study Exclusion criteria: 1. Monochorionic twins 2. Pregnancies in which the mother had been transferred as an emergency with pregnancy complications from outlying hospitals at the time of delivery 3. Fetal death 4. Major congenital fetal anomalies Intervention: Specialised diet education • Twice monthly prenatal visits to registered dietician and nurse practitioner in addition to normal prenatal visits • Additional maternal education • Modification of maternal activity • Individualised dietary prescription • Multimineral supplementation • Serial monitoring of nutritional status 180 pregnancies in intervention group, 320 in non intervention group.	PPROM Preterm Labour Delivery < 36 wk Delivery < 32 wk Delivery < 30 wk LBW VLBW Non-LBW Length of gestation (d) Average birth weight (g)	Intervention group %: 10 23 41 7 3 41 5 59 251.0±1.3 2467±37	Non Intervention group (%) 25 42 53 21 9 64 16 36 243.6±1.3 2217±36	P Value <0.0001 <0.0001 0.1 <0.0001 0.1 <0.0001 0.001 <0.0001 <0.0001 <0.0001

Dubois et al 1991	A cohort study of 177 women with twin pregnancies who received Higgins Nutritional programme and 354 non-intervention twins pregnancies Programme education which consists of: 1. An assessment ofthe risks for the presenting pregnancy 2. The determination of individual dietary requirements based on the combination of the normal requirementsof pregnancy and rehabilitation allowances for diagnosed risk, 3. Teaching of food-consumption patterns that meet individual dietary requirements while respecting preexisting food habits 4. Follow-up and supervision by the same dietician at 2 to 4-wk intervals.	Birth weight (g) Low birth weight (%) Very Low birthweight (%) Preterm(%) Very Preterm(%) IUGR(%)	Higgins Group 2468±559 47 5 40 8 14	Non intervention 2378±620 55 9 47 16 18

Luke et al 1998	Cohort study 1564 twins medical records reviewed to evaluate effect of pregnancy weight gain on Inclusion criteria: 1. both twins born alive 2. >28 weeks' gestation by last menstrual period, first trimester ultrasonography, or best obstetric estimate (a combination of clinical and ultrasonographic estimates 3. documented genders and birth weights 4. absence of major congenital anomalies as documented by normal findings	Increased mid fetal growth was associated with early maternal gain (10.91g/wk per pound per week) and mid maternal gain (15.89 g/wk per pound per week). Increased late fetal growth was associated with early maternal weight gain (16.86g/wk per pound per week) and mid maternal weight gain (23.88 g/wk per pound per week). The rate of late maternal weight gain did not contribute significantly to the rate of late fetal growth (2.51 ± 3.01 g/wk per pound per week, P = .40)and therefore was not retained in the final model of rateof late fetal growth The low pattern of maternal weight gain (5lb by 20 weeks, 13 lb by 28 weeks, and 25 lb by 36 weeks) resulted in an estimated sum of fetal weights of 2216 g (1108 g per twin, 43rd percentile for gestational age, on the basis of the twins in the study population) by 28 weeks and an estimated sum of birth weights of 4541 g(2270 g per twin, 37th percentile for gestational age) by36 weeks. With the mean pattern of maternal weight gain(15 lb by 20 weeks, 27 lb by 28 weeks, and 39 lb by 36 weeks), the estimated sum of the fetal weights was 2323 g(1162 g per twin, 56th percentile for gestational age) by28 weeks and the estimated sum of birth weights was 4811g (2405 g per twin, 54th percentile for gestational age) by 36 weeks. With the high pattern of maternal weight gain(25 lb by 20 weeks, 41 lb by 28 weeks, and 53 lb by 36weeks), the estimated sum of fetal weights was 2430 g(1215 g per twin, 68th percentile for gestational age) by 28 weeks and the estimated sum of birth weights was 5081g (2541 g per twin, 69th percentile for gestational age) by 36 weeks.

Pederson et al 1989	Cohort study of twin pregnancies to identify the optimal weight gain and pattern for normal twin gestations Optimal outcome was defined as: Pregnancy at least 37 week gestation with delivery of two living twins ≤2500g each with a 5 minute Apgar ≥7. Exclusion criteria: • Diabetes Mellitus • Major Infection • Organic Heart Disease • Drug Addiction • Accident during pregnancy • Maternal age<18 years old	Mean Weight Gain for optimal fetal outcome 20kg(44lb), the mean weight gain with less than optimum fetal outcome was 16kg(37lb) Rate noticeably declined in less than optimal groups at 30 weeks. No significant difference between zygosity

6.4.1 Twin search results

Luke et al 2003 used a large cohort of 2324 twin pregnancies to produce multiple regression curves using maternal pregravid weight, and postpartum weight within 1 week of delivery, and at least two ultrasonographic weight estimates. They used this information to decide what weight gain lead to the best outcomes, for example fetal birth weight 2850-2928g, gestation 36-38 weeks. This was subdivided based on the mothers' prenatal BMI: underweight (<19.8), normal (19.9-26.0), overweight(26.1-29.0) and obese(>29.0). It was found that in the underweight group a greater weight gain was required to achieve optimum fetal outcomes, and greater rate of weight gain was required up to 20 weeks. Not surprisingly, less weight gain was needed for the obese and overweight groups: see table 6.4.

	0-20wks lb/wk(kg/wk)	20-28wks lb/wk(kg/wk)	29wks –delivery lb/wk(kg/wk)
Overweight (26.1-29.0)	1-1.25 (0.45-0.57)	1-1.5 (0.45-0.68)	1 (0.45)
Obese (BMI>29.0):	0.75-1 (0.34-0.45)	0.75-1.25 (0.34-0.57)	0.75 (0.34)

Table 6.4. Luke et al 2003 weight gain recommendations for the overweight and obese in twin pregnancies

However these regression curves were made only using two maternal weight gains recorded prenatally or post partum after 1 delivery, this does not take into account weight that may have been gained then lost during pregnancy at critical moments of growth i.e. 20 or 32 weeks, as the mother may have gained the appropriate weight but may not have met the demands needed by the fetus later on.

This study also does not take into account where this weight gain came from, as it may come from water retention, or build up of fat or muscle. BMI is also not a good indication of the health of the mother, as it is unable to distinguish between fat and muscle mass. (Romero-Corrral et al 2008). Subsequently this muscle may have been lost during pregnancy if the mother became less active, introducing bias into the data, as it would not have shown true weight gain. Also it should be noted the fetal regression curves used ultrasonographic data, and are prospective. Operator bias could have been introduced by different levels of skill of the ultrasonographers, and therefore inaccuracies of estimates of birthweight. It does

however have a large study group which strengthens the power of the study, yet with measuring BMI at the start and the end points it does not show the full picture of maternal BMI throughout pregnancy.

Earlier work by Luke (Luke et al 2003) found that the timing of the weight gain was significant, and in underweight mothers, weight gain in the first twenty weeks made a significant impact on fetal birth weight, with the first and last trimester weight gain having the biggest influence in mothers who had an overweight pregravid BMI, and that all three trimester periods were important in normal pregnancies. This suggests that maternal nutrition in the first 20 weeks of pregnancy is very important, and has the greatest influence on fetal birth weight, as this may be due to vital 'foundations' being formed to enable optimal growth. If there is nutrient insufficiency early on it leads to poor placenta growth and therefore poor delivery of nutrients to the fetus. However it is interesting that there was no significant rate of weight gain found for underweight mothers after twenty weeks considering there was a significant difference in the normal and overweight group. This earlier weight gain is supported by another study by Luke et al 1998 concluded that a higher 20week weight gain of 10lb was associated with higher fetal birthweights (2430g compared to 2323g).

Lantz et al 1999 also looked at maternal BMI as an independent factor for the fetal outcomes. A lower rate of weight gain was associated with fetal birthweight over 2500g. However this study size was smaller than Luke et al 2003. Birthweight was the only 'optimum fetal outcome' studied – in contrast to Luke who had also included 36-37 week gestation as an optimal outcome, so this would have influenced the data. Lantz et al actually found that a lower rate weight gain than the rate recommended by Luke et al was successful to achieve over 2500g in the underweight women with a BMI<19.8 compared to those with a 19.8-26 or >26. Their rate was calculated from twenty weeks by subtracting the maternal 20 week weight from last known maternal weight before delivery, and dividing by twenty, however it is unclear what gestations these weight were recorded.

Pedersen et al 1989 also found that a weight gain of 20kg compared to 16kg led to a better fetal outcome; two fetuses over 2500g and gestation of 36-37 weeks. It was also noticed that there was a decline in the rate of growth from 30 weeks in the less than optimal weight gain group.

6.4.1.1Dietary intervention in twins

As discussed earlier the majority of studies focused on the association between maternal weight gain and fetal outcome, rather than the direct effects of nutritional advice, however two studies were found that used dietary intervention to help improve fetal outcome.
One study by Luke et al in 2003 used a specialised diet tailored to the individuals weight, and advocated a rate of weight gain dependent on their maternal pregravid BMI, 4000 kcal/day for underweight, 3500kcal/day for normal weight, 3250kcal/day for overweight and 3000kcal/day for obese. 20% of calorie intake was from protein, 40% carbohydrates, and 40% from fat. Table 6.1 gives full details of the rate of weight gain recommended. It was found that there were significant differences in between the groups, and that dietary intervention improved fetal outcomes, see table 6.3 for more details. Preterm labour was 10% in the intervention group compared to 25% in the non intervention group. There were also significantly fewer low birth weight babies in the intervention group. However there was no information on maternal health after such a high calorie diet, and what implications this might have had on their mental well being. It is difficult to say if this high weight gain was actually achieved, or if the reduction in preterm labour was a result of maternal education or of a healthy diet.

A similar intervention was tried using the Higgins Nutritional programme where a specialised diet was created dependent on the mothers' risk factors, and the demands of pregnancy. There were lower rates of low birth weight and preterm labour in the group that received the Higgins nutrition intervention programme. There were no significant differences found apart from when the results were adjusted for confounding bias, and then it was found that the odds ratio was significantly different in the intervention group for low birth weight, very low birth weight and preterm delivery, 0.73(0.55-0.99), 0.53(0.29-0.97), 0.68(0.51-0.92) respectively.

6.4.2 Triplets

Similar results were seen in the triplet search with the all the studies being cohort, and focusing on maternal weight gain rather than the factors that affect it, i.e. calorie intake, and diet composition.

Blickstein et al 2005 focused on the differences of maternal weight gain between the 5th and 1stcentile of neonatal birthweights, finding there was no significant difference between maternal weight gain between the two different centile groups. Table 6.5 shows the results of the studies found for the triplets, and gives more details of this study.

Flidel Ramon et al 2006 also found that an early weight gain was not associated with significant difference in birthweights for normal pregravid BMI triplet pregnancies, although the percentage for small for gestational age did decrease as the average weight gain increased. However rather than use each triplet's individual birth weight, the birth weight outcome was a total of all three triplets' birthweights combined, which would hide any discordancy and a triplet with a low birth weight could potentially be hidden by a triplet with a high birth weight and not show the true outcome.

Maternal BMI and the rate of weight gain was also shown to be insignificant in triplets in a cohort study evaluating 38 triplets by Luke et al 1995. It was found that maternal pregravid BMI did not influence the gestation of the triplets, and earlier maternal weight gain at a lower rate did not lead to more preterm triplets, and that the lower rate of weight gain was in the 35-37weeks gestation group. However other work by Luke et al 2002 using a larger cohort of 178 triplets found that if women had a BMI of <26.0 and had not gained 36lbs by 24 weeks gestation there was significantly reduced fetal growth and birth weight. Also a birth weight ≤25thcentile was significantly associated with a shorter gestation in all weight groups.

Eddib et al 2007 also studied the impact of maternal weight gain and BMI on fetal weight gain. Total maternal weight gain of 15kg or more significantly increased the length of gestation and the mean fetal birth weight, however maternal pregravid BMI did not significantly affect fetal outcome.

Table 6.5 Results of triplet search

Author	Method	Results		
Blickstein et al 2005	Cohort 2850 triplets	No significant difference between average maternal weight gain between the 5th and 1st decile of neonates birth weight Weight Gain greater than 0.68 kg/week 15.3% (5th decile) 13.0%(1st decile) OR 1.2 (0.7, 2.0)		
Flidel-Rimon et al 2006	Retrospective observational study of1166 triplet mothers with a normal (19.8–26) pregravid BMI. Weight gain categorized into three groups: • *Adequate* if the average exceeded a weekly weight gain of 680 g • *Average* if less than recommended weight gain 500-680g/wk • *Inadequate* if weight gain below average<500g/wk.		Weight gain (g/wk) 500(A) 500–680(B) 680(C)	Statistics(OR) A vs. B B vs. C A vs. C
		≥1 Small for gestational age	17.6% 14.2% 13.9%	1.2 (0.9, 1.9) 1.6 (0.9, 2.7) 1.3(0.8, 2.1)
		Total birth weight <4500g	26.9% 21.6 % 20.2%	1.3 (0.9, 1.8) 1.4 (0.9, 2.1) 1.4 (0.9, 2.2)

Levy 2007	Retrospective observational study of triplets: 1235 nulliparas and 705 multiparas. The difference between the pregravid body mass index (BMI) and at 16–25 weeks' gestation was averaged to obtain the weekly change in BMI, defined as slow, typical,or fast by values -1SD, "1SD, and)1SD from the mean average weekly change in BMI.	Weight Gain Gestational Age(weeks) • <28 weeks • 28-32 • >32weeks Birth weight • <1000 • 1000-1500 • >1500 SGA ≥1 Discordance>25%	Slow Typical Fast 8.0% 4.2% 3.0% 40.7% 38.6% 40.0% 50.8% 57.6% 57.2% 11.9% 6.3% 5.1% 25.7% 21.8)% 19.7% 62.3% 71.9% 75.% 25.9% 18.2% 14.6% 47.5% 45.5% 45.1%	Statistics S vs. T S vs. F T vs. F NS NS NS NS NS NS NS NS NS 2.0 (1.4–2.8) 2.5 (1.5–4.2) NS NS 1.4 (1.1–1.9) NS 2 0.6 (0.5–0.8) 0.5 (0.4–0.7) NS 1.6 (1.1–2.4) 2.0 (1.1–3.7) NS NS NS NS

Luke et al 1995	Cohort study of 38 triplets to access the effect of maternal weight gain on fetal birth weight Inclusion criteria: • Complete data of maternal height, pregravid weight, last menstrual period • Expected date of delivery, actual date of delivery • Prenatal weights at 24weeks or at 23,25 weeks gestation • Last recorded weight within 1 week of delivery • Birthweights of all three triplets	Body mass index(BMI) Underweight<19.8(%) Normal (19.8-26.0) (%) Overweight (>36.0) (%) Rate of gestational weight gain/week Before 24 weeks After 24 weeks No significance found	27-30weeks 14.3 71.4 14 1.13±0.2 1.69±0.6	31-34weeks 17.6 82.4 3 0 1.10±0.5 2.38±1.4	35-37weeks 35.7 57.1 7.1 0.97±0.3 1.99±1.2

Luke et al 2002	Cohort of 178 triplet pregnancies to evaluate maternal factors on triplets outcomes Inclusion criteria: • ≥24 gestation • 1st trimester ultrasound • Documented maternal height, pregravid weight • Prenatal weights at each visit, inc within 1 week of delivery		Underweight (BMI <19.8)	Normal weight (BMI 19.8-26.0)	Overweight (BMI >26.0)
		Change in average rate of fetal growth (g/wk)			
		Rate of maternal weight gain			
		0-24 wk	+7.4 (4.8) [.14]	+7.5 (2.0) [<.0001]	+2.5 (1.6) [.13]
		24 wk-delivery	+1.1 (2.1) [.61]	+1.7 (0.7) [.02]	+2.1 (1.1) [.07]
		<36 pounds by 24 wk	–7.7 (3.3) [.02]	–6.6 (1.9) [.001]	–2.7 (2.4) [.26]
		Change in average birth weight (g)			
		Rate of maternal weight gain			
		0-24 wk	+274.3 (166.9) [.11]	+236.9 (66.1) [.001]	+73.5 (49.2) [.15]
		24 wk-delivery	+40.2 (73.5) [.59]	+48.5 (25.3) [.05]	+62.0 (38.9) [.08]
		<36 pounds by 24 wk	–297.5 (110.5) [.01]	–222.0 (61.2) [.001]	–81.0 (73.3) [.28]
		Change in length of gestation (d)			
		Rate of fetal growth g/wk			
		≤ 25th percentile <45g/wk	–37.3 (5.8) [<.0001]	–38.6 (4.3) [<.0001]	–35.8 (8.2) [<.0001]
		≥75th percentile: >61 g/wk	+10.9 (11.9) [.37]	+20.4 (5.9) [.001]	+24.5 (8.8) [.009]
		Values are presented as β coefficients, (SE) [P value]			

Eddib et al 2007	Cohort of 56 triplet pregnancies to evaluate the relationship of Maternal BMI and weight gain on fetal outcomes	Weight Gain	Gestational Age At Delivery		Mean Triplet Birth weight	
			Median	IQR	Median	IQR
		<15.9kg	28.4	24.1-32.8	1231	624-1717
		15.9-20.5kg	33.1	31.9-33.8	1945	1495-2317
		>20.5kg	33.1	31.7-34.4	1951	1586-2279
				P value=0.004		P value=0.001

6.5 Discussion

6.5.1 Twins

Having completed the literature search, we found no randomised controlled trials, and very few of the cohort studies which focused on the effects of dietary advice rather than the association between different amounts of maternal weight gain and fetal birth weight. Maternal BMI should be taken into consideration as underweight mothers are more likely to have poor rates of fetal growth, especially in the first 20 weeks, if there inadequate weight gain. However, how much weight gain is required needs to be decided as there are varying recommendations: Luke et al (Luke et al 2003) and Lantz et al (Lantz et al 1999) both found that optimal fetal weight in the first 20weeks was achieved by a rate of maternal weight gain of 1.25-1.75 lb/wk and 1.13lb/wk respectively, and a higher rate after 20 weeks of 1.5-1.75lb/wk and 1.92lb/wk respectively. However the optimal fetal birth weight was defined as different weights, Luke et al 2850-2928g compared to >2500g by Lantz et al.

However these results were calculated by using regression curves and the mother's weight was only measured prenatally, and postpartum within one week of delivery; there was not a direct weekly measure of weight.

A high total weight gain of 53lb was also associated with a birth weight greater than 2500g by 36 weeks, with a lower weight gain of 25lb achieving a birth weight of 2270g.

The data suggests that a higher maternal weight gain results in a higher fetal birth weight, and it is important that there is adequate nutrition in the first 20 weeks, yet not all women will be aware they are having a twin pregnancy until 10+ weeks, so vital time to help growth will be lost.

The difficulty in using weight gain as a measure of growth is that it does not reflect where there is actual growth occurring; the mother maybe increasing adipose tissue rather than the fetus. It does not necessarily give the full information about the diet the mother requires in order achieving the appropriate weight gain. The maternal weight measured did not take into account the size and weight of the placenta. If there is a damaged placenta then the rate of maternal weight gain would not matter. Zygosity was also found not to be a significant issue by Pederson et al however this was focusing on normal twin pregnancy. None of the studies excluded monozygotic twins.

Two studies compared the effect of dietary intervention; either creating a specialized diet dependent of pregnancy risk factors or using a diet dependent on the mother pregravid BMI. Both found that the intervention improved fetal outcome such as preterm delivery, and low birth weight. Both interventions also required regular follow ups with the dietician and to create an individualized diet. However in the Luke group they were also given multivitamin supplements, so it is hard to clarify if these improved fetal outcomes were as a result of regular visits with the dietician, or the high daily calorie intake of 4000-3000kcal a day or the use of multivitamins. One concerning aspect of such a high calorie diet is the postpartum weight the mother will have, and how this will affect her mental well being, and future health. Many women will also suffer from severe nausea and vomiting, and it would not be practical to 'force feed' women who are already struggling to obtain a normal dietary intake. However this may indicate that other supplementation could be used such as Ensure, and other nutrient drinks in order to supply the fetus with the appropriate nutrients.
The cost of a healthy high calorie diet also needs to be taken into account, as twin pregnancies are financially demanding. Ideally, 40% of the calories intake would be from protein, which would be obtained from meat such as chicken (or lentils if vegetarian) which would also increase the costs.

6.5.2 Triplets

There is a large deficit in knowledge for triplet nutrition, there were no trials found evaluating the diet of triplets only weight gain that led to the greatest fetal birth weight. It can only be presumed that as a result of having three or more growing fetuses that there is a greater demand for nutrition than in twins and it is important to have a healthy high calorie diet. What the studies do show is that maternal pregravid BMI does not have a significant effect on fetal outcome, but a fast rate of weight gain and total weight gain of 15.9kg or more are associated with a longer gestation and larger mean birth weight. However the weight gain may not necessarily be a cause of poor rate of growth more an indication of how effective the utero-placenta unit is, showing that the fetuses are receiving enough nutrients to be able to grow from a healthy placenta.

6.6 Limitations and further work

This review showed that there was a severe shortage of studies accessing the dietary requirements required, as this limits our ability to review what current information known, and to develop appropriate guidelines. It may be that more baseline differences between multiple births and singleton physiology needs to be quantified so singleton research can be used to aid dietary advice for multiple births. However it is clear that more work needs to done to validate such a high calorie intake for twin mothers, and more maternal outcomes need to be included, as the reality of such a high calorie diet without all the dietary support will large quantities of mothers eating high fat foods in order to obtain the calories yet damaging their future health.
Also maternal education of a healthy pre-pregnancy diet will improve growth in early pregnancy also needs to evaluated as not all twin pregnancies are planned.

6.7 Conclusion

High amounts of maternal weight gain are associated with higher birth weight in twins, and that higher amounts of weight gain are required if the mother is underweight, and less if obese. During the first twenty weeks of pregnancy it is important that the mother has a healthy diet, however more research is required to in multiple births about diet content. It is also important to have a supportive network of dieticians to help educate mothers about having a healthy diet, and finding an economical way to achieve this.

Chapter 7: Final Discussion

Final Discussion

Preterm labour and preterm delivery are the greatest risks in multiple pregnancies, and therefore there are higher rates of associated perinatal mortality and morbidity. However, despite the many advances in science; the mechanism that causes early delivery in twins and triplets or even in singletons is still unknown.

Previous work has shown that twin myometrium has a longer duration (Turton et al 2008), and daily uterine monitoring systems have shown a higher basal mean frequency of contractions compared to singletons. However on comparison of *in vitro* myometrial contractile properties of multiple and singleton pregnancies there were no significant differences. It should be noted that the triplet sample number was limited. The preterm samples collected also showed no significant differences to the term group; however, these sample numbers were small, and difficult to obtain. Yet this lack of significant difference between contractile myometrial properties suggests that some other process is occurring *in vivo*.

On comparing the effects of progesterone between singleton and twin myometrium, significant differences were found at 10µM and 100µM progesterone, with progesterone having less of an effect in reducing the force of control contractions in twin myometrium with a dose response relationship. As progesterone trials have found it to be effective in singleton high risk pregnancies in reducing preterm birth (Meis et al 2003), but not in multiple pregnancies (Norman et al 2009, Caritis et al 2009, Briery et al 2009), a higher dose of progesterone may be required to have a similar effect. This difference allows us to further our understanding of the different myometrial properties and start to cast some light on the complex pathway of labour.

Other *in vitro* work has had success with progesterone metabolites to reduce the force and frequency of uterine contractions (Thornton et al 1999), as well as progesterone (Anderson et al 2009). My work has also helped support the theory of progesterone's ability to reduce the force of uterine contractions *in vitro.*

The next step maybe would be to see if a higher dose of progesterone would make a significant difference *in vivo*, however the risk-benefit ratio of giving higher doses of progesterone needs to be considered.

This difference in effect could be due to a reduction in progesterone membrane receptor expression as a result of greater amounts of stretch, thus reducing levels of cAMP and causing a more 'active uterus'. However it is likely that it is not this simple and a

combination of factors occur that reduce the expression of membrane progesterone receptors. The effect of stretch was not found to be significant; however, this finding was limited by a small sample size.

The stretch theory has not yet been fully established as a trigger for preterm delivery and future works needs to find an appropriate method to help quantify this, to help determine the effect stretch has on the uterus.

If stretch is one of the mechanisms that trigger contractile pathways leading to preterm delivery, it is interesting to consider the effect the high calorie diet recommended by Luke et al would have on preterm delivery, as it was found that a higher rate of maternal weight gain before 20 weeks led to higher rates of fetal growth, and final birth weight. These mothers that are achieving higher rates of fetal weight gain early in pregnancy are also placing having greater amounts of stretch placed on the uterus. This is another subject that there is a lack of knowledge, and requires further research. Despite the success of the intervention studies it is difficult to clarify whether or not a diet of 4000kcal or less is achievable without the necessary support, for example regular dietician meetings, or how practical and achievable it is for the patient. The maternal outcomes also need to be taken into consideration, as the high weight gain will likely impact the future health of the mother.

I hope my work has helped to answer a few of the many questions that arise around multiple pregnancy, and shed some light on a possible pathway that leads to early uterine preterm labour.

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Appendices

Appendix A: Patient characteristics of samples

Maternal Age	Gestation	P	G	BMI	Twin/Singleton	Elective/Emergency C- section
28	39	1	2	30.2	Singleton	Elective
34	39	1	4	33.9	Singleton	Elective
24	39	1	2	22.1	Singleton	Elective
28	39	1	2	42.1	Singleton	Elective
36	39.1	1	2	23.8	Singleton	Elective
23	35	0	1	19.3	Singleton	Elective
36	39	1	2	21.6	Singleton	Elective
29	39.4	2	3	18.1	Singleton	Elective
33	39	3	4	22.7	Singleton	Elective
26	39	2	1	28.7	Singleton	Elective
40	36	0	1	22	Singleton	Elective
26	39	1	2	29.3	Singleton	Elective
37	39	2	7	25.8	Singleton	Elective
27	36.2	2	3	16	Singleton	Elective
37	37.4	0	3	30.9	Singleton	Elective
28	39.6	1	1	24	Singleton	Elective
24	39	0	3	21.2	Singleton	Elective
32	39	1	3	26.2	Singleton	Elective
36	37	6	7	34.2	Twin	Elective
20	37	0	1	27.2	Twin	Elective
34	35.4	1	3	24.8	Twin	Elective
34	37.5	0	1	22.6	Twin	Elective
44	36.4	2	2	31	Twin	Elective
29	37.2	1	2	28.8	Twin	Elective
33	37	0	2	24.2	Twin	Elective
33	37.6	0	1	23.2	Twin	Elective
28	32.4	3	5	22	Triplet	Elective
40	32	0	1	25	Triplet	Elective
20	31	2	1	23	Singleton	Emergency
29	27	0	1	26.2	Twin	Emergency

Appendix B: Search strategy for dietary advice

Database: Ovid MEDLINE(R) and Ovid OLDMEDLINE(R) 1948 to Present with Daily Update
Search Strategy:

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1 Epidemiologic studies/ (4646)
2 exp case control studies/ (460485)
3 exp cohort studies/ (757520)
4 Case control.tw. (50039)
5 (cohort adj (study or studies)).tw. (45539)
6 Cohort analy$.tw. (2198)
7 (Follow up adj (study or studies)).tw. (30311)
8 (observational adj (study or studies)).tw. (22402)
9 Longitudinal.tw. (91677)
10 Retrospective.tw. (175360)
11 Cross sectional.tw. (96301)
12 Cross-sectional studies/ (109655)
13 or/1-12 (1347089)
14 exp Diet/ (155052)
15 diet$.mp. (401494)
16 exp Dietary Supplements/ (24122)
17 ((prenatal$ or antenatal$) adj2 (nutrition$ or nutrient$ or supplement$)).ti,ab. (496)
18 exp Weight Gain/ (17145)
19 exp Pregnancy, Multiple/ (24752)
20 (twin$ or triplet$).ti,ab. (43225)
21 (multiple adj pregnancy).ti,ab. (1654)
22 14 or 15 or 16 or 17 or 18 (443790)
23 19 or 20 or 21 (50768)
24 13 and 22 and 23 (211)
25 limit 24 to humans (209)
26 from 25 keep 1-209 (209)

Appendix C: Ethical approval letters

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Liverpool Women's Hospital
Crown Street
L8 7SS

Dear Professor Neilson

Study Title:	**Myometrial contractility in multiple pregnancies: exploring the pathophysiology of preterm and dysfunctional labour.**
REC reference number:	**09/H1002/64**
Protocol number:	**1**

The Research Ethics Committee reviewed the above application at the meeting held on 17 September 2009. Thank you for attending to discuss the study.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*	
Covering Letter		27 August 2009	
REC application	2.2	27 August 2009	
Protocol	1	03 August 2009	
Investigator CV	1.0	03 August 2009	
Letter from Sponsor		27 August 2009	

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1002/64	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project
Yours sincerely

Mrs Jean Harkin
Chair

Email: Ronald.Wall@liverpoolpct.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Gillian Vernon, R & D, Liverpool Women's NHS Foundation Trust*

North West 3 Research Ethics Committee - Liverpool East

Attendance at Committee meeting on 17 September 2009

Committee Members:

Name	*Profession*	*Present*	
Dr Paul Baines	Vice Chair / Consultant Intensivist	No	
Mr Colin Birch	Charge Nurse	No	
Mr John Bridson	Lecturer	Yes	
Mr Colin Bruce	Consultant Paediatric Orthopaedic Surgeon	Yes	
Mrs Elizabeth Gilkes	Lay Member	Yes	
Ms Andrea Gill	Senior Pharmacist	Yes	
Ms Carole Griffith	Physiotherapist Case Manager	Yes	
Mrs Jean Harkin	Chair / Lay Member	Yes	
Mrs G J Hunt	Lay Member	No	
Mr Ed Ladusans	Consultant Cardiologist	Yes	
Dr Omnia Marzouk	Consultant Paediatric A&E Medicine	No	
Professor Neil Pender	Professor of Orthodontics	Yes	

Mrs Jean Pownceby	Lay Member	Yes	
Dr Richard Sarginson	Consultant (Anaesthesia/PICU)	Yes	
Dr Peter Walton	Lay Member	Yes	

Written comments received from:

Name	*Position*	
Mr John Bridson	Lecturer	
Mr Colin Bruce	Consultant Paediatric Orthopaedic Surgeon	
Mrs Elizabeth Gilkes	Lay Member	
Ms Andrea Gill	Senior Pharmacist	
Ms Carole Griffith	Physiotherapist Case Manager	
Mrs Jean Harkin	Chair / Lay Member	
Mr Ed Ladusans	Consultant Cardiologist	
Professor Neil Pender	Professor of Orthodontics	
Dr Richard Sarginson	Consultant (Anaesthesia/PICU)	
Dr Peter Walton	Lay Member	

Appendix D: Copy of consent form

Consent form
Collection and Storage of Tissue for Research

Myometrial Research Tissue Bank, University Department, Liverpool Women's NHS Foundation Trust, Liverpool L8 7SS. Tel: 0151 702 4100

I (print name):……of

Address: …… …..

	Please initial
I have read and understood the Information Leaflet (version 1) about the use of tissue removed at my caesarean section for research purposes and have been given a copy to keep.	
I agree that Liverpool Women's NHS Foundation Trust will become the custodian of this tissue and that it will be used in regulated research projects following anonymisation (I will not be identifiable to the researcher) and subject to separate ethical approval.	
I give permission for medical records that relate to my pregnancy / labour to be periodically examined and relevant information taken from them to be used in approved research projects.	
I understand that any information about me will be treated confidentially and stored securely.	
Neither the Research Tissue Bank nor I will benefit financially if this research leads to new treatments or medical tests.	
I understand that I may, at any time, withdraw my approval for tissue and information to be stored and used for research without giving any reason and without it affecting my medical treatment.	

Signed ……Date……..

I have explained the request for research purposes and answered such questions as the patient has asked.

Clinical Practitioner

Signed ……..

Print Name……

Date……