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MYOMETRIAL CONTRACTILITY STUDIES IN DIABETIC PREGNANT WOMEN

A DISSERTATION SUBMITTED TO THE FACULTY OF MEDICINE IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

BY

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List of Abbreviations

ADP	Adenosine diphosphate
AMAs	Animal tissue microarrays
ATP	Adenosine triphosphate
au	Arbitrary units
AUC	Area under the curve
BAY K8644	Methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-Trifluoromethylphenyl)-pyridine-5-carboxylate
BK_{Ca}	Large conductance K_{Ca} channels
BMI	Body mass index
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
Cl^-	Chloride ion
CS	Caesarean section
DPX	1,3-diethyl-8-phenylxanthine
FFAs	Free fatty acids
FPG	Fasting blood glucose
GCT	Glucose Challenge Test
GDM	Gestational diabetes mellitus
GLUT	Facilitative glucose transporter
GPCRs	G-protein coupled receptors
GS	Glycogen synthase

GSK3	Glycogen synthase kinase-3
G6PDH	Glucose 6 phosphate dehydrogenase
G-1-P	Glucose 1-phosphate
G-6-P	Glucose 6-phosphate
HLAs	Human leukocyte antigens
HMAs	Human tissue microarrays
IK_{Ca}	Intermediate-conductance K_{Ca} channels
Indo-1	Indo-1 acetoxymethyl ester
IRS	Insulin receptor substrate
IP_3	Inositol 1,4,5-triphosphate
IP_3R	IP_3 receptor
K^+	Potassium ion
K_{ATP}	ATP-sensitive potassium channels
K_{Ca}	Calcium-activated potassium channels
K_{IR}	Inward rectifier potassium channels
K_V	Voltage-dependent potassium channels
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
Na^+	Sodium ion
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of $NADP^+$
NCX	Na^+/ Ca^{2+} exchanger
OTRs	G-protein coupled oxytocin receptors
OGTT	Oral glucose tolerance test

pH_i	Intracellular pH
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PMCA	Plasma membrane Ca^{2+}-ATPase
pre-GDM	Pre-gestational diabetes mellitus
PSS	Physiological Salt Solution
RyR	Ryanodine receptors
ROCCs	Receptor-operated calcium channels
RT	Room temperature
SEM	Standard error of mean
SERCA	Sarcoplasmic reticulum Ca-ATPase pump
SK_{Ca}	Small-conductance K_{Ca} channels
SR	Sarcoplasmic reticulum
TBS	Tris-buffered saline
TEA	Tetraethyl Ammonium Chloride
TG	Triglycerides
TMAs	Tissue microarrays
UDP-glucose	Uridine diphosphoglucose
VDCCs	Voltage-dependent Ca^{2+} channels
WHO	World health organization
$[ATP]_i$	Intracellular ATP concentration
$[Ca^{2+}]_i$	Intracellular calcium concentration

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ABSTRACT

Myometrial Contractility Studies in Diabetic Pregnant Women

Diabetic women are known to encounter more problems with pregnancy and labour than non-diabetic women. In particular, they are more likely to require an emergency caesarean delivery, even when their blood glucose is well controlled and their babies are of normal weight or if oxytocin is used to augment labour. The reasons for this are unknown. I have explored the hypothesis that the myometrium of diabetics may be less able to contract compared to non-diabetics. However, very little is known about the intrinsic properties of myometrium from diabetic women, or the response to changes in insulin and glucose concentrations. Similarly, there are few studies and measurements of the glucose storage molecule, glycogen, in the two groups. To date, no studies have determined the type of glucose transporters in the myometrium.

I compared *in vitro* contractility and calcium signalling in women with and without diabetes and examined the effects of glucose and insulin on spontaneous contractions and insulin on oxytocin-induced muscle contractions of myometrium. In addition, myometrial glycogen content was measured. I also examined in the myometrium of pregnant women; non-diabetic or diabetic, the expression of glucose transporter 1, 3 and 4 (GLUT-1, GLUT-3 and GLUT-4).

Diabetic samples contracted poorly and showed reduced intracellular calcium transients compared to the non-diabetics. Insulin (700 pM) significantly decreased spontaneous and oxytocin-induced contractions in both groups. High glucose (25 mM) significantly and equally reduced the contractions and calcium transients in both groups. In 0-glucose solution, a more rapid cessation of contractility was observed in the diabetics. In response to Bay K8644 (10 mM) and 40 mM KCl, the diabetic myometrium responded less strongly and showed significantly lower force and calcium transients compared to non-diabetics. Insulin reduced both Bay K8644- and TEA-induced myometrial contractions. There was significantly less glycogen in diabetic samples. The GLUT-1 and GLUT-3 staining density in myometrium of pregnant women at term were lower in diabetics. However, the staining for GLUT-4 was higher in diabetics.

These findings suggest that the diabetic myometrium contracts less strongly than non-diabetic. This appears related to differences in intracellular calcium, as the calcium transients were also smaller in diabetics, suggesting changes to L-type calcium channel entry. My results suggest also, that insulin can induce relaxation of myometrial cells via inhibition of calcium influx through L-type calcium channels and/or stimulation of potassium channels. Hyperglycaemia is inhibitory for uterine contractions. There may be differences in uterine contractility associated with diabetes, with them perhaps being less able to withstand the metabolic demands of labour, as blood flow and hence glucose falls with contraction. This may relate to differences in glycogen utilisation, which is lower in diabetics due to the lower expression of GLUT-1 and GLUT-3. All of these changes seem to contribute to poor uterine contraction and, hence, may well contribute to the higher rate of CS seen in diabetes.

Chapter 1

General introduction

1.1 The Uterus

1.1.1 Anatomy of the uterus

The following account is based on standard anatomical texts (William, 2005; Tortora & Nielsen, 2008; Graaff *et al.*, 2009). The uterus is a thick-walled muscular organ situated deep in the abdominal cavity. It is located in the pelvic cavity between the bladder and the rectum. The uterus has a number of important functions that are fundamental in order for successful pregnancy to occur. It provides an appropriate environment for implantation of the fertilised ovum, after which time it undergoes alterations in size and structure to accommodate to the needs of the growing embryo. During pregnancy, the uterus provides nourishment for the foetus and also serves as a mechanical barrier throughout the entire developmental stage. This female reproductive organ is divided into fundus, body and cervix (Figure 1.1). At the fundus, the uterus narrows to form the uterine tubes, which terminate at the ovaries. The body of the uterus narrows below to form the cervix.

Humans have a single-chambered simplex uterus which has a similar cross-sectional morphology throughout. The wall of uterus is divided into three distinct regions; endometrium, myometrium and perimetrium. The innermost layer is the endometrium which lines the uterine cavity. It is the area where the fertilized egg is implanted upon its arrival in the uterus. In the event of pregnancy, the endometrium continues to grow and eventually forms part of the placenta. The endometrium sloughs off in the form of menstruation if implantation does not occur. The middle layer

of the uterine wall is known as myometrium. This layer is the predominating region of the uterus both in terms of size and function, consisting mainly of poorly defined muscle bundles. They arranged in circular, longitudinal and diagonal orientations and are held together by connective tissue. The myometrium is able to contract without hormonal or nervous stimulation, so that uterus is considered a myogenic organ. During labour, the myometrium contracts in co-ordinated way and strongly enough to provides the driving force for the expulsion of the foetus and placenta. The perimetrium is a thin epithelial layer which covers the outside of the uterus.

1.1.2 Uterine myometrial cells

The major components of the uterus are the smooth muscle myocytes. Although similar to other smooth muscle cells, they are considerably larger, ranging in size from 300-600 μM in length and 5-10 μM in diameter during pregnancy (Broderick & Broderick, 1990). It has been reported that the average volume and total surface area of a human myometrial cell are about 21,000 μm^3 and 23,000 μm^2 respectively (Kao, 1977; Izumi *et al.*, 1994).

The uterine myocyte contains thick myosin and thin actin filaments arranged in long, random bundles which occupy most of the myocyte volume and constitute the contractile machinery (Broderick & Broderick, 1990). The myometrial smooth muscle cells communicate with each other by means of gap junctions (Garfield *et al.*, 1992). These cell-to-cell contacts mediate the rapid spread of electrophysiological activity between cells, and they are upregulated before spontaneous labour in humans (Garfield *et al.*, 1977; Garfield *et al.*, 1978; Broderick & Broderick, 1990; Garfield *et al.*, 1992; Young, 2000).

The plasma membrane of the myometrial cells is believed to be occupied by high density of flask-shaped invaginations, known as caveolae that increase the surface of plasma membrane

(Wray & Noble, 2008). The uterine caveolae are thought to be involved in signalling pathways in the uterus (Lee *et al.*, 2001).

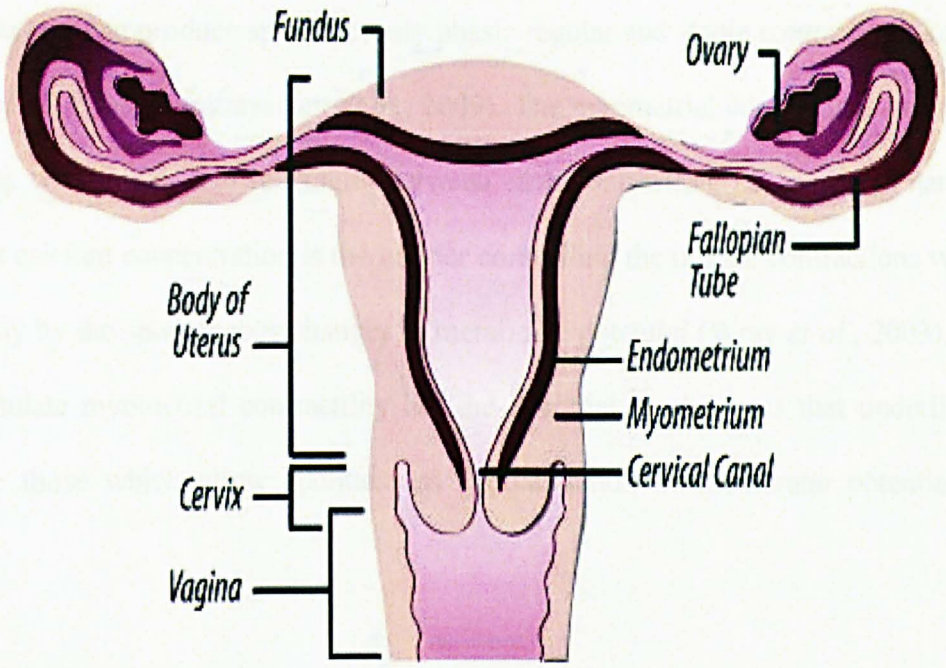

Figure 1.1: The structure of human uterus. Picture was taken and adapted from National Uterine Fibroids Foundation website (http://www.nuff.org/images/NormalUterusRGB.jpg).

1.2 The excitability of uterine myocytes

1.2.1 An overview

The uterus is a myogenic organ; means that its smooth muscle is able to contract spontaneously without the need for hormonal or nervous stimulation (Wray, 1993). Indeed, it has been reported that *in vitro* the uterus can produce spontaneously phasic regular and stable contractions for several hours (Gullam *et al.*, 2009; Kupittayanant *et al.*, 2009). The myometrial contractions are relatively fast, short lasting with a period of relaxation between each contraction, i.e. phasic in nature. The free intracellular calcium concentration is the key for controlling the uterine contractions which are modulated mainly by the spontaneous changes in membrane potential (Wray *et al.*, 2003). Several factors can modulate myometrial contractility but the essential mechanisms that underlie phasic contractions are those which allow spontaneous depolarization of membrane potential of the myometrium.

1.2.2 Myometrial membrane potential and contraction

The uneven distribution of ions in the extracellular and intracellular milieu is needed to generate the resting membrane potential (Wray *et al.*, 2001; Wray *et al.*, 2003). Like other human tissues, the resting membrane potential in myometrial cells is largely determined by the concentrations of calcium (Ca^{2+}), potassium (K^{+}), sodium (Na^{+}) and chloride (Cl^{-}) ions on either side of the membrane (Parkington & Coleman, 1988; Jones *et al.*, 2004; Young, 2007). The differences in ions across the myometrial cell membrane are driven by the activity of array of proteins in the plasma membrane such as potassium and calcium channels (Khan *et al.*, 1997; Noble *et al.*, 2009).

Most of the knowledge concerning membrane potential in myometrium comes from the work of Parkington and Coleman (Parkington & Coleman, 2001). The value of resting membrane

potential in myometrial cells ranges from -80 to -55 mV, depending upon the species (Parkington *et al.*, 1999). Rhythmic oscillations of membrane potential (slow waves) can be recorded from the uterine smooth cells (Coleman & Parkington, 1990; Parkington *et al.*, 1999). Spontaneous contraction can be initiated when some of these spontaneous depolarizations reach the threshold for an action potential (Parkington & Coleman, 1990). It is not obvious how these spontaneous depolarizations are triggered in the myometrium. Similar slow waves were identified in smooth muscles of urethra and gastrointestinal tract, in which specialized pacemaker cells were reported (Sanders & Publicover, 1994; Sergeant *et al.*, 2000). These cells exhibit spontaneous depolarizations, which are thought to initiate slow waves in adjacent smooth muscle cells (Sanders & Publicover, 1994; Sergeant *et al.*, 2000). It is not known if the uterus exhibits similar specialised pacemaker cells or that the spontaneous depolarization is an intrinsic property of all smooth cells of uterus (Parkington & Coleman, 1988; Parkington & Coleman, 1990; Wray *et al.*, 2003). However, it has been reported recently that ATP may be involved in the pacemaking mechanism in human myometrium (Hutchings *et al.*, 2009).

Whenever the cell membrane depolarizes, opening of voltage gated Ca^{2+} channels takes place with a threshold for activation at -60 to -30 mV and peak activity (current flow) occurring at -30 to +10 mV (Jmari *et al.*, 1986; Amedee *et al.*, 1987; Triggle, 1998; Perez-Reyes, 2003). Calcium entry into the cell, which occurs down its concentration and electrochemical gradient, is the major component of action potential upstroke and causing further membrane depolarization (Wray, 1993; Wray *et al.*, 2003). Inactivation of the Ca^{2+} current and activation of the outward K^+ current lead to repolarisation; the down stroke of action potential (Wray, 1993; Wray *et al.*, 2003). It has been reported that there are cycles of rising and falling of $[Ca^{2+}]_i$ (Sanborn, 2000) which are accompanied by similar changes in force or spontaneous phasic activity (Wray, 1993). The crucial involvement of Ca^{2+} entry via L-type voltage-dependent Ca^{2+} channels (VDCCs) to this process in

the myometrium has been shown in human and animal studies (Young *et al.*, 1993; Wray *et al.*, 2002; Lee *et al.*, 2009). Both Wray et al. (2002) and Lee et al. (2009) found that spontaneous phasic activity was inhibited by nifedipine, a specific inhibitor of VDCC.

1.2.3 L-type voltage-gated Ca^{2+} channels

L-type voltage-dependent Ca^{2+} channels are formed as a complex of several different subunits (α_1, α_2, β, γ and δ); α_1 forms the ion conducting pore and α_2, β, γ and δ are auxiliary subunits (Klockner *et al.*, 1996; Serysheva *et al.*, 2002). There are two different types of voltage-dependent Ca^{2+} channels in human uterine smooth muscle cells; T- and L-type calcium channel (Parkington & Coleman, 1988; Parkington & Coleman, 1990; Wray *et al.*, 2003). Calcium current of both channels have been reported in myometrial cells of pregnant women (Young *et al.*, 1993). However, L-type Ca^{2+} current is the major source of calcium entry during depolarization of uterine myocytes (Shmigol *et al.*, 1998b). Each phasic myometrial contraction is accompanied by a Ca^{2+} transient in which both the transients and contractions are abolished if L-type channels are blocked (Wray *et al.*, 2003; Lee *et al.*, 2009). Further, it has been found that the L-type channels are more sensitive to inhibitory effect of dihydropyridine such as nifedipine than T-type channels (Young *et al.*, 1993). Moreover, it has been reported that T-type Ca^{2+} channels may be involved in the generation of spontaneous Ca^{2+} transients and the modulation of the frequency of spontaneous Ca^{2+} transients in the myometrium (Lee *et al.*, 2009).

Cumulative studies showed that calcium channels activity is modulated by hormonal stimulation. It has been revealed that oestrogen increases Ca^{+} entry in rat myometrium which may due to increment in the density of calcium channels (Batra, 1987). However, previous studies showed that oestrogen inhibits Ca^{+} channels currents in cultured (Yamamoto, 1995) and freshly

dispersed (Okabe *et al.*, 1999) myometrial cells isolated from pregnant rats. Further, one study has shown that the density of the Ca^{2+} current in the myometrium of non-pregnant rats is increased by progesterone (Rendt *et al.*, 1992).

1.2.4 Potassium channels

The resting membrane potential of myometrium is mainly set by the K^+ conductance of the membrane (Parkington & Coleman, 1990). The major K^+ channels that have been recorded in smooth muscles are voltage-dependent potassium (K_V) channels, calcium-activated potassium (K_{Ca}) channels, inward rectifier potassium (K_{IR}) channels and ATP-sensitive potassium (K_{ATP}) channels. Moreover, the channel subtypes have been characterized based on physiological and pharmacological properties. For instance, there are three different subtypes of $K_{Ca;}$ large conductance K_{Ca} (BK_{Ca}), intermediate-conductance K_{Ca} (IK_{Ca}) and small-conductance K_{Ca} (SK_{Ca}).

The main effect of increased K^+ channels conductance in excitable tissues such as myometrium is to decrease excitability, while decreased K^+ conductance results in depolarising force and increased membrane excitability. This has led to the specific study of K^+ channel activity in the myometrium at the end of pregnancy where the uterus undergoes a dramatic increment in its contractile activity. It has been reported that BK_{Ca} channels are the predominant K^+ channel type encountered in non-pregnant and pregnant human myometrium (Tritthart *et al.*, 1991; Anwer *et al.*, 1993; Khan *et al.*, 1993a). These channels play an important role in the maintenance of uterine tone by regulating membrane potential and intracellular Ca^{2+} (Anwer *et al.*, 1993). Furthermore, using Tetraethyl Ammonium Chloride (TEA) or iberiotoxin to inhibit these channels elicits or augments uterine smooth contraction suggesting that under physiological conditions at least a proportion of BK_{Ca} channels are open opposing contractile activity (Anwer *et al.*, 1993; Khan *et al.*, 1997). In

human myometrium, BK_{Ca} channels may play a key role by providing an outward current throughout pregnancy which may maintain the inexitability to some extent and preventing the premature strong uterine contractions seen in active labour (Khan *et al.*, 1993b). Moreover, Noble et al. found that SK_{Ca} channels contribute more to quiescence than BK_{Ca} channels (Noble *et al.*, 2009). However, it has been reported that K_V channels plays a crucial role in regulating basal contractility in rats myometrial smooth muscle particularly during mid and late pregnancy whereas BK_{Ca} channels have little or no effect (Aaronson *et al.*, 2006; Noble *et al.*, 2009).

1.3 Regulation of $[Ca^{2+}]_i$

1.3.1 An overview

The free intracellular calcium concentration is a major determinant of smooth muscle contractility; an increase of $[Ca^{2+}]_i$ from 10^{-7}M to 10^{-6}M is needed for contraction to take place (Horowitz *et al.*, 1996). The increase of $[Ca^{2+}]_i$ during contractions occurs as a result of influx of Ca^{2+} from the extracellular space and/or release of Ca^{2+} from intracellular stores; sacroplasmic reticulum (SR). Although Ca^{2+} influx through VDCC is crucial to spontaneous contractility, Ca^{2+} can also enter the cells through receptor-operated calcium channels (ROCCs) and store-operated calcium channels (capacitative calcium entry). Intracellular free Ca^{2+} can be extruded from the cell by the reverse process; sequestration into SR and/or extrusion through the cell membrane by plasma membrane Ca^{2+}-ATPase (PMCA) and/or Na^{+}/ Ca^{2+} exchanger (NCX) .

1.3.2 Ca^{2+} influx through plasma membrane

As mentioned above, L-type Ca^{2+} current is the major source of calcium entry in uterine myocytes which occurs during depolarization (Matthew *et al.*, 2004b). However, activation of ROCCs, which are located in the plasma membrane, can lead to a calcium influx that is independent of membrane depolarization (Bolton & Imaizumi, 1996). These channels open in response to the binding of an agonist to specific channel-associated receptors. Receptor-operated currents were described in a number of smooth muscle types following activation of a range of receptors and are of non-selective for cations and have different selective permeability to calcium. G-proteins are essential in the transduction pathway in the myometrium. Uterine activity can be modulated by several G-protein coupled receptors (GPCRs). For instance, G-protein coupled oxytocin receptors (OTRs) which have been studies extensively as oxytocin receptors are abundant in the

pregnant uterine tissues (Ku *et al.*, 1995; Sanborn *et al.*, 1995). Binding of oxytocin to OTRs leads to activation of phospholippase C (PLC), which librates inositol 1,4,5-triphosphate (IP_3), and hence release of Ca^{2+} from SR (Ku *et al.*, 1995; Sanborn *et al.*, 1995). However, it has been reported that oxytocin increases $[Ca^{2+}]_i$ via activation of L-type Ca^{2+} channels (Kupittayanant *et al.*, 2002). Indeed, the current through ROCCs can be increased by a range of intracellular signalling pathways, such as calcium-calmodulin and MLCK (McFadzean & Gibson, 2002). In addition, it has been noted that activations of ROCCs can alter the membrane potential and thus in turn activates or inhibits VOCCs (Wray, 1993).

1.3.3 Extrusion of Ca^{2+} through the cell membrane

Extrusion of Ca^{2+} in smooth muscle cells occurs via two proteins spanning the plasma membrane; the PMCA and the NCX (Liu *et al.*, 1996; Shmigol *et al.*, 1998a). The PMCA uses energy of hydrolysis of adenosine triphosphate (ATP) for Ca^{2+} transport (Kosterin *et al.*, 1991), while the NCX utilises the Na^+ gradient for active Ca^{2+} transport (McCarron *et al.*, 1994). By the operation of these mechanisms, intracellular calcium levels are maintained at levels 10,000 fold lower than external calcium. The PMCA has a higher affinity but lower capacity system for Ca^{2+} compared with NCX (Blaustein *et al.*, 2002; Bradley *et al.*, 2002). In the myometrium, both PMCA and NCX have been recognized (Kosterin *et al.*, 1944; Guerini, 1998) and play an important role in Ca^{2+} extrusion with the PMCA contributing up to 70% and NCX 30% (Shmigol *et al.*, 1998a; 1999; Tribe *et al.*, 2000).

1.3.4 Sarcoplasmic reticulum Ca^{2+} release and ion channels

Uptake and release of Ca^{2+} from Sarcoplasmic reticulum (SR) have been noted in human and animal uterine myocytes (Taggart & Wray, 1998; Luckas *et al.*, 1999). The release of calcium

from SR is mediated by both IP_3 and Ca^{2+} (ryanodine) gated receptors Ca^{2+} release channels; IP_3R and RyR respectively (Martin *et al.*, 1999), while the SR Ca-ATPase (SERCA) pumps Ca^{2+} to the SR lumen (Tribe *et al.*, 2000). The IP_3R are mainly activated by the binding of IP_3, but also sensitive to changes in $[Ca^{2+}]_i$ (Bultynck *et al.*, 2003). The increase in local $[Ca^{2+}]$ activates, while its phosphorylation by protein kinase C (PKC) inhibits RyR (Bonev *et al.*, 1997). The opening of IP_3R or RyR could open the neighbor SR channels in a process called calcium induced calcium release (CICR) (Matthew *et al.*, 2004a).

1.3.5 Excitation-contraction coupling

Myometrial contractions are initiated by an increase in intracellular $[Ca^{2+}]_i$. Depolarization of the membrane from around −55 to −40 mV (Shmigol *et al.*, 1998b) opens L-type Ca^{2+} channels leading to influx of calcium; the major source of Ca^{2+} for contractions. This depolarization can be generated spontaneously (Duquette *et al.*, 2005) or in response to hormones binding to receptors and opening channels (e.g. nonspecific cation channels; (Arnaudeau *et al.*, 1994; Miyoshi *et al.*, 2004)) and chloride channels (Jones *et al.*, 2004). Excitation–contraction coupling is a sequence of events that link between the generation of the action potential and initiation of the contraction. In the cytoplasm, calcium ions bind to calmodulin and subsequent activation of myosin light chain kinase (MLCK) takes place, leading to the phosphorylation of serine 19 on regulatory light chains of myosin. In this phosphorylated state, there is a significant interaction of myosin with actin forming cycles of cross bridges, the hydrolysis of ATP and contraction of the myometrium (Taggart *et al.*, 1997). Thus, under physiological conditions the Ca^{2+}–calmodulin–MLCK pathway is essential in producing uterine force. It has been reported that several kinases can phosphorylate MLCK (Horowitz *et al.*, 1996; Weber *et al.*, 1999), and such phosphorylation diminishes its activity and therefore desensitises the contractile machinery.

Dephosphorylation of the myosin light chains by myosin light chain phosphatase (MLCP) reverses the Ca^{2+}–calmodulin–MLCK pathway. Subsequently Ca^{2+} falls as L-type Ca^{2+} channels close and Ca^{2+} efflux mechanisms are stimulated and hence inactivation of MLCK occurs as a result of the dissociation of Ca^{2+} from calmodulin leading to myometrial relaxation (Wray *et al.*, 2001; Wray *et al.*, 2003). MLCP can be phosphorylated, particularly by rho-associated kinase, resulting in a reduction in its activity (Somlyo & Somlyo, 1998) and thus sensitises the contractile machinery, Figure 1.2.

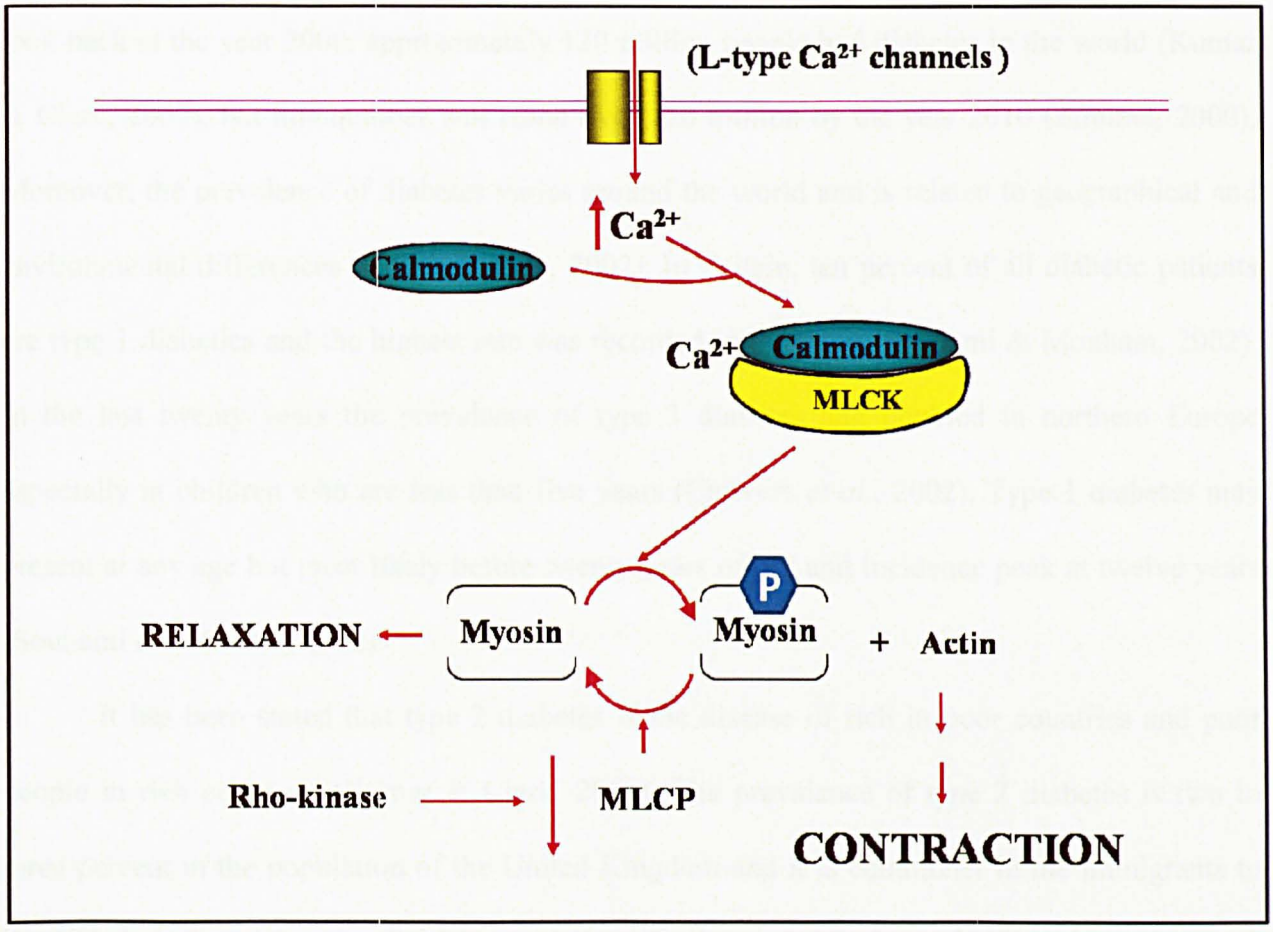

Figure 1.2: The link between Ca^{2+} entry through VDCC and uterine contraction. Ca^{2+}-calmodulin activates MLCK (myosin light chain kinase) which in turn phosphorylates myosin, stimulating cross-bridge cycling and thereby promotes contraction. MLCP (myosin light chain phosphatase) dephosphorylates myosin, and hence, relaxation. Its activity is inhibited by rho-kinase.

1.4 Diabetes mellitus

1.4.1 Overview

Diabetes Mellitus is a world-wide disease characterized by the chronic elevation of the blood glucose with relative insulin deficiency, resistance or both together (Kumar & Clark, 2002). A look back at the year 2000, approximately 120 million people had diabetes in the world (Kumar & Clark, 2002), but this number will reach over 220 million by the year 2010 (Zimmet, 2000). Moreover, the prevalence of diabetes varies around the world and is related to geographical and environmental differences (Chilvers *et al.*, 2002). In Britain, ten percent of all diabetic patients are type 1 diabetics and the highest rate was recorded in Scotland (Souhami & Moxham, 2002). In the last twenty years the prevalence of type 1 diabetes had doubled in northern Europe especially in children who are less than five years (Chilvers *et al.*, 2002). Type 1 diabetes may present at any age but most likely before twenty years of old and incidence peak at twelve years (Souhami & Moxham, 2002).

It has been stated that type 2 diabetes is the disease of rich in poor countries and poor people in rich countries (Kumar & Clark, 2002). The prevalence of type 2 diabetes is two to three percent in the population of the United Kingdom and it is commoner in the immigrants to the UK than the indigenous British population (Souhami & Moxham, 2002). Although type 2 diabetes can be presented at an earlier age in some populations such as Afro-American (Chilvers *et al.*, 2002), most patients develop this disease at forty years of age (Souhami & Moxham, 2002). Diabetes is an irreversible disease that leads to a reduction in the life expectancy due to its retinopathy, neuropathy and nephropathy complications (Kumar & Clark, 2002).

Diabetes can be classified according to the latest world health organization's aetiological classification into type 1 diabetes, previously known as insulin dependent diabetes mellitus, type 2 diabetes, previously known as non insulin dependent diabetes mellitus, gestational diabetes

mellitus (GDM) and other specific types due to, for example genetic defects of beta cell function, diseases of exocrine pancreas and infections like rubella (Souhami & Moxham, 2002).

1.4.2 Diagnostic Criteria, Manifestations and Treatment of Diabetes

According to the criteria postulated by the World Health Organization (WHO) in 2003 the diagnosis of diabetes will confirmed in symptomatic patients with single venous random plasma blood glucose ≥11.1 mmol/L (200 mg/dL), asymptomatic patients with two fasting blood glucose samples taken in subsequent days ≥ 7 mml/L (126 mg/dL) or asymptomatic patients with two random blood glucose samples taken in subsequent days ≥11.1 mmol/L (200 mg/dL) (anonymous, 2003b). In patients with fasting blood glucose (FPG) reading between 6.1 mmol/l (110 mg/dL) and 7.0 mmol/l (126 mg/dl) and in patients with random blood glucose reading between 7 mml/L (126 mg/dL) and 11.1 mmol/L (200 mg/dL), oral glucose tolerance test (OGTT) is recommended (anonymous, 2003b). Patients are informed to have unrestricted carbohydrate diet for three days before the test, fasted overnight then plasma glucose is measured before 75 g glucose load and at two hours after (anonymous, 2003b). The diagnosis of diabetes is confirmed if the FBG ≥ 7 mml/L (126 mg/dL) and two hours after glucose load ≥11.1 mmol/L (200 mg/dL). The diagnosis of diabetes will be excluded if the FBG < 7 mml/L (126 mg/dL) and two hours after glucose load < 7.8 mmol/L (140 mg/dL) (anonymous, 2003b). Impaired glucose tolerance occurs when the FBG < 7 mml/L (126 mg/dL) and two hours after glucose load reading ranges between 7.8 mmol/L (140 mg/dL) and 11.1 mmol/L (200 mg/dL) and as a result patient will be at high risk to develop diabetes and regular follow up is recommended to such a candidate (Chilvers *et al.*, 2002).

Thirst, nocturia, polyuria and rapid weight loss are the classical symptoms of type 1 diabetes (Chilvers *et al.*, 2002). Many of type 2 diabetes remain asymptomatic for long time

before they discovered to have diabetes or have non-specific symptoms like fatigue and malaise (Kumar & Clark, 2002). Further, weight loss is the common physical sign of type 1 diabetes patients while about 70% of type 2 diabetes patients are overweight at presentation and 50% of them have hypertension (Kumar & Clark, 2002).

Insulin is the only treatment of type 1 diabetes whereas in type 2 diabetes diet control and physical activity is the first line of the management (Kumar & Clark, 2002). Uncontrolled diabetics will need the addition of the oral hypoglycemic medications, sulfonylurea for thin and metformin for obese patients (Chilvers *et al.*, 2002). If still uncontrolled, the combination of both sulfonylurea and metformin can be introduced, however, other drugs like acarbose (ɯ-glucosidase inhibitors) and thiazolidinediones might added to reach euglycaemia (Souhami & Moxham, 2002). Furthermore, insulin should replace the oral medication if patient glucose levels are still uncontrolled or if signs of neuropathy, nephropathy or retinopathy complications started to present in the diabetic patients (Kumar & Clark, 2002).

1.4.3 Pathogenesis of diabetes

Type 1 Diabetes

Type 1 diabetes is a chronic autoimmune disease resulting from a cellular-mediated autoimmune destruction of the pancreatic ß-cells (Atkinson & Maclaren, 1994). Genetic and environmental factors work together to precipitate the disease. Certain human leukocyte antigens (HLAs) show a strong association with the development of type 1 diabetes (Huang *et al.*, 1996; Notkins, 2002). Some genes within the HLA complex particularly class I genes have been linked to type 1 diabetes; however, the strongest linkage by far is with the DQ and DR class II genes (Huang *et al.*, 1996; Notkins, 2002). Besides that, the role of the cell autoantibodies as a contributory factor in the destruction of ß-cells and developing of type 1 diabetes has been identified. These

autoantibodies include the islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase and autoantibodies to the tyrosine phosphatases IA-2 and IA-2ß (Baekkeskov *et al.*, 1982; Atkinson *et al.*, 1986; Kaufman *et al.*, 1992). Furthermore, autoantibodies against insulin are among the first autoantibodies to appear in the pre-diabetic state and are usually found in very young children (Notkins, 2002). Between 30 and 50% of young children with type 1 diabetes have autoantibodies against insulin (Atkinson & Eisenbarth, 2001). Environmental factors, stresses and some viral infections may play a role in developing type 1 diabetes (Chilvers *et al.*, 2002).

Type 2 Diabetes

Normally, postprandial increases in serum glucose levels induce insulin synthesis and release from ß-cells of the pancreas. Secreted insulin binds to its receptors on target organs; skeletal muscle, adipose tissue and liver (Bell & Polonsky, 2001). Subsequently, insulin binding triggers signal transduction pathways that inhibit glucose production in the liver, suppress lipolysis in adipose tissue and stimulates glucose uptake in skeletal muscle and adipose tissue (Saltiel & Kahn, 2001; Notkins, 2002).

Type 2 diabetes is characterized by two major defects: impaired insulin secretion and resistance to its actions (Kahn, 1994). Indeed, insulin resistance occurs before the onset of diabetes and the initial response of the pancreatic ß cell is to increase insulin secretion (van Haeften *et al.*, 2000; Bell & Polonsky, 2001). Increased insulin levels can be detected, also, before the onset of frank diabetes (Stumvoll *et al.*, 2005). As diabetes progresses, insulin release decreases leading to progressive hyperglycaemia (Kahn, 1994). Hyperglycaemia itself aggravates insulin resistance and impairs insulin secretion. The causes of insulin resistance and pancreatic ß-cell failure are not fully understood. However, a number of factors including elevated levels of

free fatty acids, glucotoxicity, chronic inflammation and genetic changes have been reported to have a role (Buchanan, 2003; Kashyap *et al.*, 2003)

It has been reported that free fatty acids (FFAs) are implicated in the pathogenesis of insulin resistance (Nurjhan *et al.*, 1992; Stumvoll & Jacob, 1999). Lipolysis is the process responsible for FFAs generation which takes place in adipocytes. Plasma FFAs level is high in obese and type 2 diabetic patients and this can be explained by the reduction of antilipolytic effect of insulin (Dhindsa *et al.*, 2005; Oprescu *et al.*, 2007; Ye, 2007). FFAs work against the insulin effects by inhibiting the uptake oxidation of glucose in skeletal muscle and increasing the process of gluconeogenesis in liver (Felley *et al.*, 1989; Gonzalez-Manchon *et al.*, 1989; Gonzalez-Manchon *et al.*, 1992). In addition to its role as a source of excess circulating FFAs, adipose tissue is a source for leptin (an adipokine) and tumour necrosis factor-α (an inflammatory factor) which have been postulated to involve in the mechanism of insulin resistance (Zhang *et al.*, 1994; Peraldi & Spiegelman, 1998).

White et al. (1985) described the first substrate of the insulin receptor. Subsequently, this substrate was named insulin receptor substrate-1 (IRS-1) after it has been cloned by Sun et al. (1991). IRS-1 and other cloned IRS proteins (IRS-2, -3, -4) have adaptor function between the insulin receptor and other cellular substrates such as the phosphatidylinositol-3-kinase (PI3K) (Sun *et al.*, 1991; Sun *et al.*, 1995; Lavan *et al.*, 1997a; Lavan *et al.*, 1997b). PI3K controls the expression of a number of genes implicated in lipid and glucose homeostasis (Agati *et al.*, 1998; Kotani *et al.*, 1999). It has been postulated that PI3K activates the expression of the glucose transporter-4 (GLUT-4) in muscle and adipose tissue (Laville *et al.*, 1996; Roques & Vidal, 1999). The insulin receptor, IRS-1 and IRS-2 and the PI3K mediate several effects of insulin actions and because of that the possible role of these genes in pathogenesis of insulin resistance that occurs in type 2 diabetes has been examined. Decrease in the protein expression level of

IRS-1 and PI3K were observed in the skeletal muscle of lean and obese type 2 diabetic patients (Goodyear *et al.*, 1995; Bjornholm *et al.*, 1997). Approximately 1–5% of type 2 diabetic patients have insulin receptor mutations (O'Rahilly *et al.*, 1991; Cocozza *et al.*, 1992; Hart *et al.*, 1996). Indeed, some researchers suggested impaired insulin action by these mutations (Almind *et al.*, 1993; Bernal *et al.*, 1998). Moreover, it has been observed in many studies that activation and/or a decline in expression of PI3K in response to insulin occur in different insulin-resistant rodent models as well as in patients with type 2 diabetes (Anai *et al.*, 1998; Cusi *et al.*, 2000).

1.5 Diabetes in pregnancy

1.5.1 Overview

Pregnancy complicated by diabetes is a medical challenge which associated with maternal and fetal illnesses. Diabetes in pregnancy is associated with significant fetal and maternal morbidity and mortality. Indeed, there are two types of diabetes affecting pregnancy; pre-gestational (pre-GDM) and gestational diabetes (GDM). Diabetes is considered pre-gestational if the diabetes was diagnosed before the onset of pregnancy. Pre-GDM is further subdivided into type 1 and type 2 diabetes. GDM is defined as diabetes that develops or first recognized during pregnancy. There are approximately 650,000 births in England and Wales each year and of these, 2-5 % of pregnancies involve women with diabetes (NICE, 2008). Most of these pregnancies complicated by diabetes are caused by GDM (NICE, 2008). Furthermore, the prevalence of births complicated by pre-existing diabetes has increased by about 50 % in less than a decade (Bell *et al.*, 2008).

1.5.2 Pathogenesis

Normal pregnancy is considered as a diabetogenic state as it is characterized by an increased production of insulin combined with resistance to its action. Indeed, the resistance to the glucose lowering effects of insulin is a normal phenomenon of pregnancy. The placental hormones namely, human placental lactogen, cortisol and prolactin are contributed to this resistance via decreasing phosphorylation of insulin receptor substrate-1 (IRS-1) which occurs mainly during the second trimester (Ryan & Enns, 1988; Kuhl, 1998; Friedman *et al.*, 1999). Insulin release increases to compensate for this demand, but if resistance becomes dominant the women become hyperglycaemic. GDM occurs when there is insufficient insulin secretion to counteract the pregnancy-related reduction in insulin sensitivity. As in Type 2 diabetes, GDM is associated

with both a deterioration of beta cell function, may due to autoimmune process (Damm *et al.*, 1992) or enzymatic defect like glucokinase (Di Cianni *et al.*, 2003), and insulin resistance (Ryan *et al.*, 1985; Kuhl, 1991; Catalano *et al.*, 1993). In pre-GDM, pregnancy worsens the severity of diabetes whereby the insulin resistance becomes more prominent during late pregnancy due to the increase levels of human placental lactogen, cortisol and prolactin.

1.5.3 Screening and diagnostic tests for GDM

Among the several screening tests for GDM are screening for risk factors and the 50 gram Glucose Challenge Test (GCT) (Stephenson, 1993; Berger *et al.*, 2002; Brody *et al.*, 2003). They are the most widely used ones. If risk factors are found to be present and/or the GCT is positive, the woman is eligible for a diagnostic test. There are several internationally recognized diagnostic tests for GDM, which in general, performed between 24 and 28 weeks where the diabetogenic effect of pregnancy is evident (Benjamin *et al.*, 1986). At present, the most commonly used diagnostic test is OGTT. Internationally, 75 gram OGTT is used in many parts of the world including Europe and is recommended by the WHO (Alberti & Zimmet, 1998). The 100 gram OGTT (used commonly in USA) and 50 gram OGTT (used in some hospitals in Australia) are not commonly used (Oats & Beischer, 1986; Scott *et al.*, 2002).

1.5.4 Medicines management of pregnancy complicated with diabetes

The only treatment for type-1 diabetes during pregnancy is insulin. For type-2 diabetes and GDM, different modalities of management are taking place. Originally, whenever type-2 diabetic women become pregnant, oral hypoglycaemic medications are replaced with insulin and GDM is started with diet therapy and if it is failed insulin is introduced to maintain blood glucose levels within the normal range. Moreover, using oral hypoglycaemic instead of insulin is

now widely acceptable as a treatment of diabetes during pregnancy; both type-2 and GDM. However, there are many conflicting reports of the safety of these medications during pregnancy. Many oral hypoglycaemic drugs are known to cross placenta and may have a harmful effect on fetus (Anonymous, 2003a) . For instance, metformin is a biguanide oral hypoglycaemic drug that able to cross placenta and enter the fetus circulation. US-FDA rates metformin as pregnancy risk category B. However, a recent Australian randomized trial comparing insulin with metformin in women with GDM has shown that metformin alone or with supplemental insulin is not associated with higher perinatal complications as compared with insulin (Rowan *et al.*, 2008). In addition, this trial showed that women used the combined treatment of insulin and metformin required less insulin compared with women used insulin alone. The same study reported a similar rate of macrosomia and CS in metformin and insulin groups. Glyburide (also known as glibenclamide), a second generation hypoglycemic sulfonylurea, is considered as a safe drug to be used to achieve euglycaemia during pregnancy in women with type-2 or GDM. Compared to metformin and other sulfonylureas, glyburide does not cross the placenta in significant amount (Elliott *et al.*, 1991; Langer *et al.*, 2000; Koren, 2001) and has no effect on the rate of fetal anomalies (Langer, 2002). It has been reported that using glyburide to achieve euglycaemia in patients with GDM is as effective as the use of insulin (Langer *et al.*, 2000). several studies reported that in women diagnosed with GDM there was no significant difference in the rate of caesarean sections in group treated with insulin compared with those treated with glyburide (Langer *et al.*, 2000; Bertini *et al.*, 2005; Jacobson *et al.*, 2005; Ramos *et al.*, 2007). Both metformin and glyburide are the two oral hypoglycaemic agents in the WHO Model List of Essential Medicines (WHO, 2007). Sulfonylureas appear to act by inhibiting potassium ATP channels in pancreas and this leads to depolarization of membrane potential and as a result, calcium channels open leading to an increment in calcium entry that stimulates insulin release

(Groop *et al.*, 1987; Gedeon & Koren, 2006). Sulfonylureas work primary by enhancing insulin secretion, which suppresses hepatic glucose production; the main cause of fasting hyperglycaemia commonly seen in diabetics (Groop *et al.*, 1987; Groop *et al.*, 1991). Glyburide also enhances insulin sensitivity in peripheral organs (DeFronzo & Simonson, 1984; Simonson *et al.*, 1984), therefore, less insulin will be needed for uptake of glucose and less glucose will be produced by liver. The ongoing clinical studies on issue of oral hypoglycaemic medications safety in pregnancy will open a new era in the management of type-2 and GDM.

1.5.5 The effect of diabetes on pregnancy

Diabetes in pregnancy adversely affects both mothers and their babies. A number of pregnancy complications due to GDM and pre-GDM have been reported such as macrosomia, fetal distress, neonatal hypoglycemia, congenital malformation, caesarean section (CS) and preeclampsia (Casey *et al.*, 1997; El Mallah *et al.*, 1997; Jensen *et al.*, 2004; Kjos *et al.*, 2004; Clausen *et al.*, 2005; Yogev *et al.*, 2009; Yogev & Visser, 2009). It has been postulated that the incidence of these adverse pregnancy outcomes is seen similarly in GDM and pre-GDM pregnancies (El Mallah *et al.*, 1997). Several studies reported that the maternal, fetal and neonatal outcomes in pre-gestational type 1 diabetes are similar to those in pregnancies with pre-gestational type 2 diabetes (Farrell *et al.*, 2002; Boulot *et al.*, 2003; Clausen *et al.*, 2005; Balsells *et al.*, 2009).

Several studies have reported that the caesarean CS rate is higher in women with diabetes compared to non-diabetics (Dunne *et al.*, 2003; Ehrenberg *et al.*, 2004; Jensen *et al.*, 2004). CS rates vary between 10 to 60 % (Dunne *et al.*, 2003; Ehrenberg *et al.*, 2004; Jensen *et al.*, 2004; Crowther *et al.*, 2005; Bell *et al.*, 2008; Metzger *et al.*, 2008). The odd ratio (OR) for risk of emergency CS in pregnancy complicated by a diabetic pregnancy has been reported as 3-4 (Ehrenberg *et al.*, 2004; Evers *et al.*, 2004). Caesarean section in women with diabetes in

pregnancy is associated with a 2.5 increased risk of wound infection (Takoudes *et al.*, 2004), an increased risk of thrombosis and post-partum haemorrhage (Dunne *et al.*, 2003). Despite the significant attendant morbidity associated with CS in diabetic pregnancies, the high CS rate in diabetic pregnancies is not fully understood. Increased fetal insulin (an anabolic hormone) levels in response to the maternal hyperglycaemia result in development of macrosomic infant and predispose to fetal hypoglycaemia during neonatal period (Catalano *et al.*, 1995). As macrosomia is associated with birth asphyxia and shoulder dystosia (Keller *et al.*, 1991), patients are at increased risk of CS delivery (Naylor *et al.*, 1996; Casey *et al.*, 1997; Boulot *et al.*, 2003). Macrosomia (a birth weight > 4000 g) is one of the most common abnormalities associated with pregnancies complicated by diabetes (Di Cianni *et al.*, 2003; Grissa *et al.*, 2007; Suhonen *et al.*, 2008; D *et al.*, 2009; O'Brien, 2009). Indeed, macrosomia has been reported as a risk factor for CS in women with diabetes (Bo *et al.*, 2003; Ehrenberg *et al.*, 2004; Kjos *et al.*, 2004; Grissa *et al.*, 2007; Suhonen *et al.*, 2008; D *et al.*, 2009; O'Brien, 2009). However, a previous trial found that a reduction in macrosomia did not result in a concomitant reduction in CS rate (Crowther *et al.*, 2005). Moreover, two other studies also found that an increased CS rate occurred in treated diabetic women even in the absence of macrosomia (Naylor *et al.*, 1996; Remsberg *et al.*, 1999). Fetal distress is another common complication seen in pregnant diabetic women and cited as a common reason for emergency CS in diabetes (Platt *et al.*, 2002; Dunne *et al.*, 2003; Evers *et al.*, 2004; Jensen *et al.*, 2004). Langer et al. (2005) has reported that CS rates were similar in normal and overweight diabetics controlled with insulin or diet regardless the achievement of targeted levels of glycaemic control. The same study suggests that the increased CS rate is due to the confounding factor of obesity; however other analyses have found diabetes to be an independent risk for CS in labour (Bo *et al.*, 2003; Ehrenberg *et al.*, 2004; Kjos *et al.*, 2004). Diabetes is known to be associated with prolonged labour and failed induction of labour which accounted

for 51 % of emergency CS (Evers *et al.*, 2004). Therefore poor myometrial contractility may be an important factor in diabetic pregnancies. Further evidence to implicate poor myometrial contractility is that post partum haemorrhage has been reported as being six times more common in diabetic women (Dunne *et al.*, 2003). Figure 1.3 summarizes some common complications associated with diabetes in pregnancy.

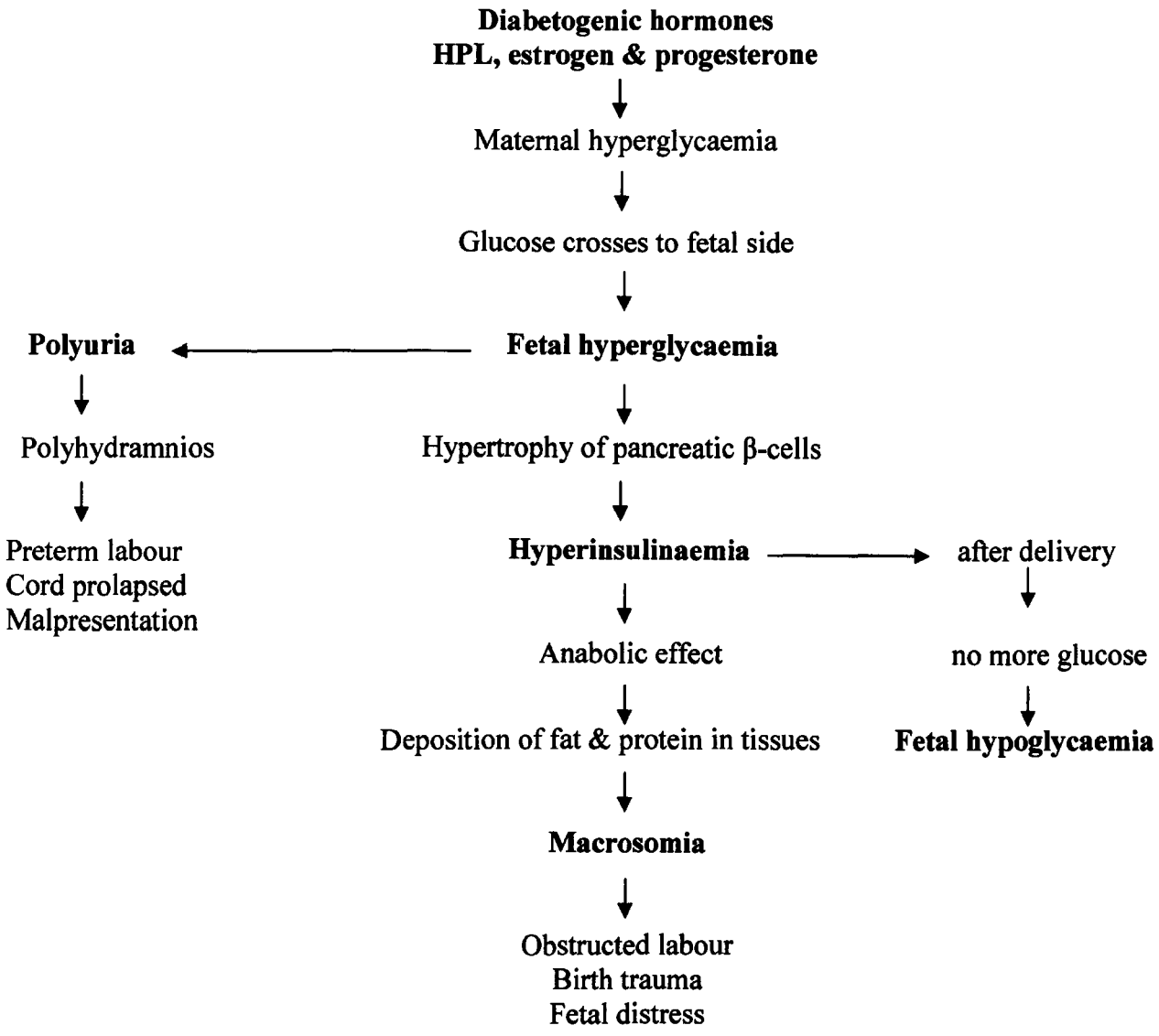

Figure 1.3: Common complications associated with diabetes during pregnancy.

1.6 Effect of diabetes on smooth muscle contractions

1.6.1 Uterine smooth muscle

There have been few studies investigating myometrial contractility in diabetes. There are very little data concerning the effect of diabetes in the myometrium of any species and none on Ca signalling (Goldraij *et al.*, 1975; McMurtrie *et al.*, 1985; Franchi *et al.*, 1988; Jawerbaum *et al.*, 1996). The previous studies used streptozotocin-induced diabetic animal models and found either no effect (Franchi *et al.*, 1988) or inhibition of contractility (McMurtrie *et al.*, 1985; Jawerbaum *et al.*, 1996). However, an earlier report on human myometrium from diabetic women noted that the stability of contractions, as measured by their decrement within 1 hour, was reduced in diabetics, i.e. force was reduced faster (Kaya *et al.*, 1999). Unfortunately Kaya et al. (1999) did not compare contractions amplitude or any other parameters of contraction between the two groups. Calcium entry, which occurs mainly via the L-type VDCC, is crucial for initiation of myometrial contractions (Wray *et al.*, 2003). None of the above studies measured the changes in intracellular calcium concentrations that could occur in the myometrium of diabetics.

The myometrium of diabetic is exposed to high levels of insulin to face the intermittent high levels of plasma glucose seen during pregnancy to achieve glycaemic control (Balsells *et al.*, 2000; Lepercq *et al.*, 2008; Stenninger *et al.*, 2008). Kuznetsova et al. (2006) has reported that insulin interferes with uterine smooth muscle contractions in rat and human. In another study, it has been reported that the ability of insulin to induce myometrial relaxation in diabetic pregnant women is less compared to non-diabetic controls (Kuznetsova *et al.*, 2005). This may be due to the insulin resistance phenomenon which usually occurs in diabetes. However, two other studies have showed that insulin enhances the contractility of the myometrium in rats (Goldraij *et al.*, 1979, 1982). Moreover, insulin attenuates KCl-induced myometrial contractions

in human and rats (Kuznetsova *et al.*, 2006), indicating that insulin may hyperpolarize the smooth muscles of uterus. Further, a previous study has indicated that insulin enhances the oxytocin-induced myometrial contraction in rat (McMurtrie *et al.*, 1985). Unfortunately, none of these studies have measured the calcium transients. Furthermore, to the best of my knowledge no previous work has investigated the effect of hyperglycaemia on uterine contractility.

Oxytocin is a nanopeptide hormone that is synthesized in hypothalamic neurons (Silvermann & Zimmerman, 1983). Once released in the circulation, oxytocin stimulates powerful uterine contractions especially around the onset of labour (Kimura *et al.*, 1992; Fuchs *et al.*, 1995; Furuya *et al.*, 1995). Indeed, several studies have reported that oxytocin increases contractility in normal myometrium at term (Kimura *et al.*, 1992; Fuchs *et al.*, 1995; Furuya *et al.*, 1995). Synthetic oxytocin may be used to augment uterine contractions at labour in normal pregnancy (Mozurkewich, 2006). In many diabetics, augmentation of labour using oxytocin fails (Evers *et al.*, 2004). To the best of my knowledge no previous work investigated the effect of oxytocin on human diabetic myometrial contractility in simultaneous with calcium transients during late pregnancy. However, in rats, one previous study showed that compared to controls the response of the myometrium of diabetics to oxytocin was less at day 21 but similar at day 22 of pregnancy (Jawerbaum *et al.*, 1996). Another study revealed that oxytocin-induced myometrial contraction in diabetic rats was less as compared to control animals (McMurtrie *et al.*, 1985).

There is a cumulative body of evidence which indicate that diabetes and the changes associated with it; hyperinsulinaemia and hyperglycaemia are capable of altering the contractility in different types of smooth muscle.

1.6.2 Other smooth muscles

Clinical conditions linked to altered smooth muscle function with diabetes in tissues other than myometrium include hypertension, gastric paresis, constipation, enlarged bladder and urine retention and vas deferens dysfunction. *in vitro* studies have found tissue specific alterations in smooth muscle contractility in response to diabetes (Pfaffman *et al.*, 1982; White & Carrier, 1990; Inazu *et al.*, 1991; Fleischhacker *et al.*, 1999; Waring & Wendt, 2000a; Forrest *et al.*, 2005). It has been reported that in rats, diabetes enhances urinary bladder smooth muscle contractions (Longhurst *et al.*, 1991), but impairs vascular smooth muscle contractility (Pfaffman *et al.*, 1982). Waring & Wendt (2000b) have reported that the contraction enhancement of bladder smooth muscle from diabetic rats was due to the increased sensitivity to $[Ca^{2+}]_i$. In the same species, it has been indicated that diabetes decreases (Pfaffman *et al.*, 1982), increases (Owen & Carrier, 1980) or has no effect (MacLeod & McNeill, 1985) on agonist-induced contraction of aortic smooth muscle cells. In responses to various agonists, streptozotocin-induced diabetic rats showed hyperreactivity of smooth muscles contractions in vas deferens and gastric fundus (Sakai & Honda, 1987; Aihara & Sakai, 1989). In a similar diabetic rat model, the smooth muscles of aorta respond differently to agonists. Endothelium-1 induced stronger while noradrenaline induced similar contractions compared with controls (Fulton *et al.*, 1991).

Calcium entry is crucial for initiation of smooth muscle contraction in vascular tissues which occurs predominantly through L-type calcium channels that are activated by membrane depolarization (Linas *et al.*, 1988; Nelson *et al.*, 1990). *in vitro*, L-type calcium channels can be activated either by membrane depolarization using high KCl concentration or by direct stimulation with BAY K8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate). Studies of the response of diabetic smooth

muscle cells to high KCl and BAY K8644 are often conflicting. Some reported that in vascular smooth muscle (VSM) of diabetic rats the contractile responses to high KCl and Bay K 8644 were increased (White & Carrier, 1990; Inazu *et al.*, 1991), while others showed that the responses were diminished (Carmines *et al.*, 1996; Hattori *et al.*, 1996). Further, Wang et al (1998) has found that the effect of KCl-induced increased [Ca2+]i in VSM of diabetic and non-diabetic control rats was similar. In addition, it has been reported, using the patch-clamp technique, that the densities of L-type VDCC currents are reduced in VSM of diabetic rats (Wang *et al.*, 2000). Direct measurement of potassium currents in VSM showed functional defect in voltage dependent potassium channels in diabetic rats (Bubolz *et al.*, 2005; Bubolz *et al.*, 2007). It seems that diabetes may alter the membrane potential and the activity and/or expression of L-type calcium and potassium channels, which affect the excitability, in smooth muscle cells.

It has been stated that insulin attenuates the contractility of vascular and urinary bladder smooth muscles in human and animals (Longhurst *et al.*, 1991; Kim & Zemel, 1993; Muniyappa *et al.*, 1997). A previous study reported that insulin decreased the contractility of rabbit cavernous smooth muscle induced by Bay K8644, L-type calcium channel agonist (Myung *et al.*, 2006). In contrary, one author has reported that insulin is capable of enhancing VSM contractions (Pfaffman *et al.*, 1982). Kuznetsova et al. (2006) showed that KCl-induced smooth muscle contractions in rats trachea is decreased in response to insulin, which may indicate a role of insulin in membrane hyperpolarization. Indeed, insulin hyperpolarizes the membrane potential of mouse diaphragm smooth muscle as demonstrated by Zemkova et al. (1982). It has been reported that insulin is able to inhibit calcium influx controlled by both receptor- and voltage-operated calcium channels (Muniyappa *et al.*, 1997). Insulin is known to activate the Na^+-K^+-ATPase in VSM (Omatsu-Kanbe & Kitasato, 1990; Hundal *et al.*, 1992) leading to

hyperpolarization of the these cells, blocking of voltage-operated calcium channels, and relaxation takes place (Kahn & Song, 1995). Furthermore, insulin activates plasma membrane Ca^{2+}-ATPase–mediated Ca_i^{2+} efflux (Zemel *et al.*, 1992) and inhibits IP_3-induced Ca^{2+} release from intracellular stores (Saito *et al.*, 1993). Insulin hyperpolarizes membrane potential of different types of muscles in animal models (Hazlewood & Zierler, 1967; Zierler & Rogus, 1981; Zemkova *et al.*, 1982; Zierler & Moore, 1984; Iannaccone *et al.*, 1989; Li & Sperelakis, 1993), which may mediated by activation of the electrogenic Na^+ pump. Insulin decreases TEA-induced muscle contraction in rabbit cavernous smooth muscle (Myung *et al.*, 2006). Potassium channel currents are reduced in smooth muscle cells from insulin-resistant rats as reported by Dimitropoulou et al (2002).

Some previous studies have revealed that hyperglycaemia induces smooth muscle relaxation. Reduced smooth muscle contractions were reported in stomach (Horowitz *et al.*, 1991), jejunum (Byrne *et al.*, 1998), gallbladder (de Boer *et al.*, 1994) and vascular cells in response to hyperglycaemia (Williams & Schrier, 1992, 1993; Wang *et al.*, 2000). In VSM calcium entry is crucial for initiation of VSM contractions (Nelson *et al.*, 1990; Pelzer *et al.*, 1990). Many previous reports have shown that hyperglycaemia induces muscle relaxation in VSM via inhibiting calcium entry through these channels (Williams & Schrier, 1992; Kam *et al.*, 1993; Williams & Schrier, 1993; Wang *et al.*, 2000). It has been reported that hyperglycaemia reduced current density of L-type Ca^{2+} in SMCs which may due to the alteration in these channels activity (Wang *et al.*, 2000).

1.7 Diabetes and glucose transporters

1.7.1 Overview

Glucose is the primary energy source for all human cells under normal physiological circumstances. It also serves as a precursor to a variety of metabolites in many, if not all, tissues. Glucose is released into the circulation from the liver in the fasting state as a result of glycogen breakdown and is used (oxidized) for the energetic needs of cells. The postprandial clearance of glucose from the circulation is tightly regulated by insulin. Since many cell membranes are impermeable to glucose, it is transported by specific carrier proteins or transporters that span the cell membrane and allow the binding and transfer of glucose across the hydrophobic lipid bilayer.

1.7.2 Glucose transport pathways

Two general classes of glucose carriers have been described in mammalian cells that belonged to two protein families, the facilitative glucose transporters (GLUTs), which work in the direction of the glucose gradient transport and the sodium-glucose cotransporters, which can concentrate glucose against a gradient (Hediger *et al.*, 1987; Bell *et al.*, 1993; Scheepers *et al.*, 2004).

The facilitative glucose transporters family

The human GLUT family consists of fourteen members, of which 11 have been shown to catalyze sugar transport (Scheepers *et al.*, 2004). The GLUTs utilise the diffusion gradient of glucose across plasma membranes and exhibit different tissue expression (Mueckler, 1994). Structurally, GLUTs possess 12 membrane-spanning helices with intracellular located amino- and carboxyl-termini. These facilitative glucose transporters vary in their affinity for glucose.

The GLUT family has been divided on the basis of sequence similarities into three subclasses; Class I, II and III.

Class I includes the high-affinity GLUT-1, GLUT-3 and GLUT-4 and the low-affinity GLUT-2. GLUT-1 has a wide tissues distribution; in erythrocytes, brain, liver, kidney, mammary gland, placenta, endometrium, adipose tissues and skeletal and smooth muscles (Birnbaum *et al.*, 1986; Jansson *et al.*, 1993; Camps *et al.*, 1994; Dudek *et al.*, 1994; Younes *et al.*, 1996; Nie *et al.*, 2000). The GLUT-1 provides glucose uptake under basal conditions in many cells. GLUT-3 is expressed in tissues with high glucose demand such as brain and placenta (Gould *et al.*, 1991; Haber *et al.*, 1993). It is the ideal GLUT for neuronal cells that are incapable of glycogen storage (Bell *et al.*, 1993). GLUT-3 is also expressed in macrophages, platelets, testes, muscles and endometrium (Stuart, 2000; Von Wolff *et al.*, 2003). Unlike GLUT-1 and GLUT-3, GLUT-4 is the major insulin-responsive transporter, which is expressed predominantly in skeletal muscle and adipose tissue and plays an important role in whole-body glucose homeostasis (Buse *et al.*, 1992; Liu *et al.*, 1992; Ezaki, 1997). Stimulation of glucose uptake by insulin involves translocation of GLUT-4 from its intracellular storage sites to the plasma membrane (Shepherd & Kahn, 1999; Watson & Pessin, 2001; Bryant *et al.*, 2002). Insulin is known to induce GLUT-4 synthesis in insulin-sensitive cells such as adipocytes (Sargeant & Paquet, 1993). Furthermore, the biochemical mechanism by which this is accomplished has been a subject of intense experimentation, although clarification of the pathways has remained elusive. In recent years, numerous signalling molecules and cascades modulated by insulin have been identified, although few have been definitively established as important to the metabolic actions of the hormone. Figure 1.4 shows a simplified scheme for the secretion and action of insulin that result in translocation of GLUT-4 to the plasma membrane, which facilitates glucose transportation into the cell (Notkins, 2002). Although it is a low-affinity transporter, GLUT-2 has a high

glucose transport capacity compared with GLUT-1, thus, it is present in tissues exposed to large glucose fluxes, such as pancreatic β-cells, liver, kidney and intestine (Hogan *et al.*, 1991). This feature allows GLUT-2 to be involved in β-cell glucose-sensing mechanisms which detect changes in glucose levels (Levitsky *et al.*, 1994; Noel & Newgard, 1997). Hyperinsulinaemia decreases (in liver) while hyperglycaemia increases (in pancreas and liver) GLUT-2 expression (Tiedge & Lenzen, 1991; Burcelin *et al.*, 1992).

Class II transporters, including GLUT-5, GLUT-7, GLUT-9 and GLUT-11, have a very low-affinity for glucose. GLUT-5 and GLUT-7 are fructose transporters and are expressed predominantly in the small intestine (Davidson *et al.*, 1992; Li *et al.*, 2004; Cheeseman, 2008). GLUT9 is distributed mainly in the liver and the kidneys (Doege *et al.*, 2000a; Phay *et al.*, 2000). GLUT11 is expressed primarily in heart and skeletal muscle (Doege *et al.*, 2001), but It is also found in liver, lung, trachea and brain (Wu *et al.*, 2002).

Class III transporters comprise: GLUT-6, GLUT-8 (previously named GLUT-X1), GLUT-10 and GLUT-12. GLUT-6 is expressed in the brain, spleen and leucocytes (Doege *et al.*, 2000a), while GLUT-8, GLUT-10 and GLUT-12 are predominantly found in insulin-sensitive tissues such as muscle, adipose tissue and liver (Ibberson *et al.*, 2000; Dawson *et al.*, 2001; Rogers *et al.*, 2002). GLUT-8 is also present in the testis and brain (Doege *et al.*, 2000b; Ibberson *et al.*, 2000).

The sodium-glucose cotransporters

The sodium-glucose cotransporters transport glucose into cells against its concentration gradient (Hediger *et al.*, 1987). They are mainly distributed across the luminal membrane of cells lining the small intestine and the proximal tubules of the kidneys (Hediger *et al.*, 1987; Wells *et al.*, 1992).

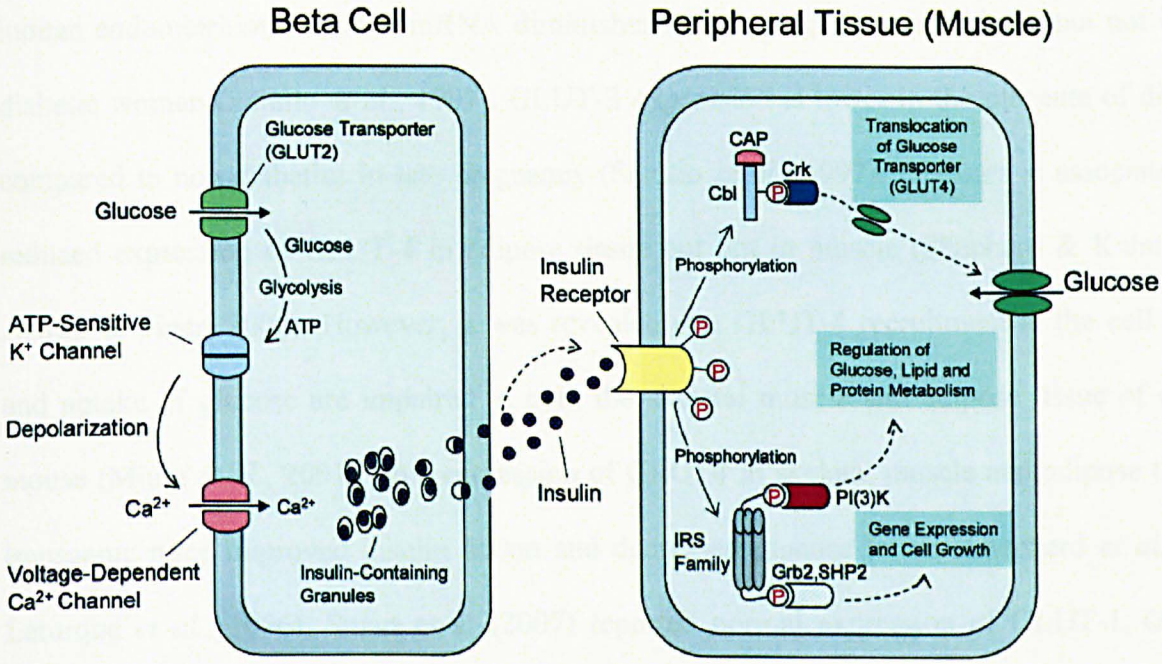

Figure 1.4: Insulin release and action. Glucose enters beta cells via the glucose transporter (GLUT2) and ATP is generated by glycolysis. This result in closure of ATP-sensitive K^+ channels, depolarization of the plasma membrane, and opening of voltage-dependent Ca^{2+} channels. The influx of Ca^{2+} leads to the release of insulin (Bell & Polonsky, 2001), which is carried in the bloodstream to cells throughout the body where it binds to insulin receptors. This results in autophosphorylation of insulin receptors and phosphorylation of tyrosines on a variety of cellular proteins including members of the insulin receptor substrate (IRS) family and Cbl-CAP (Saltiel & Kahn, 2001). The phosphorylated proteins provide docking sites for SH2 domains of several proteins (e.g. phosphatidylinositol 3-kinase (PI(3)K); Grb2 and SHP2; and Crk) that activate different signalling pathways (dashed lines). This results in translocation of the glucose transporter (GLUT4) and uptake of glucose by the cell; alterations in glucose, lipid, and protein metabolism; and changes in gene expression and cell growth (Notkins, 2002).

1.7.3 Effect of diabetes on glucose transporters

Many authors reported changes in GLUT expression with diabetes. In diabetic rats, GLUT-2 expression is reduced in the pancreatic beta cells, but unaltered in liver (Girard *et al.*, 1992). In human endometrium, GLUT-3 mRNA diminishes in late pregnancy in diabetic but not in non-diabetic women (Sciullo *et al.*, 1997). GLUT-3 expression is lower in the placenta of diabetics compared to non-diabetics in late pregnancy (Sciullo *et al.*, 1997). Diabetes is associated with reduced expression of GLUT-4 in adipose tissue but not in muscle (Shepherd & Kahn, 1999; Astrup & Finer, 2000). However, it was revealed that GLUT-4 recruitment at the cell surface and uptake of glucose are impaired in both the skeletal muscle and adipose tissue of diabetic mouse (Miura *et al.*, 2001). Overexpression of GLUT4 in skeletal muscle and adipose tissue in transgenic mice improved insulin action and decreased glucose levels (Shepherd *et al.*, 1993; Leturque *et al.*, 1996). Stuart et al (2007) reported normal expression of GLUT-1, GLUT-3, GLUT-4, GLUT-8, GLUT-11, and GLUT-12 in the skeletal muscle of diabetics. However, the same study demonstrated that GLUT-5 expression is increased in diabetics compared with non-diabetic subjects.

1.8 Diabetes and Glycogen

1.8.1 Overview of glycogen metabolism

Glycogen, the storage form of glucose, is a very large, branched polymer of glucosyl units of glucose residues. Although many cells store glycogen, the primary sites are liver and skeletal muscles. The balance between synthesis and breakdown of glycogen is critical to glucose homeostasis. The liver and skeletal muscle convert excess glucose into glycogen for future use in a process called glycogenesis. Breakdown of glycogen (glycogenolysis) takes place when the glucose levels fall especially between meals, Figure 1.5. Defects in both glycogenesis and glycogenolysis are frequently reported in diabetes.

An increase in blood-glucose concentration is needed for the stimulation of glycogenesis (Katz & McGarry, 1984). There are several enzymes and regulatory proteins involved in the synthesis of glycogen. In liver, the GLUT-2 transporter mediates the transport of glucose into hepatocytes in bidirectional ways maintaining the intracellular glucose concentration in near-equilibrium with the extracellular concentration, while in skeletal muscle, GLUT-4 is the main glucose transporter. The first reaction of glycogenesis is the phosphorylation of glucose to glucose 6-phosphate (G-6-P) by hexokinase, and then to glucose 1-phosphate (G-1-P) by phosphoglucomutase. G-1-P is then converted to uridine diphosphoglucose (UDP-glucose) by UDP-glucose pyrophosphorylase. The last steps in glycogen synthesis are catalyses by glycogen synthase (GS) and branching enzyme. In this step these enzymes transfer and attach glucosyl units from UDP-glucose to nascent glycogen (Hers, 1992; Braithwaite *et al.*, 1995). The metabolism of the storage glycogen is linked with insulin action and blood glucose homeostasis. Glycogen synthase, the rate limiting enzyme, is activated by insulin and in response to glycogen depletion (Fernandez-Novell *et al.*, 1996; Ferrer *et al.*, 1997; Halse *et al.*, 2003). Glycogen synthase kinase-3 (GSK3) maintains low activity GS under basal conditions by continuous

phosphorylation of three specific serine residues, collectively termed site 3 (Parker *et al.*, 1983) . Insulin activates GS mainly through the inhibition of GSK3, by its phosphorylation, via PI3K/ protein kinase pathway (Cross *et al.*, 1995; Lawrence & Roach, 1997). The mechanism which underlies the activation of GS in response to glycogen depletion has not been elucidated. However, it has been suggested that an insulin-independent pathway leading to inactivation of GSK3 may take place (Markuns *et al.*, 1999). Another study proposed that this phenomenon could occur by a GSK3-independent mechanism (Wojtaszewski *et al.*, 2001). However, glycogen depletion may directly activate GS (Halse *et al.*, 2003). Insulin and high glucose levels are known to activate the hexokinase enzyme, too (Parker *et al.*, 1983; Lawrence & Roach, 1997; Pendergrass *et al.*, 1998; Halse *et al.*, 2003).

Glycogenolysis pathway starts with the release of G-1-P from glycogen using glycogen phosphorylase and debranching enzyme. G-1-P is then converted by phosphoglucomutase to G-6-P for further metabolism. The conversion of G-6-P to glucose, which is mediated by glucose-6-phosphatase, occurs in the liver, kidney and intestine but not in skeletal muscle as it lacks this enzyme. Therefore, any glucose released from glycogen stores of muscle will be oxidized in the glycolytic pathway to provide energy for contraction within the muscle tissue itself (Bouche *et al.*, 2004) In contrast; the presence of glucose-6-phosphatase in liver, kidney and intestine allows glycogenolysis to generate free glucose for maintaining blood glucose levels. Glucagon and adrenaline are the major activators of glycogenolysis in liver and skeletal muscle respectively (Hendrickx & Willems, 1996).

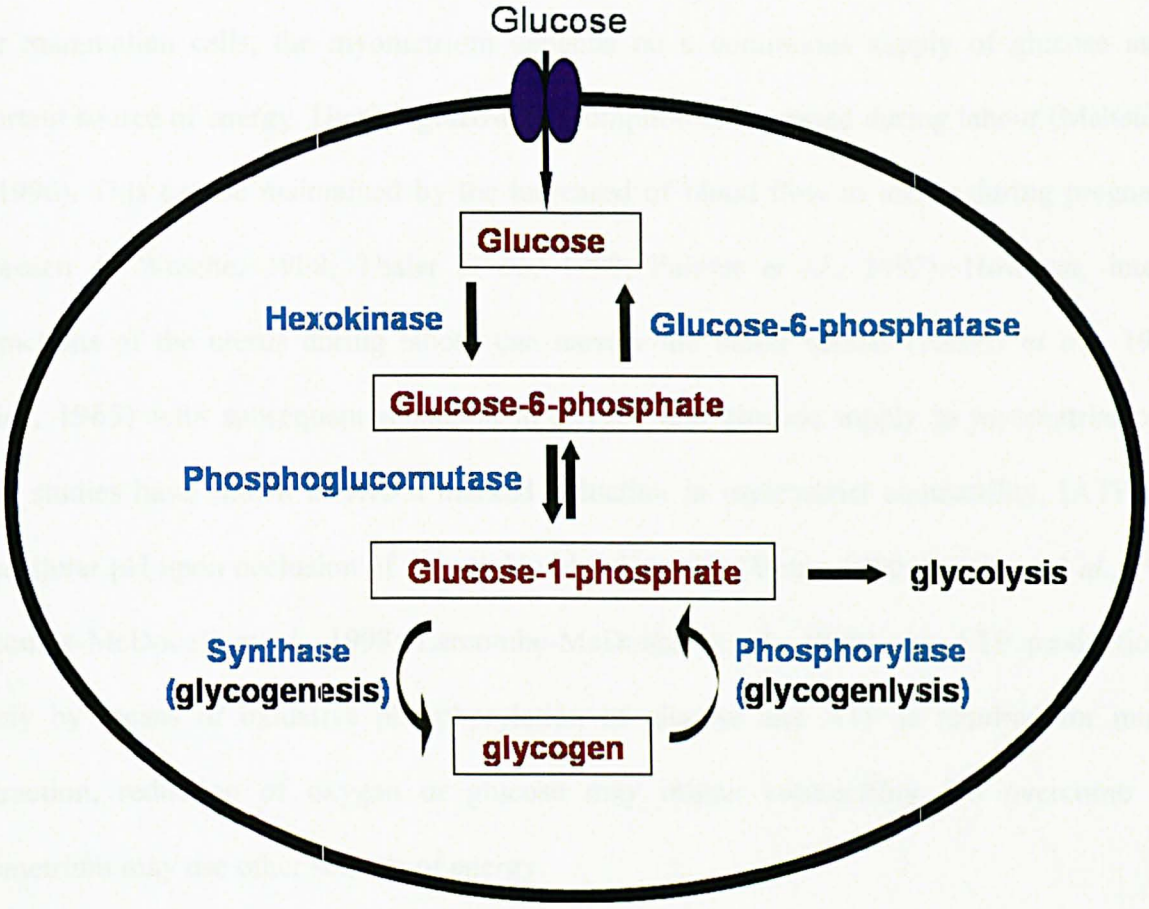

Figure 1.5: Glycogen synthesis and breakdown pathway. A simple scheme which demonstrates the main steps involved in glycogenesis and glycogenolysis.

1.8.2 Glycogen store in myometrium

During pregnancy there are many metabolic alterations in the uterus including increases in the concentration of ATP in uterine smooth cells (Dawson & Wray, 1985). It has been suggested that these changes occur to ensure adequate contractile activity at labour (Wray, 1990). Like other mammalian cells, the myometrium depends on a continuous supply of glucose as an important source of energy. Uterine glucose consumption is increased during labour (Maheux *et al.*, 1996). This can be maintained by the increased of blood flow to uterus during pregnancy (Thoresen & Wesche, 1988; Thaler *et al.*, 1990; Palmer *et al.*, 1992). However, intense contractions of the uterus during labour can narrow the blood vessels (Assali *et al.*, 1958; Greiss, 1965) with subsequent reduction in oxygen and glucose supply to myometrial cells. Many studies have shown *in vivo* a marked reduction in myometrial contractility, [ATP] and intracellular pH upon occlusion of the uterine blood vessels (Wray, 1990; Harrison *et al.*, 1994; Larcombe-McDouall *et al.*, 1998; Larcombe-McDouall *et al.*, 1999). As ATP production is mainly by means of oxidative phosphorylation of glucose and ATP is required for muscle contraction, reduction of oxygen or glucose may impair contractility. To overcome this, myometrium may use other sources of energy.

Similar to liver and skeletal muscle, myometrium is capable of storing glycogen for future metabolic needs (Hers, 1992; Braithwaite *et al.*, 1995). Previous studies have shown that in compared to the myometrium of non-pregnant, glycogen stores are higher in pregnant animal and human (Milwidsky & Gutman, 1983; Sakamoto *et al.*, 1987; Wedenberg *et al.*, 1990). Further, glycogen content in rat myometrium increased prior to labour to levels comparable to those in skeletal muscles (Chew & Rinard, 1979). The same study reported that glycogen depleted immediately after delivery. Thus, glycogen can be considered as another source of energy for the contracting uterus and this may become required during the strong contractions

which take place during labour when the blood supply is decreased (Assali *et al.*, 1958; Greiss, 1965). Indeed, it has been reported that glycogen is a considerable energy source for the contracting uterus during parturition in rats (Chew & Rinard, 1979; Sterin *et al.*, 1991). This means that glucose released from glycogen stores in the myometrium will be oxidized in the glycolytic pathway to provide energy in the form of ATP.

Triglycerides (TG) are another possible energy source for labouring uterus. Although it has been reported that in normal pregnant rats TG increase toward the end of pregnancy (Argiles & Herrera, 1981), previous studies have shown that uterine TGs are not used as energy sources during pregnancy in diabetics and non-diabetics (Gonzalez *et al.*, 1993; Jawerbaum *et al.*, 1994; Jawerbaum *et al.*, 1996).

1.8.3 Effect of diabetes on glycogen stores

As the major stores of glycogen are liver and skeletal muscles, most studies of effect of diabetes on glycogen stores were conducted in these tissues. Diabetes is associated with the impairment of glycogen synthesis in skeletal muscle (Eriksson *et al.*, 1989; Shulman *et al.*, 1990). Increased GSK3 protein levels and activity in human skeletal muscles was reported in diabetics, which inversely correlated with GS activity (Hansen *et al.*, 1997). Impaired glucose transport and elevated plasma fatty acids in diabetes can be attributed to the decrement in glycogen disposal, too (Kelley *et al.*, 1992; Boden *et al.*, 1994). Indeed, it has been reported that insulin resistance can be attributed to diminished insulin-stimulated glucose uptake and decreased hexokinase expression and GS activities (Pendergrass *et al.*, 1998; Cline *et al.*, 1999; Halse *et al.*, 2001; Hojlund & Beck-Nielsen, 2006). Therefore, insulin resistance, which is the major feature of diabetes, can be attributed to defects in muscle glycogen storage. However, it has been suggested that the defect in GS seen in diabetes occurs secondary to hyperglycaemia (Pratipanawatr *et al.*, 2002) These changes lead to reduction in glycogen synthesis. Further, the activity of glucose-6 phosphatase is increased in diabetes, which can explain the lack of suppression of hepatic glucose production in the setting of hyperglycaemia seen in this disease (Mevorach *et al.*, 1998; Clore *et al.*, 2000), therefore, less glucose is available to be stored as glycogen. Glycogenolysis is accelerated in diabetics due to lack of glucagon suppression and this may decrease the glycogen stores in liver (Fery & Balasse, 1994; Shah *et al.*, 2000). To the best of my knowledge no previous study has investigated the effect of diabetes on myometrial glycogen stores.

1.9 Aims of my thesis

In this thesis I will examine the following hypothesis: The increased CS rate in women with diabetes in pregnancy is partly due to the poor uterine contractility. Specifically, I will examine the following possibilities:

- Both the force and calcium transients of the spontaneous myometrial contractions are less in diabetics compared with non-diabetics controls.
- Changes in L-type VDCC and K^+ channels activity and/or function may underlie the changes in myometrial contractility in diabetes.
- Both hyperglycaemia and hyperinsulinaemia decrease myometrial contractions and calcium transients.
- Insulin decreases contractions by affecting the L-type VDCC and K^+ channels in myometrium.
- Myometrium from women with diabetes have less glycogen compared with non-diabetics.
- Diabetic uterus is more susceptible to glucose deprivation, therefore contracting less strongly.
- The expression of glucose transporters is lower in diabetics compared with non-diabetics.
- The glucose transporter 1, 3 and 4 are expressed in myometrium and their expression is changed as pregnancy progress and in response to diabetes.
- There are no differences in the structural appearance of the myometrium from diabetics compared with non-diabetics.

Chapter 2

General Materials and Methods

2.1 Human tissue

Human myometrial samples were obtained from non-diabetic (n = 67) and diabetic (n = 31) pregnant women undergoing elective CS with Local Ethical Committee approval and informed consent at Liverpool Women's Hospital.

2.1.1 Non-diabetics

The total number of patients who underwent CS under spinal anaesthesia was 56 while 11 women were under general anaesthesia. The median gestational age was 39 weeks (range: 37-41) while the median mother age was 34 years (range: 24-44). The median body mass index was 26.3 (range: 18.5-44.9). The indications for caesarean deliveries were: previous CS (38 patients), breech presentation (18), previous difficult vaginal delivery (4), placenta previa (3), maternal request (2), unstable lie (1) and previous Rhesus disease (1), Table 2.1.

2.1.2 Diabetics

Twenty one caesarean deliveries were performed with the patients under spinal anaesthesia while 10 women were under general anaesthesia. The median gestational age was 38 weeks (range 34-39). The median age of the patients was 35 years (range 24-44) and the median body mass index (BMI) was 30 (range 19.4-43). Similar to non-diabetic patients, all diabetic samples were obtained from patients who had elective Caesareans. Six patients were diet-controlled while

rest were insulin-controlled. Out of 31 diabetics, seven patients were poorly-controlled throughout their pregnancy. Nineteen patients had one previous Caesarean, four had two Caesareans, two had three previous caesarean sections, one diabetic had three previous vaginal deliveries, one patient had two previous vaginal deliveries and one Caesarean and five diabetics were primiparous. Other details of diabetic patients are demonstrated in Table 2.2.

2.1.3 Tissue preparation

Each biopsy, measuring about 1 x 2 cm, was excised immediately after delivery of the infant from the middle of the upper edge of the lower segment uterine incision. Then, straight away placed in Hank's balanced salt solution at room temperature (RT) and transferred to the laboratory. The biopsy was then placed in a shallow dissecting dish containing physiological salt solution (PSS) at RT. While viewing under a dissecting microscope, excess blood, peritoneum and any foetal membranes were carefully removed. Small strips of the myometrial fibres measuring approximately 3 mm x 1 mm x 5mm (width x thickness x length) were dissected, before loading with calcium-sensitive fluorescent indicator, Indo-1/AM (see later), or attaching to force transducer for study (see below).

Table 2.1: Demographic data of non-diabetic patients. This table summarises some information about non-diabetic pregnant women.

Patient's number	Age	Birthweight (Kg)	Gestation age	BMI
1	34	4.310	39 + 1	26.7
2	44	3.200	36+4	30.8
3	36	3.260	39+4	37.6
4	35	3.460	38+6	37.1
5	28	3.630	39	41.5
6	28	3.910	39	21.8
7	36	3.620	39+2	24.6
8	39	3.665	39+2	29.2
9	42	3.910	41+5	25.1
10	41	2.690	39	18.7
11	38	2.305	37+5	19.3
12	37	4.100	39	28.7
13	32	3.101	39+2	23.3
14	27	4.310	41+3	25.9
15	35	4.480	40	30.7
16	27	2.413	37+3	23.2
17	29	3.635	39+3	27.2
18	33	2.883	37+1	41.5
19	37	3.260	38+3	30.8
20	35	3.260	39	24.5
21	36	3.755	39	24.4
22	36	3.260	38+1	23.1
23	38	3.190	38+4	27.2
24	32	3.415	38+4	38.3
25	31	3.740	39+1	26.7
26	26	3.940	39+3	22.1
27	30	3.350	38	22.3
28	34	3.090	39+1	36
29	28	3.850	39	31.4
30	35	3.740	39	22.5
31	34	3.350	39	28.8
32	25	3.200	39	23.7
33	33	3.280	39+1	31.5
34	35	2.830	37+3	26
35	36	3.880	39	24
36	34	3.540	38+5	31.2
37	28	3.400	38+2	38.7
38	44	3.450	38+4	22.8
39	41	3.230	38+4	24
40	31	2.920	38+3	19.2

Continue of Table 2.1.

Patient's number	Age	Birthweight (Kg)	Gestation age	BMI
41	35	3.120	37+5	26.8
42	28	3.860	39	27.1
43	41	3.455	38	30.1
44	38	4.054	39+1	27.6
45	25	3.300	39+1	23.2
46	29	3.710	39+5	34.1
47	39	3.460	38+3	27.6
48	24	3.180	38+5	20.2
49	32	2.643	37+3	18.5
50	38	3.060	39+1	27.7
51	36	3.600	38+6	44.9
52	26	3.860	38+6	19.9
53	31	3.575	40	41.2
54	30	2.890	39	34.9
55	41	3.020	38+6	23.3
56	30	3.460	39+1	21.3
57	33	3.010	39+3	25
58	37	3.530	39+5	27.4
59	38	3.350	38+6	27.9
60	29	2.805	37	23.7
61	24	3.570	39+4	25.6
62	31	3.602	39+4	24.7
63	25	3.520	38+5	25.6
64	26	3.020	37+2	20
65	41	3.820	39	30.1
66	36	3.290	39+4	24.9
67	38	3.570	38	24.3

Table 2.2: Demographic data of diabetic patients. This table summarises some information about diabetic pregnant women. DM1 = type 1 diabetes mellitus previously known as insulin dependent diabetes mellitus (IDDM), GDM = gestational diabetes mellitus, Y = yes, N = no, UTIs = urinary tract infections, CS = caesarean section, PSOS = polycystic ovary syndrome, CSII = continuous subcutaneous insulin infusion, FMS = fetal movements, CTG = cardiotocograph, PET = pre-eclampsia.

Patient's Number	Age	BMI	DM1	GDM	Gestational Age	Duration of DM (y)	HbA1C range (%)	Antenatal Control
1	40	31.6	Y	N	38	25	6.1-6.2	moderate
2	26	35.4	N	Y	38	0	n/a	good
3	40	22.3	Y	N	38	25	5.9 - 6.4	good
4	26	28.9	Y	N	35+2	20	6.4-6.6	moderate
5	40	30.8	N	Y	36+6	0	5.7-6.0	good
6	24	30.1	Y	N	38	20	7.9-8.1	poor
7	29	42.5	N	Y	38 +1	0	6.9 - 7.2	good
8	36	24.6	Y	N	37+4	25	6.0-7.0	moderate
9	31	27.3	Y	N	38	20	6.8-7.8	poor
10	30	40.7	N	Y	38	0	5.1 - 5.6	good
11	33	26.5	N	Y	38	0	5.3-5.6	good
12	36	22.4	Y	N	36+4	27	6.5-6.8	moderate
13	27	41.9	Y	N	35 +3	10	7.9 - 9.7	poor
14	38	28.9	N	Y	39	0	5.1-5.3	good
15	37	35.6	N	Y	38	0	5.6-5.7	good
16	39	30	Y	N	34	27	6.4-8.2	poor
17	33	29.8	N	Y	37+6	0	4.4-5.8	good
18	38	27.8	N	Y	36+5	0	5.4-7.0	good
19	31	24	N	Y	38+6	0	5.1	good
20	38	38	N	Y	38+1	0	7.0-9.3	poor
21	36	31.5	N	Y	38+3	0	7.4	good
22	35	33.7	N	Y	38 +1	0	4.1-6.2	good
23	32	31.6	Y	N	38+1	10	5.0-7.1	good
24	39	35.8	Y	N	37+4	6	7.1-9.0	poor
25	35	19.4	N	Y	38+2	0	n/a	good
26	44	31.2	Y	N	36+4	25	6.3	good
27	29	22.3	Y	N	38	10	n/a	good
28	40	22.2	Y	N	37+4	0	n/a	good
29	29	24.8	N	Y	38	0	5.8-6.0	good
30	30	21.5	N	Y	37+5	0	n/a	good
31	28	43	Y	N	35+2	20	7.1-7.5	poor

Continue of Table 2.2.

Patient's number	Medications	Birthweight (Kg)	Diabetic complications	Obstetric history	Type of insulin	Antenatal complications
1	none	3.69	none	1 x cs	humulin;humalog	None
2	none	3.71	none	1 x cs	novomix	None
3	none	3.49	none	primiparous	humalog; lantus	None
4	none	2.18	dilated fundi	primiparous	CSII	hypoglycaemia, hyperemesis & Severe UTIs
5	inhalers	3.62	none	1 x cs	insulin	None
6	thyroxine	3.34	microalbuminuria	1 x cs	novorapid;glargine	hypoglycaemia, hypertension & microalbuminuria
7	metformin	4.16	none	1 x cs	novomix	None
8	none	4.50	none	1 x cs	novorapid, lantus; CSII	None
9	none	4.05	none	primiparous	lantus	Hypoglycaemia
10	none	3.2	none	1 x cs, PCOS	metformin; lantus	None
11	none	3.57	none	1 x cs	none; diet-controlled	None
12	nifedipine	4.42	previous nephropathy	1 x cs	CSII	CTG failed at 35 weeks
13	labetalol	4.48	none	2 x cs	humalog; lantus	vomitting; PET
14	none	3.57	none	1 x cs	none;diet-controlled	None
15	none	3.74	none	1 x cs	none;diet-controlled	None
16	none	2.58	retinopathy	primiparous	Humalog;Humulin	hypoglycaemia & reduced FMS
17	none	4.83	none	2 x cs	none:diet-controlled	None
18	none	2.78	none	2 x cs	none;diet-controlled	abnormal dopplers
19	none	3.03	none	1 x cs	Lantus	None
20	none	4.76	none	1 xcs	novorapid; lantus	None
21	inhalers	3.90	none	1 x cs	metformin	None
22	none	3.30	none	2 x cs	novorapid; lantus	None
23	none	3.06	none	1x cs	humalog: lantus	None
24	none	4.76	none	3 vaginal deliveries	novrapid:lantus	hypoglyceamia & abnormal dopplers
25	none	3.25	none	3 x cs	none;diet-controlled	None
26	none	2.72	vascular	1 x cs, 2 x vaginal delivery	insulin	None
27	none	3.26	none	1 x cs	insulin	None
28	none	2.58	none	1 x cs	insulin	None
29	none	3.98	none	primiparous	novarapid: lantus	None
30	none	4.15	none	1 x cs	novorapid	None
31	amlodipine	4.37	nephropathy	3 X cs	novorapid; lantus	high dosages of insulin required

2.2 Force and calcium measurement

2.2.1 The calcium fluorophore, Indo-1

Indo-1 acetoxymethyl ester (Indo-1/AM, Molecular Probes, Oregon, USA) is a dual wavelength ratiometric dye used to monitor the changes in the intracellular calcium concentration $[Ca^{2+}]_i$. When excited by light at wavelength 340 nm, it emits light of wavelength 400 nm and 500 nm. The ratio of these two wavelengths can be used as an indicator of changes in $[Ca^{2+}]_i$ (Wahl *et al.*, 1990).

2.2.2 Loading the tissue with Indo-1

Dissected strips were loaded with Indo-1/AM at 15 μM. A 1 mM stock solution was made by dissolving the anhydrous form of Indo-1 (50 μg vial) in 50 μl dimethyl sulphoxide (DMSO) containing 20% by weight of the non-ionic detergent pluronic acid (F-127 Molecular Probes, Oregon, USA) to aid dispersal of the AM esters in aqueous solution (Haugland, 1996). A 7 μm/l solution Indo-1 was then prepared by adding 15 μl stock to 2 ml of PSS. The strips then kept in this solution for 3 hours at RT, then rinsed and placed in PSS for one hour at RT before the onset of the experiment to ensure equilibrium and complete hydrolysis of the AM groups of the Indo-1 molecules.

2.2.3 Simultaneous measurements of calcium and tension or tension alone

Strips loaded with Indo-1 were mounted in a 150 μl chamber on an inverted Nikon microscope. The bath was perfused with PSS at 3 ml/min and maintained at 35°C. The myometrial strips were attached at each end to metal hooks by means of aluminium foil clips. One hook was attached to

a tension transducer and the other to a fixed point. Tissue was stretched to 1.5 X its resting length (2mN force), as this had been shown to give a steady baseline of force and optimal conditions. The tissue was excited using a xenon arc lamp (75W) with light of wavelength 340 nm. Subsequently, emitted light at 400 nm and 500 nm was detected with photo multiplier tubes and digitally recorded at sampling rate of 10 Hz (Figure 2.1). In all experiments, changes in the Indo-1 ratio were accompanied by shifts in the opposite direction of emission signals at 400 nm and 500 nm. Figure 2.2 shows an example of simultaneous measurement of intracellular calcium and force.

Experimental Protocol and analysis:

Data were obtained on muscle strips that been left to equilibriate for one hour and were generating stable spontaneous contractions. After stable contractions were established, a control period of 60 minutes was obtained before the physiological solution was altered. The frequency of contractions was obtained over this control period. Contractile activity measured in the last three contractions of this control period was used to determine peak amplitude and duration at half-height. The integral of force (area under the curve (AUC), in arbitrary units, au) was obtained over the last 20 minutes of the control period. The same parameters were applied to measure calcium transients.

The exact parameters analysed was determined by the contractile response to the manoeuvres. Frequency, amplitude, duration and AUC were used to compare spontaneous contractions and contractions produced in response to insulin between non-diabetic and diabetic groups. The same parameters were used apart from AUC, to compare the contractility in diabetic subgroups (good-, poor-, insulin- and diet-controlled) and in response to 0-glucose. The effect of high glucose was examined by measuring frequency, amplitude and AUC, while AUC was used

to examine the effects of oxytocin, Bay K8644, TEA and insulin on Bay K8644- and TEA-induced muscle contraction. Amplitude and AUC were the parameters used to investigate the effect of insulin on oxytocin-induced myometrial contractions.

In some experiments, the tissues were exposed to 40 mM KCl solution (K^+ substituted for Na^+ in the physiological saline) for 2 minutes. The amplitude, duration and AUC were obtained during this period and compared between non-diabetic and diabetic groups.

For comparison of contractility between non-diabetics and diabetics, all samples obtained during the period of this thesis were analysed. For specific experiments, detailed in the subsequent chapters, the *n* number was determined by the size of the response and the availability of tissues during the period of these experiments. For the mechanistic studies described in the final result Chapter (6), several drugs and compounds had to be investigated and the data obtained was for the purpose of guiding further future studies. Therefore in that chapter some of the *n* numbers were smaller than elsewhere in the thesis.

The result data was analysed using Microcal Origin Software (Massachusetts, USA). Data are expressed as median (first quartile, third quartile) for non-normally distributed data or as mean ± SEM for normally distributed data and significance was tested using the appropriate statistical tests; Mann-Whitney U, Wilcoxon , student's *t* tests or analysis of variance (ANOVA). Differences were taken as significant if *p* values were <0.05 and *n* is the number of samples. Results are expressed as percentages of control contractions (i.e. the control is 100%) unless stated otherwise.

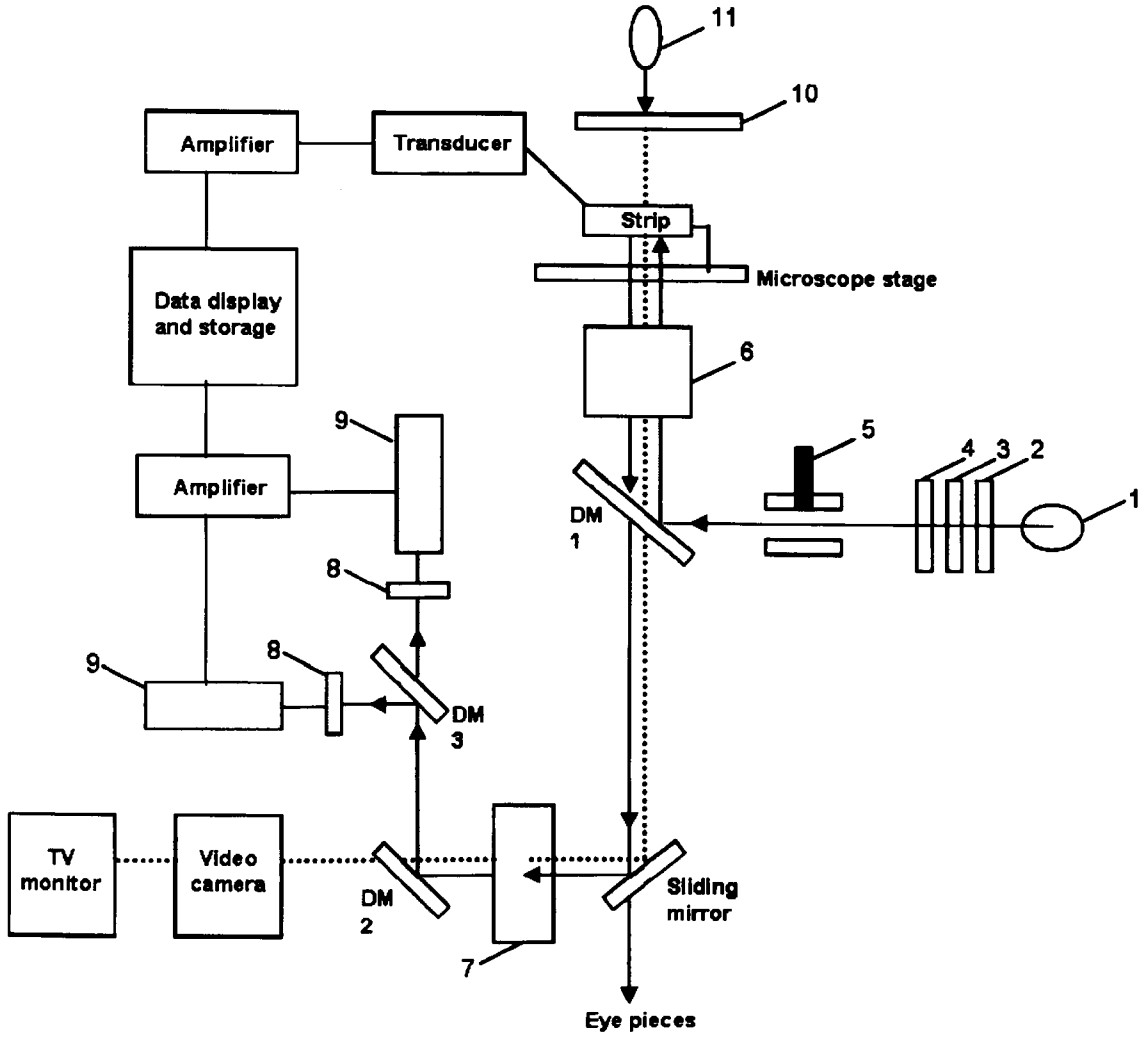

Figure 2.1: Schematic diagram of apparatus based around an inverted microscope. Ultraviolet illumination is provided by a **xenon lamp (1). Heat filter (2) and natural density filter** (3) are placed in the light path to prevent the excess of heat and to reduce the intensity of the excitation light respectively. The appropriate excitation wavelength is selected by using an interference **filter** (4) centre at 340nm. The illumination time of tissue are kept to minimum by the usage of an **electromechanical shutter** (5) in the excitation path. The exciting light is then reflected and directed upwards by a 400 diachronic mirror (DM1) and focus onto the preparation by a **microscope objective (6).** The light emitted by the fluorescence indicator passed back through the objective is transmitted by the diachronic mirror (DM1), reflected by a sliding mirror and directed through an **adjustable diaphragm** (7). The light then hit a 610nm diachronic mirror (DM2) mounted at 45°, which directed the longer wavelength emitted light (greater than 610nm) to form an image on a video camera, which is relayed to a monochrome monitor, and shorter wavelength light to the **photomultipliers (8, PMTs).** The shorter wavelength light reflects and is split by a 450nm diachronic mirror (DM3), passing to either the 400 nm or 500 nm PMTs. In front of each PMT there is an **emission filter (9)** centre at the appropriate wavelengths. To avoid interference from the

microscope light with the fluorescence measurements a **long pass filter (10)** is placed in front of the **microscope lamp (11).**

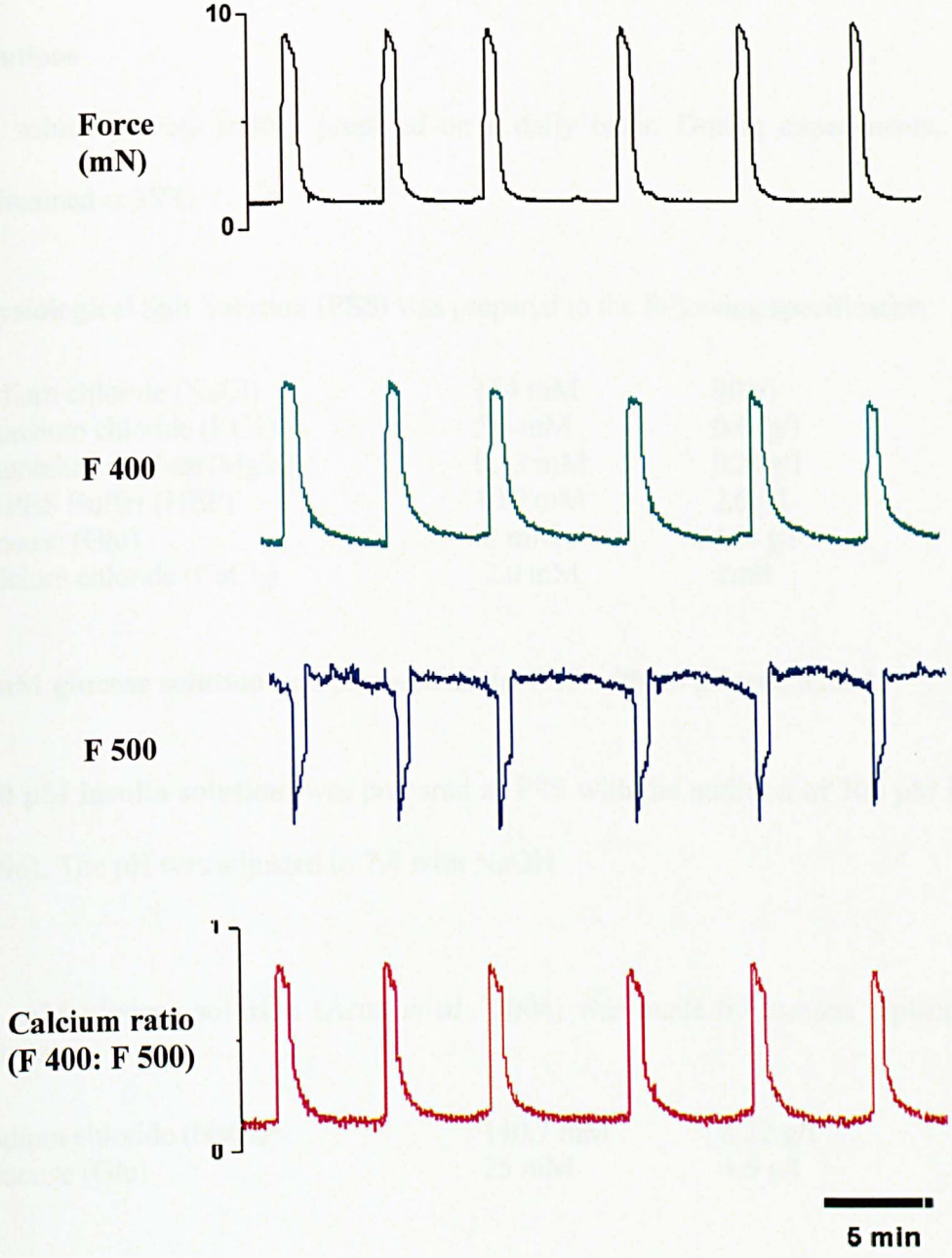

Figure 2.2: A recording of simultaneous measurement of fore and intracellular calcium. The black trace is the force, the green trace is 400 nm emission signal and the blue trace is 500 nm emission signal, showing opposite directions. The red trace is the ratio of the 400 nm and 500 nm emission signals.

2.2.4 Solutions and chemicals

All chemicals used were obtained from Sigma (Dorset, UK) unless otherwise states.

Solutions

All solutions were freshly prepared on a daily basis. During experiments, the solutions were maintained at 35°C.

Physiological Salt Solution (PSS) was prepared to the following specification:

Sodium chloride (NaCl)	154 mM	9.0 g/l
Potassium chloride (KC1)	5.6 mM	0.42 g/l
Magnesium sulphate ($MgSO_4$)	0.12 mM	0.29 g/l
HEPES Buffer (HEP)	10.9 mM	2.6 g/l
Glucose (Glu)	8 mM	1.44 g/l
Calcium chloride ($CaCl_2$)	2.0 mM	2 ml/l

0 mM glucose solution was prepared as the PSS with no glucose added.

700 pM insulin solution was prepared as PSS with the addition of 700 pM insulin (Tack *et al.*, 1996). The pH was adjusted to 7.4 with NaOH

25 mM glucose solution (Arun *et al.*, 2004) was made by osmotic replacement of NaCl with glucose:

Sodium chloride (NaCl)	140.7 mM	8.22 g/l
Glucose (Glu)	25 mM	4.5 g/l

Mannitol (used as an osmotic control for 25 mM glucose) was prepared as for the 25 mM glucose solution, but with 8 mM glucose and 17 mM mannitol.

High potassium solution (40 mM KCl) (Perusquia *et al.*, 2005) was made by osmotic replacement of NaCl with KC1:

Sodium chloride (NaCl)	114 mM	6.99 g/l
Potassium chloride (KCl)	40 mM	2.98 ml/l

Hank's balanced salt solution contained the following specification:

Sodium chloride (NaCl)	140 mM	8.18 g/l
Potassium chloride (KC1)	5.4 mM	0.403 g/1
Potassium dihydrogen phosphate (KH_2PO_4)	0.44 mM	0.06 g/1
HEPES Buffer (HEP)	5 mM	1.19 g/l
Glucose (Glu)	5.5 Mm	0.99 ml/l
Sodium hypophosphite ($NaPO_2H_2$)	0.24 mM	0.058 g/l
Calcium chloride (CaC12)	0.1 mM	0.1 ml/l

The pH of all solutions was adjusted using sodium hydroxide (NaOH) to 7.4, which was the pH for all experiments.

Insulin

10 ml of acidified H_2O (pH = 2), prepared by addition of 0.1ml glacial acetic acid, was added to bovine insulin (lyophilized powder, approximate 25 IU/mg solid, FW: 5733) to prepare 10 mg/ml stock solution. A concentration of 700 pM was used in the experiments obtained by diluting the stock solution with PSS (Tack *et al.*, 1996). Insulin vehicle control (acidified H_2O) experiments were not necessary as the pH of PSS was maintained at 7.4 after adding insulin.

Oxytocin

Oxytocin (lyophilized powder, approximate 50 IU/mg solid, FW: 1007) was dissolved in 5% acetic acid at a concentration of 1 nM. The concentration used for experimentation was 10 nM (McKillen

et al., 1999) as oxytocin half-maximal effect on myometrial contractility occurs at this concentration (Szal *et al.*, 1994). Oxytocin vehicle control (acetic acid) was not use as the pH of PSS was maintained at 7.4 after adding oxytocin.

Tetraethyl Ammonium Chloride (TEA)

TEA (FW: 165.71) was used to block K^+ channels. It was applied at concentration of 10 mM by dissolving 160 mg of TEA into 100 ml PSS (Raymond & Lapied, 1999; Myung *et al.*, 2006). The pH of PSS was maintained at 7.4 after adding TEA.

Bay K8644

Bay K8644 was dissolved in ethanol (63 mg/ml) to produce a 10 mM stock solution and then diluted to give a final concentration of 1 μM which was used for experimentation (Artalejo *et al.*, 1991a; Artalejo *et al.*, 1991b; Burghardt *et al.*, 1999; Kupittayanant *et al.*, 2009). Bay K8644 binds specifically to the dihydropyridine binding site within the L type voltage dependent calcium channel to activate this channel in a variety of tissues (Williams & Schrier, 1993). The solvent (ethanol) concentration never exceeded 0.1% (v/v). Several studies have shown that ethanol at 0.1% has no effect on contractility in non-pregnant (Kupittayanant *et al.*, 2009) and pregnant women uterus (Burghardt *et al.*, 1999; Perusquia *et al.*, 2005). In addition, it has been reported that this concentration has no effect on spontaneous uterine contractility in rats (Perusquia & Navarrete, 2005). Moreover, this concentration also had no effect on calcium currents when tested directly in bovine chromaffin cells (Artalejo *et al.*, 1991a; Artalejo *et al.*, 1991b). As stated earlier the pH of PSS was maintained at 7.4 after adding Bay K8644. Ethanol (0.01%) was used as vehicle control for Bay K8644.

2.3 Glycogen measurement

2.3.1 Human samples

Biopsies taken from diabetic (n = 13) and non-diabetic (n = 19) pregnant women undergoing elective CS were immediately immersed in cold Hank's balanced salt solution and transferred to the laboratory. These tissues obtained with Local Ethical Committee approval and informed consent at Liverpool Women's Hospital.

2.3.2 Glycogen assay

The glycogen is hydrolyzed in HCL into its constituent glucose residues and measured enzymatically. The method is the hexokinase/glucose-6-phosphate dehydrogenase reaction for measuring glucose based on the method developed by Lowry and Passonneau (1972). The NADPH produced is read on a spectrophotometer at 340 nm and reflects the amount of glycogen in the tissues:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-phosphogluconic acid} + \text{NADPH}$$

Once the biopsy was obtained it was weighed to the nearest milligram, then frozen immediately in liquid nitrogen and stored in an eppendorf container at -80° C. The frozen samples were ground under liquid nitrogen and placed into polypropylene tube. The following procedure was followed for measuring muscle glycogen:

STAGE 1 - Release of glycogen

1. The muscle was homogenized on ice in 1 ml of 0.02 M HCl.
2. Samples spun down in floor centrifuge, transferred to 2 ml eppendorfs with holes in lids and boiled for 10 minutes.
3. Following the addition of 100 µl of 1 M NaOH the samples were immersed in boiling water (100°C) for a further 10 minutes.

STAGE 2 - Degradation of glycogen

4. 100 µl of 1.5 M acetic acid and 200 µl of 0.1 M acetate buffer were added to the samples and mixed. This gave a total of 1.4 ml in each tube.
5. 500 µl of the muscle extract, 500 µl of acetate buffer (blanks 1 and 2) and 500 µl of standards (S1-S7) were transferred to Eppendorf tubes.
6. 100 µl of the amyloglucosidase solution was added to all blanks, standards and samples left to incubate at RT for 2 hours.
7. The tubes were centrifuged at 4°C for 5 minutes at 14,000 revolutions per minute.

STAGE 3 - Glucose assay

8. 200 µl of the supernatant was transferred to another Eppendorf tube. Then, 15 µl of the 1 M NaOH and 400 µl of the glucose reagent were added, mixed and left at RT for 5 minutes.
9. 200 µl of the above placed into 96 wells microplate and absorbance was measured at 340 nm (Powerwave X340, BioTek Instruments Inc, USA).
10. 2 µl of the hexokinase solution was then added, mixed and the reaction proceeded to its endpoint (20 minutes) at RT.
11. Endpoint absorbance measured and the blank subtracted from the final values. Post - pre

gives change in absorbance.

12. Calculation:

A standard curve (linear regression line) was constructed from the absorbance of the different standards to calculate the glycogen concentration in μmoles/g.

Experimental protocol and analysis

Glycogen content in samples frozen immediately after elective caesarean sections (obtained from both non-diabetic and diabetic women) were measured and compared. In other experiment, glycogen stores in myometrial biopsies frozen immediately after elective caesarean sections (non-diabetic controls) and those frozen after they had been left to contract *in vitro* until contractions disappeared (non-diabetic tests) were measured and compared.

Data were analyzed using student *t* tests. Differences were taken as significant if *p* values were <0.05. Results are expressed as mean ± SEM and *n* is the number of samples.

2.3.3 Chemicals and solutions

All reagents were obtained from Sigma (Sigma-Aldrich Company Ltd, UK). The concentrations and volumes of the solutions used in this assay were:

Tris-buffered saline (TBS): prepared by adding 50 mg $MgCl_2$ and 1.51 g Trizma into 500 ml H_2O (pH 8.1)

Glucose reagent: prepared by dissolving 1 mM $MgCl_2$, 500 μM ATP, 500 μM $NADP^+$ and 25 μl G6PDH (172 U/ml stock) into 50 ml TBS.

Hexokinase: made by adding 10 μl of stock (551 U/ml) to 1.7 ml TBS.

1 M NaOH: 4g NaOH was dissolved in 100 ml H_2O

1.5 M acetic acid: 1.17 ml H2O was added to 100 μL concentrated stock acetic acid.

0.1 M acetate buffer: 2.86 ml stock acetic acid and 6.8 g sodium acetate added to 1 litre H_2O (pH 4.5).

0.02 M HCl: 1 ml in 597 ml H_2O

Amyloglucosidase: 10 mg (738 U/mg Stock) added to 12.3 ml acetate buffer. For experimentation 1 ml of above was dissolved in 9 ml acetate buffer.

Glucose standards:

5 mM glucose standard: 9 mg glucose in 10 ml acetate buffer.

0.5 mM glucose standard (S1): 1 ml of 5 mM and 9 ml acetate buffer.

0.4 mM glucose standard (S2): 4 ml of 0.5 mM and 1 ml acetate buffer.

0.3 mM glucose standard (S3): 3 ml of 0.4 mM and 1ml acetate buffer.

0.2 mM glucose standard (S4): 2 ml of 0.3 mM and 1ml acetate buffer.

0.1 mM glucose standard (S5): 1 ml of 0.2 mM and 1ml acetate buffer.

0.05 mM glucose standard (S6): 1 ml of 0.1 mM and 1ml acetate buffer.

0.01 mM glucose standard (S7): 1 ml of 0.05 mM and 4 ml acetate buffer.

2.4 Immunohistochemistry

2.4.1 Tissue microarrays (TMAs)

Human tissue microarrays

Human tissue microarrays (HMAs) contained formalin-fixed paraffin embedded myometrial samples were obtained from non-diabetic (n = 9), diabetic (n = 9) pregnant women undergoing elective caesarean section. Another five samples were taken from women undergoing hysterectomy as part of another project. These samples obtained with Local Ethical Committee approval and informed consent at Liverpool Women's Hospital (see the Appendix).

Animal tissue microarrays

Animal tissue microarrays (AMAs) contained formalin-fixed paraffin embedded samples were obtained from myometrium of pregnant rat at 12, 16, 20, 21 days and labouring uterus (n = 1 for each). Another sample was taken from non pregnant rat. Samples from rat's skeletal muscle and kidney were used as a control samples. These microarrays were prepared by Dr. Rachel Floyd (Physiology department, Liverpool University).

2.4.2 Tissue preparations

Human and animal samples were dissected and fixed in buffered neutral formalin for 24 hours before paraffin wax embedding. Tissue microarrays cores were made using the techniques outlined in Mobasheri & Marples (2004). Briefly, paraffin embedded tissue blocks to act as donors were warmed to 43°C for 10-15 minutes to allow the wax to soften slightly. Small cores of tissue were removed from donor blocks and transferred into a pre-made chamber in a recipient block of paraffin wax. Once all chambers were filled, the block was heated and sectioned at 5 μm and mounted onto aminopropyltriethoxysilane (APES) coated slides.

2.4.3 Immunohistochemistry

GLUT staining

The immunohistochemical protocol was optimized firstly by using "test" human and animal tissue microarrays. These arrays were used to titrate immunohistochemical assay parameters and antibody dilutions before use of the more comprehensive tissue microarrays. The slides were heated at 40°C for 30 minutes to improve tissue adhesion to the slides. TMA slides were deparaffinized in 100% xylene for 20 min to remove embedding medium and washed in graded series (100%, 90% and 70%) of fresh alcohol baths in order to rehydrate the sections. Antigen retrieval was performed by immersing slides in boiling 1.5 L citrate buffer (pH = 6) and left in the pressure cooker for one minute from when the steam starts to vent. The slides were dried then rinsed twice in TBS for 5 minutes after drawing "DAKOpen" circles to define the staining area. They were moved to humidified chamber before the incubation with DAKO EnVision peroxidise block for 5 minutes at RT. Sections were rinsed twice in TBS for 5 minutes then incubated overnight at 4°C with goat anti-GLUT1 or anti-GLUT-3 polyclonal antibodies (Ericsson *et al.*, 2005) (1:50, Santa Cruz Biotechnology, Inc) or for 1 hour with mouse anti-GLUT-4 monoclonal antibody (Ericsson *et al.*, 2005) (1:200, AbD Serotec, MorphoSys UK Ltd). Tissues stained with GLUT-4 were washed twice for 5 minutes with TBS before the incubation with labelled polymer-HRP anti-mouse (DakoCytomation, UK) for 30 minutes at RT. Tissues stained with GLUT-1 and 3 were incubated for 1 h in rabbit anti-mouse secondary antibody (1:250, Sigma, UK). All stained tissues with GLUT-1, 3 and 4 antibodies were washed twice for 5 minutes with TBS then incubated with liquid DAB+ Chromogen (3, 3'-diaminobenzidine solution, DakoCytomation, UK) for 10 minutes at RT. The development of the brown-colored reaction was stopped by rinsing in tap water. The stained slides were immersed for 1 minute in a bath of aqueous hematoxylin (VWR International Ltd, UK) to counterstain cell nuclei. Slides were

dipped briefly in acid alcohol then washed for 5 min in running tap water. Sections were dehydrated in a series of graded ethanol baths before being rinsed in two xylene baths and mounted in DPX; 1,3-diethyl-8-phenylxanthine (BIOS EUROPE, UK).

Initially, immunohistochemistry experiments were undertaking by doing the antibodies titrations for GLUT-1, GLUT-3 and GLUT-4. Different concentrations were used first to come up with most staining and least background. Different primary antibody and secondary antibody concentrations were examined for GLUT-1 and they were either 1:25 or 1:50 primary with 1:250, 1:500 or 1:1000 secondary. The optimal staining was 1:50 primary with 1:500 secondary. Similarly, for GLUT-3 antibodies titration was done to get best staining with least background. The concentrations were similar to that used with GLUT-1 but the best couple concentrations were 1:50 primary and 1:250 secondary. For GLUT-4 I did 1:100, 1:200, 1:400 and 1:800 for the primary antibody and the staining was the best with 1:200.

Positive controls included staining of erythrocytes (GLUT-1), endometrium (GLUT-3) and skeletal muscle (GLUT-4). Negative controls included staining without the primary antibody and the substitution by rabbit IgG (1:100). All antibodies were diluted in TBS/0.5% BSA.

Data acquisition and analysis.

The stained TMA slides were examined using a Nikon Eclipse microscope and scored in a blinded fashion whereby the observed was not aware of the origin of the samples. A second observer reviewed a proportion of all slides, also blinded to the origin of the samples, to ensure minimal intra-observer error occurred. Three images per sample images were captured using a Nikon Digital Sight DS-5M camera connected to a PC running Eclipse net software (v 1.20, Laboratory Imaging for Nikon, Kingston Upon Thames, UK).

The staining for GLUT-1 was assessed as follow: 0= no staining seen, (+) = low intensity (++) = moderate intensity , (+++) = high intensity, and (++++) = very high intensity.

An average value for the three high power fields of view was taken. The immunohistochemistry was also performed on positive and negative control sections.

The staining intensity for GLUT-3 was scored as follow: 0= no staining, (+) = light staining (light intensity), (++) = less than 5 cells with dark staining (moderate intensity), (+++) = more than 5 cells with dark staining (high intensity) and (++++) = all cells stained darkly (very high intensity). An average value for the three high power fields of view was taken.

For GLUT-4 as only a proportion of the cells were stained, the number of immunopositive cells was expressed as a percentage of the total cell population for that examined field of the sections.

Histology: Paraffin embedded 5 μM sections were made from control and diabetic myometrium and stained with haematoxylin and eosin to allow measurement of muscle bundles, which could be clearly seen at lower magnification, and uterine histology to be made. Samples were viewed at x 400 magnification by two independent observers blind as to the origin of the samples. Muscle bundles were assessed in semi-quantitative fashion in terms of muscle bundle size, organisation and separation. Data were analyzed using student *t* tests. Differences were taken as significant if p values were <0.05. Results are expressed as mean ± SEM and n is the number of samples.

Chapter 3

The influence of insulin and oxytocin on intracellular calcium and force of contraction in non-diabetic and diabetic pregnant women

3.1 Introduction

There is a consensus amongst researchers that the CS rate is higher in women with diabetes compared to non-diabetics (Dunne *et al.*, 2003; Ehrenberg *et al.*, 2004; Jensen *et al.*, 2004; Crowther *et al.*, 2005; Bell *et al.*, 2008; Metzger *et al.*, 2008). Poor myometrial contractility may be an important contributing factor for higher rate of CS in diabetic pregnancies. The evidence to implicate poor myometrial contractility is that post partum haemorrhage has been reported as being six times more common in diabetic women (Dunne *et al.*, 2003).

Previous studies have shown that diabetes has an inhibitory effect on myometrial contractility during late pregnancy (Jawerbaum *et al.*, 1996) and in non-pregnant (McMurtrie *et al.*, 1985) rats. However, one study has reported that diabetes has no effect on myometrial contractility in non-pregnant rats (Franchi *et al.*, 1988). In other smooth muscle cells of rats, it has been reported that diabetes impairs vascular smooth muscle contractility (Pfaffman *et al.*, 1982) and enhances urinary bladder smooth muscle contractions (Longhurst *et al.*, 1991). It has been reported that diabetes decreases (Pfaffman *et al.*, 1982), increases (Owen & Carrier, 1980) or has no effect (MacLeod & McNeill, 1985) on agonist-induced contraction of smooth muscle cells of rat aorta. In many cases the myometrium of diabetics are exposed during pregnancy and labour to high levels of insulin to achieve glycaemic control (Balsells *et al.*, 2000; Lepercq *et al.*,

2008; Stenninger *et al.*, 2008), but it is not known if insulin interferes with oxytocin-induced myometrial contraction.

The purposes of this study were to elucidate whether the high CS rate in diabetic pregnancies may be explained by poor myometrial contractility and to investigate the effect of insulin and oxytocin on uterine contractility. In addition, the effect of insulin on the oxytocin-induced myometrial contraction was examined.

3.2 Methods

In this chapter human myometrial samples were obtained from non-diabetic (n = 67) and diabetic (n = 26) pregnant women undergoing elective CS. Details of the tissue preparation and measurement of fore and calcium transients and histological slides preparation have been explained in chapter 2.

Acidified H_2O and acetic acid were used to prepare insulin and oxytocin stock solutions, respectively. As changes in pH are known to be able to cause changes in uterine contractility (Parratt *et al.*, 1995a, b; Kupittayanant & Kupittayanant, 2009), it is important to note that, when these drugs were diluted for experimentation, the pH remained at 7.4, i.e. identical to that of control PSS, as HEPES buffer was present in both. This was verified every time insulin or oxytocin were used. Therefore, there is no effect of insulin or oxytocin vehicle on myometrial activity. Some drugs were dissolved in the solvent DMSO, which at high concentrations may affect cell membranes. However, at the concentrations used in this thesis no such effects have been reported in all cell types examined including the myometrium, see for example the following references: (Kahn *et al.*, 1998; Noble & Wray, 2002; Gullam *et al.*, 2009).

Table 2.1 and Table 2.2 give the medical details of non-diabetic and diabetic groups involved in this chapter's experiments. The patients numbers involved in this chapter are: 1) Comparing spontaneous contraction; non-diabetics (all in Table 2.1) vs diabetics (all in Table 2.2 except patients number 1, 4, 8, 12 and 24), poor-controlled (6,9,13,16,20,31) vs good-controlled (2, 3, 5, 7, 10, 11, 14, 15, 17-19, 21-23, 25-30), diet-controlled (11, 14, 15, 17, 18, 25) vs insulin-controlled (2, 3, 5-7, 9, 10, 13, 16, 19, 20, 22, 23, 26-31). 2) In response to High KCl; non-diabetic (10-23) vs diabetics (3, 6, 7, 10, 11, 13, 14, 16, 17, 20-22, 25, 31). 3) In response to oxytocin; non-diabetic (24-30) vs diabetics (5, 7, 10, 11, 14, 15). 4) Effect of insulin on oxytocin-induced muscle contraptions; non-diabetics (25-30) vs diabetics (7, 10, 11, 14, 15). 5)

In response to insulin; non-diabetics (24-34) vs diabetics (5, 7, 10, 11, 14, 15, 17). 6) Myometrial histology; non-diabetics (31-39) vs diabetics (18-25).

Non-diabetics

Eleven women underwent CS under spinal anaesthesia and fifty six under general anaesthesia. The median gestational age was 39 weeks (range: 37-41) while the median mother age was 34 years (range: 24-44). The median body mass index was 26.3 (range: 18.5-44.9).

Diabetics

Nineteen caesarean deliveries were performed with the patients under spinal anaesthesia while seven women were under general anaesthesia. The median gestational age was 38 weeks (range: 34-39). The median age of the patients was 34 years (range: 24-44) and the median BMI was 30 (range: 19.4-43). Six patients were poor-controlled while twenty were good-controlled. Out of 26 diabetics, six were diet-controlled and the rest were insulin-controlled.

Data were analyzed using Mann-Whitney U, Wilcoxon or student *t* tests or analysis of variance (ANOVA) test. Differences were taken as significant if p values were <0.05. Results are expressed as median (first quartile, third quartile) or mean ± SEM and n is the number of samples.

Table 3.1: Demographics of diabetic and non-diabetic groups. The table summarises the differences in gestational age, mother age and body mass index.

	Non-diabetic (n=67)	Diabetic (n=26)
Gestational age median (range)	39 weeks (37-41)	38 weeks (34-39)
Mother age median (range)	34 years (24-44)	34 years (24-44)
Body mass index median (range)	27 (18.5-44.9)	30 (19.4-43)

3.3 Results

3.3.1 Patients

There were no significant differences in maternal age; 34 (28, 38) vs 34 (31, 38), gestational age; 39 (38, 39) vs 38 (37, 38) and BMI; 27 (23, 31) vs 30 (27, 35) between non-diabetic (n=26) and diabetic patients (n=67); Table 3.1.

3.3.2 Spontaneous uterine contraction in diabetic and non-diabetic pregnant women

Strips from 67 non-diabetic and 26 diabetic pregnant women were left to contract for one hour while perfused with PSS, then frequency, amplitude, and duration and area under the curve (AUC) of contractions and calcium transients were measured, Figure 3.1 and Table 3.2.

Frequency

As shown in Figure 3.1, under control conditions there were significant differences in the median frequency of calcium transients and contractions between non-diabetic and diabetic; 9 (6, 12) *vs* 5 (3, 11) contractions/hour.

Amplitude

Contraction amplitude of force and calcium transients was significantly less in diabetics 1.4 mN (0.7, 2) and 0.7 mN (0.3, 1.0) than non-diabetics 2.5 mN (1.2, 4.5) and 0.2 mN (0.1, 0.5). As another method of analysing contraction amplitude, the spontaneous contractions were normalized to a 40 mM KCl contraction. The amplitude of force and calcium transients relative to high-K contraction (100%) was significantly lower in the diabetics 58.% (50%, 79%) compared to non-diabetic controls 80% (61%, 90%), n=15 and 14 respectively.

Duration

The duration of contraction and calcium transient at 50% amplitude was significantly lower in diabetics 3.1 min (2.4, 6.0) and 3.03 min (2.3, 5.2), n= 26, compared to non-diabetics 5.3 min (3.5, 8.7) and 5.9 min (3.5, 10.0).

Area under the curve

The significant decreases in contractility resulted in the AUC of force and calcium transients also being significantly less in diabetics, 2.1 au (1.1, 3.5) and 1.1 au (0.6, 1.7, compared to non-diabetic controls, 8.7 au (4.0, 14.5) and 4.4 au (2.0, 7.2).

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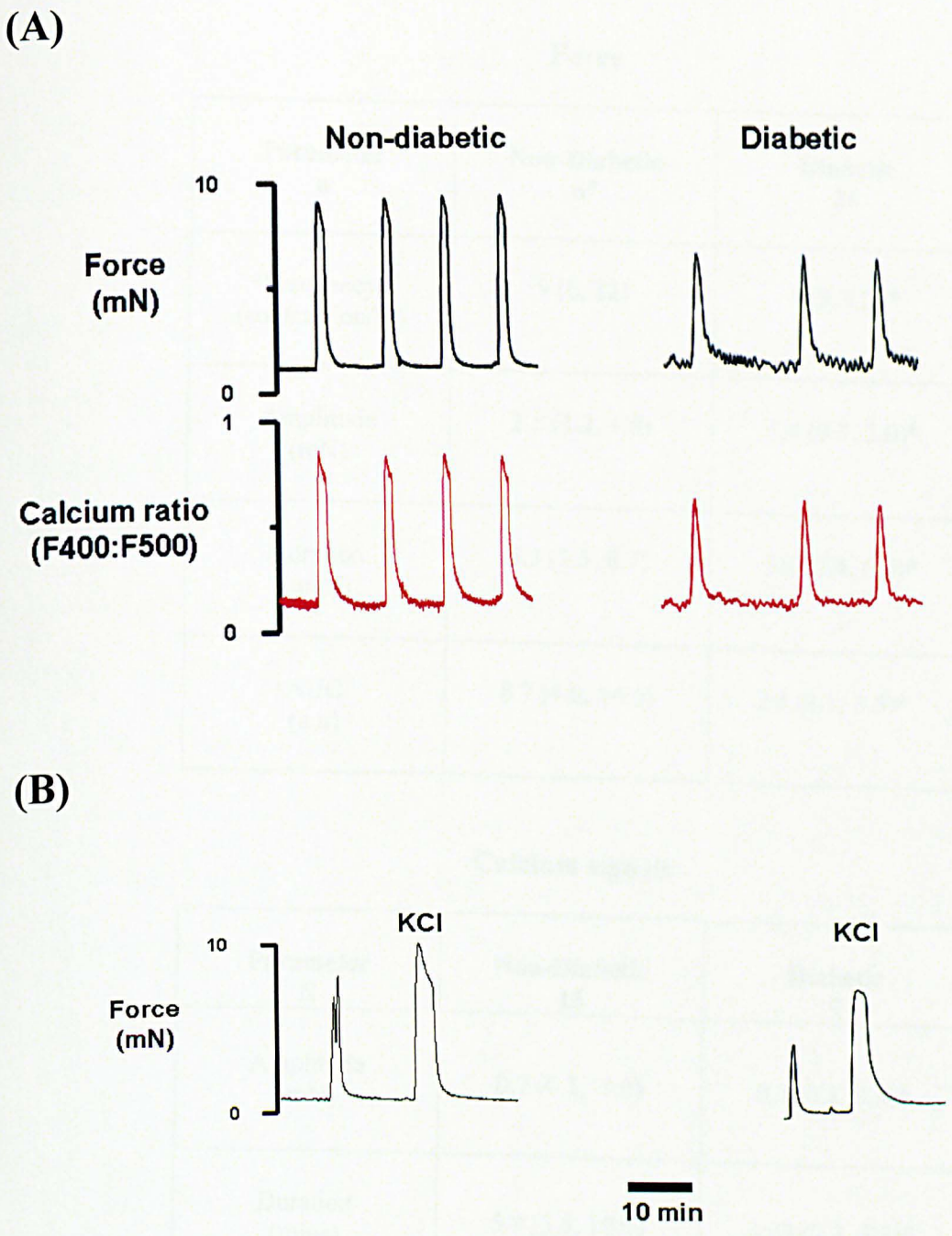


Figure 3.1: Spontaneous contractions and calcium signalling. A): Typical example of simultaneous calcium transients (Indo-1 florescence) and contractions in control (non-diabetic) and diabetic myometrium. B): Contractions related to 40 mM KCl. In this and subsequent Figures, temperature was 35°C and the flow rate was 3 ml/min.

Table 3.2: Parameters of spontaneous contractions in diabetic and non-diabetic women. Median data with first and third quartile in parenthesis. * significantly different ($P<0.05$).

Force

Parameter **n**	**Non-Diabetic** **67**	**Diabetic** **26**
Frequency (contraction/hr)	9 (6, 12)	5 (3, 11) *
Amplitude (mN)	2.5 (1.2, 4.5)	1.4 (0.7, 2.0)*
Duration (mins)	5.3 (3.5, 8.7)	3.1 (2.4, 6.0)*
AUC (a.u)	8.7 (4.0, 14.5)	2.1 (1.1, 3.5)*

Calcium signals

Parameter **N**	**Non-Diabetic** **15**	**Diabetic** **9**
Amplitude (mN)	0.7 (0.3, 1.0)	0.2 (0.1, 0.5)*
Duration (mins)	5.9 (3.5, 10.0)	3.03 (2.3, 5.2)*
AUC (a.u)	4.4 (2.0, 7.2)	1.1 (0.6, 1.7)*

3.3.3 Spontaneous uterine contraction in good-controlled *vs* poor-controlled and insulin-controlled *vs* diet-controlled diabetic subgroups.

The differences in frequency, amplitude, and duration of myometrial contractility and calcium transients between good-controlled (n=20) *vs* poor-controlled (n=6) and between insulin-controlled (n=20) *vs* diet-controlled (n=6) diabetic patients were investigated, Figure 3.2.

The frequency of contractions and calcium transients was not significantly different in good-controlled; 5 contractions/hour (3.3, 12.3) compared to that in poor-controlled diabetic patients; 6 contractions/hour (2, 13). No significant difference in amplitude of contractions and calcium transients between good-controlled and poor-controlled diabetic women; 1.4 mN (0.8, 2.9) vs 1.38 mN (0.6, 2). Similarly, contractions duration at 50% amplitude in myometrium of good-controlled diabetic samples 3.5 min (1.9, 5.4) was not different compared with poor-controlled group 3.1 min (2, 4.3).

The frequency, amplitude and duration of 50% amplitude showed no significant differences between insulin-controlled *vs* diet-controlled groups. The medians of frequency, amplitude and duration were 5.5 contractions/hour (3.8, 8.5) *vs* 5 contractions/hour (3.5, 11.8), 1.5 mN (1, 1.9) *vs* 1 mN (0.4, 2.1) and 3.2 min (2.2, 4.4) *vs* 2.9 min (2.1, 4.1) respectively.

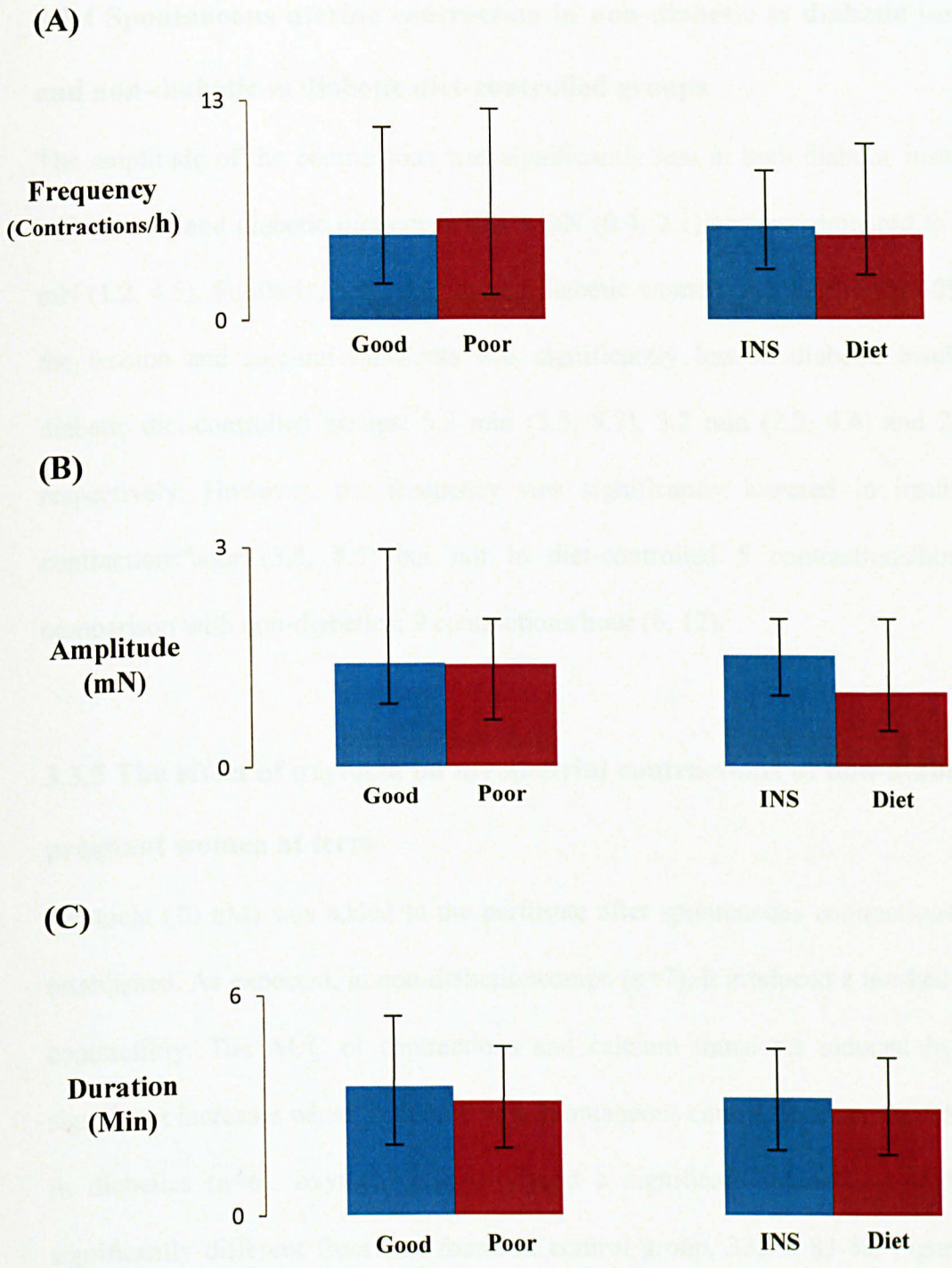


Figure 3.2: Comparisons of contractility in good-controlled (n=20) *vs* poor-controlled (n=6) and insulin-controlled (n=20) *vs* diet-controlled diabetic (n=6) groups. A): No differences in frequency of contractions in good-controlled (good) *vs* poor-controlled (poor) and in insulin-controlled (INS) *vs* diet-controlled (diet) groups. B): The amplitude of contractions showed no significant changes in good *vs* poor and in INS *vs* diet groups C): Similarly, the duration of contractions was not significantly different in good *vs* poor and in INS *vs* diet groups. The p value is > 0.05 for all comparisons.

3.3.4 Spontaneous uterine contraction in non-diabetic *vs* diabetic insulin-controlled and non-diabetic *vs* diabetic diet-controlled groups

The amplitude of the contractions was significantly less in both diabetic insulin-controlled 1.5 mN (1, 1.9) and diabetic diet-controlled 1 mN (0.4, 2.1) groups compared to non-diabetics 2.5 mN (1.2, 4.5). Similarly, compared to non-diabetic women the duration at 50% of amplitude of the tension and calcium transients was significantly less in diabetic insulin-controlled and diabetic diet-controlled groups; 5.3 min (3.5, 8.7), 3.2 min (2.2, 4.4) and 2.9 min (2.1, 4.1), respectively. However, the frequency was significantly lowered in insulin-controlled; 5.5 contractions/hour (3.8, 8.5) but not in diet-controlled 5 contractions/hour (3.5, 11.8) in comparison with non-diabetics; 9 contractions/hour (6, 12).

3.3.5 The effect of oxytocin on myometrial contractions of non-diabetic and diabetic pregnant women at term

Oxytocin (10 nM) was added to the perfusate after spontaneous contractions (100%) had been established. As expected, in non-diabetic women (n=7), it produced a marked increase in uterine contractility. The AUC of contractions and calcium transients induced by oxytocin showed significant increases when compared with spontaneous contractions; mean value was 337± 46 %. In diabetics (n=6), oxytocin also produced a significant increase in AUC which was not significantly different from that found in control group, 332 ± 83 %, Figure 3.4. However as initial contractility was lower in the diabetic samples their contractility even when stimulated with oxytocin remained significantly reduced when compared with oxytocin stimulation in diabetics.

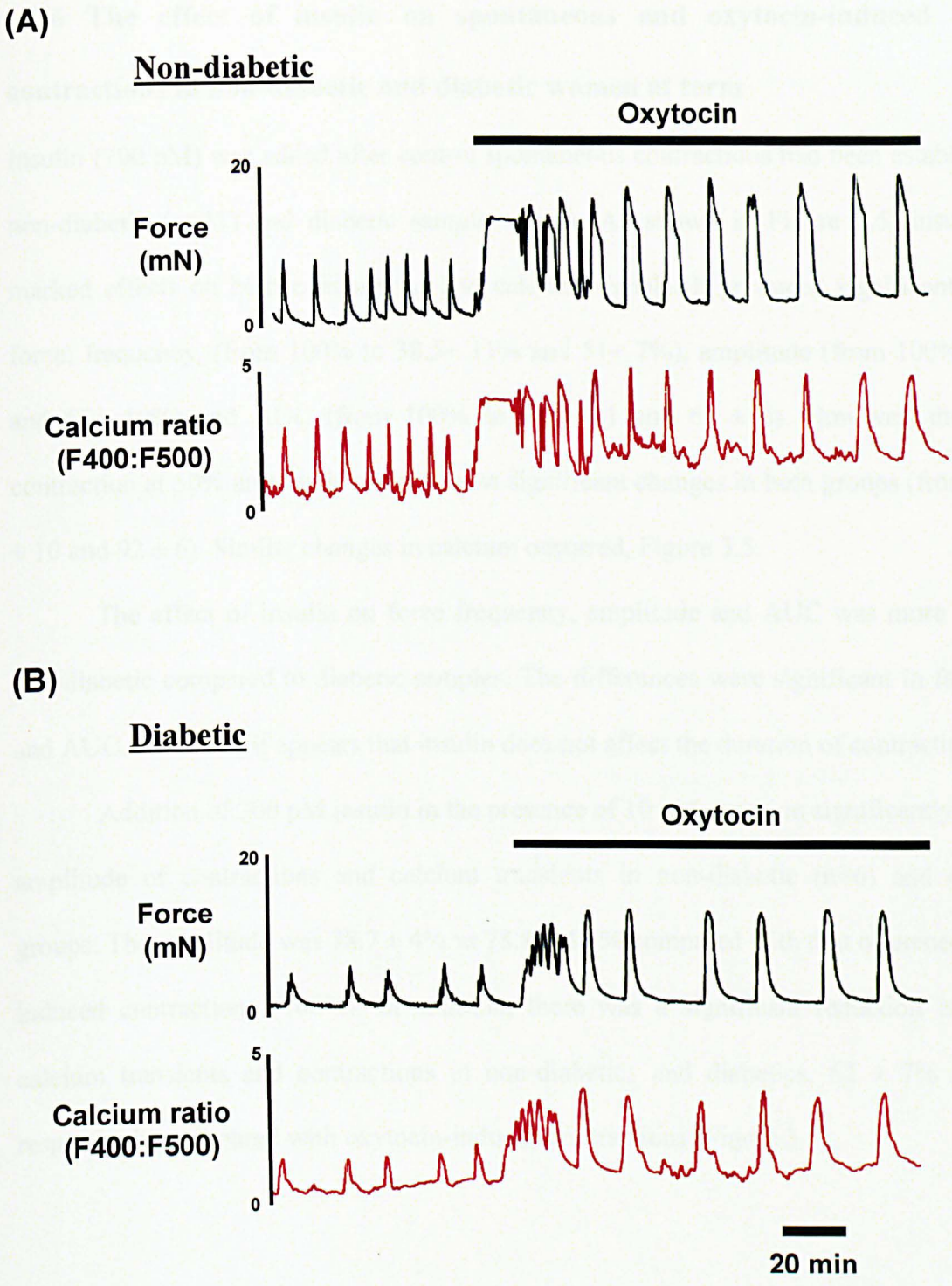


Figure 3.4: Representative traces of the effect of 10 nM oxytocin on the spontaneous myometrial contractions and calcium signalling measured simultaneously in non-diabetic (A) and diabetic (B) myometrium of pregnant women; n= 7 and 6 respectively. The AUC of contractions and calcium transients increased significantly in both groups in response to 10 nM oxytocin.

3.3.6 The effect of insulin on spontaneous and oxytocin-induced myometrial contractions in non-diabetic and diabetic women at term

Insulin (700 pM) was added after control spontaneous contractions had been established to both non-diabetic (n=11) and diabetic samples (n=7). As shown in Figure 3.5, insulin produced marked effects on both contractions and calcium signals. It produced significant reduction in force; frequency, (from 100% to 38.5+ 11% and 51+ 7%), amplitude (from 100% to 38+14 % and 65+ 10%) and AUC (from 100% to 38 ± 11 and 63 ± 6). However, the duration of contraction at 50% amplitude did not show significant changes in both groups (from 100% to 90 ± 10 and 92 ± 6). Similar changes in calcium occurred, Figure 3.5.

The effect of insulin on force frequency, amplitude and AUC was more prominent on non-diabetic compared to diabetic samples. The differences were significant in force amplitude and AUC. However, it appears that insulin does not affect the duration of contraction.

Addition of 700 pM insulin in the presence of 10 nM oxytocin significantly decreased the amplitude of contractions and calcium transients in non-diabetic (n=6) and diabetic (n=5) groups. The amplitude was 78.7 ± 4% *vs* 78.8 ± 4.7% compared with that of preceding oxytocin-induced contractions (100%). In addition, there was a significant reduction in the AUC of calcium transients and contractions in non-diabetics and diabetics, 62 + 7% *and* 72 + 5% respectively, compared with oxytocin-induced contractions (Figure 3.6).

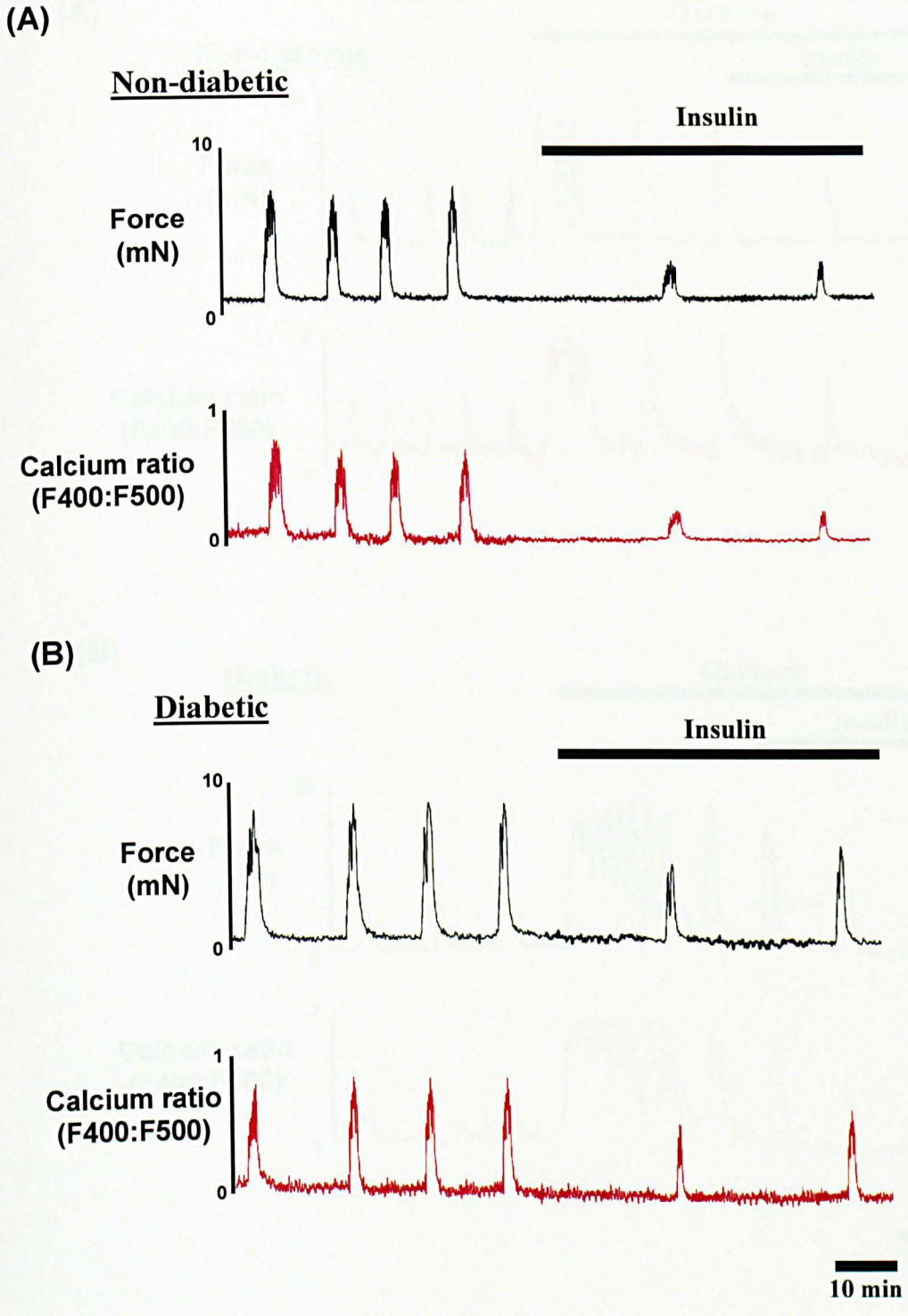


Figure 3.5: The effect of 700 pM insulin on spontaneous calcium transients and contractions from non-diabetic; n=11, (A) and diabetic; n=7, (B) myometrium of pregnant women. Insulin induces muscle relaxation in both groups; however, the effect is more prominent in non-diabetic group.

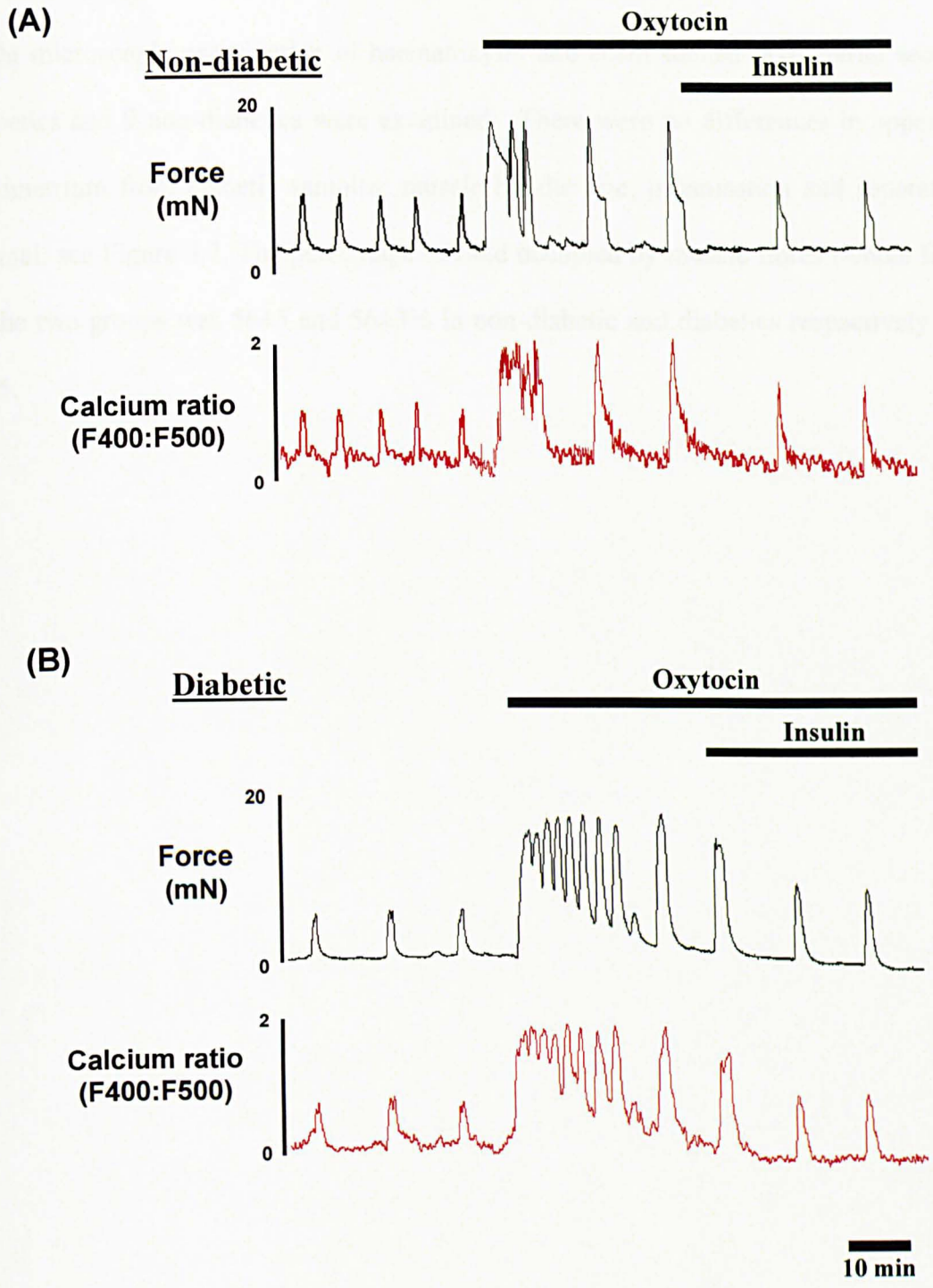


Figure 3.6: The effect of 700 pM insulin on oxytocin-induced calcium signals and force. Simultaneous recording of the changes of myometrial contractions and calcium transients of non-diabetic; n=6, (A) and diabetic; n=5, (B) myometrium of pregnant woman in response to 10 nM oxytocin alone and in the presence of 700 pM insulin. Insulin decreased oxytocin-induced myometrial contractions in both groups significantly.

3.3.7 Histology

Light microscopic examination of haematoxylin and eosin stained myometrial sections from 8 diabetics and 9 non-diabetics were examined. There were no differences in appearance of the myometrium from diabetic samples; muscle bundle size, organisation and separation were all normal, see Figure 3.7. The percentage of field occupied by muscle fibres (whole field = 100%) in the two groups was 56±5 and 56±3% in non-diabetic and diabetics respectively and p was > 0.05.

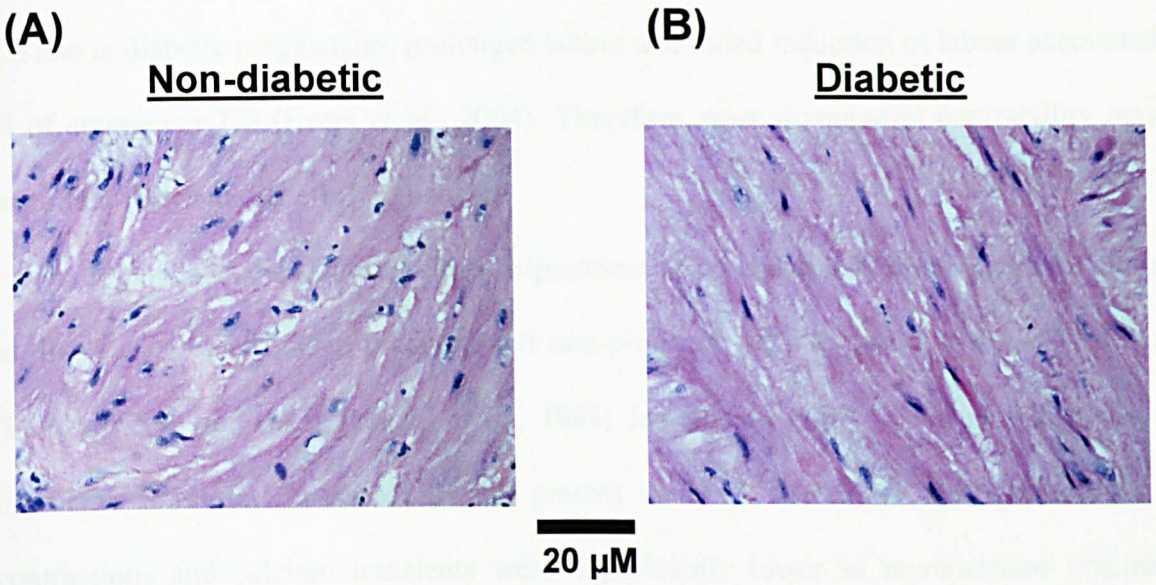


Figure 3.7: Myometrial histology. Haematoxylin and Eosin stained uterine sections from A; non-diabetic; n=9 and B; diabetic myometrium of pregnant women (n=8). The percentage of field occupied by muscle fibres (whole field = 100%) in the two groups showed no significant difference and p value was > 0.05.

3.4 Discussion

3.4.1 The impact of diabetes on myometrial contractility

As discussed earlier, several studies worldwide have shown a higher CS rate in diabetic compared to non-diabetic women (Dunne *et al.*, 2003; Ehrenberg *et al.*, 2004; Jensen *et al.*, 2004). While fetal distress, macrosomia and obesity may contribute to the increased emergency CS rate in diabetic pregnancies, prolonged labour and failed induction of labour accounted for 51 % of emergency CS (Evers *et al.*, 2004). Therefore, poor myometrial contractility may be an important factor in diabetic pregnancies.

Previous studies showed either impairment or no changes in the myometrial contractility of diabetic myometrium in pregnant and non-pregnant rats compared to non-diabetic controls (McMurtrie *et al.*, 1985; Franchi *et al.*, 1988; Jawerbaum *et al.*, 1996) in which the calcium transients were not measured. In the present study, I found that the spontaneous uterine contractions and calcium transients were significantly lower in myometrium obtained from diabetics compared to that obtained from non-diabetics. These effects were marked in contraction frequency, amplitude, duration and AUC; they were half that of the non-diabetics. This may indicate that L-type VDCC activation may be impaired in the myometrium of diabetic pregnant women. Further investigation of this will be produced in chapter 6. In addition, I found in the current work that no differences in contractility between diabetic subgroups; good-controlled vs poor-controlled and insulin-controlled vs diet-controlled. Further, I found that in compare to non-diabetics, both insulin-controlled and diet-controlled showed significant reduction in tensions. These results may explain the findings of Langer et al. (2005) who has reported similar CS rates in normal and overweight diabetic women controlled during their pregnancy by insulin or diet regardless of established levels of glycaemic control.

My data clearly show that *in vitro* the diabetic uterus contracts poorly at late pregnancy. This impaired contractility if translated to *in vivo* condition, would lead to poor labour contractions and failure to progress in labour. This in turn would be an indication for CS and thus my findings may help explain the increased rate of CS in diabetics. So, what is the reason for this poor uterine contractility in diabetes?

Myometrial contractility is dependent mainly on calcium influx via the voltage-gated L-type calcium channels, and hence in turn, on membrane potential (Wray *et al.*, 2003). In the current study, I found calcium transients in myometrium from women with diabetes are less than that detected in myometrium obtained from non-diabetics under normal conditions. Therefore, a defect in calcium channel function or expression may be present in myometrium obtained from women with diabetes. These suggestions are supported by previous studies in diabetic cardiac muscles.

Cardiomyocytes contractility is also initiated by calcium entry through the voltage-dependent L-type calcium channels (Henderson *et al.*, 2004; Ebert *et al.*, 2008). Diabetes is known to associate with impairment of cardiomyocytes contraction (Wang *et al.*, 1995; Bracken *et al.*, 2004; Bracken *et al.*, 2006). Many studies have reported that the intracellular calcium is less in diabetic cardiomyocytes compared to controls (Lagadic-Gossmann *et al.*, 1996; Bracken *et al.*, 2006; Lu *et al.*, 2007). This can be explained by the reduction in L-type calcium current (ICa) (Wang *et al.*, 1995; Bracken *et al.*, 2004; Pereira *et al.*, 2006) which may due to the decrease in calcium channels function or expression (Bracken *et al.*, 2006; Pereira *et al.*, 2006; Lu *et al.*, 2007). However, other mechanisms that govern the intracellular calcium availability may be involved. It would be of interest to determine the magnitude and kinetics of the L-type calcium current in isolated voltage-clamped uterine myocyte, from diabetic and non diabetic women, to better understand the underlying mechanism. Experiments with modulators of L-type

calcium entry are described in chapter 6, and they suggested a reduction in calcium entry through L-type calcium channels.

The poor contraction and calcium signalling in diabetic women has presumably resulted from the chronic diabetic condition. However, in addition I also wanted to investigate two clinically relevant managements the administration of oxytocin and insulin. Diabetes mellitus is associated with a rise in glucose and insulin levels in the blood compared to non-diabetics (DeFronzo *et al.*, 1982; Bogardus *et al.*, 1984; Perriello *et al.*, 1997). A growing body of evidence has implicated both hyperglycaemia and hyperinsulinaemia in inducing muscle relaxation in vascular and non vascular smooth muscle cells (Omatsu-Kanbe & Kitasato, 1990; Horowitz *et al.*, 1991; Zemel *et al.*, 1992; Mene *et al.*, 1993; de Boer *et al.*, 1994; Muniyappa *et al.*, 1997; Byrne *et al.*, 1998; Weber & Ehrlein, 1998). Although during labour at Liverpool Women's Hospital good glycaemic control is achieved by continuous infusion of intravenous glucose and insulin, to protect the fetus from neonatal hypoglycaemia (Balsells *et al.*, 2000; Taylor *et al.*, 2002; Lepercq *et al.*, 2008). Nevertheless I asked the question could hyperglycaemia (discussed in chapter 4) and hyperinsulinaemia exert an inhibitory effect on myometrial contractility?

In future studies it would be useful to undertake regression analysis of HbA1c and BMI against contraction parameters to determine if there is any association between changes in HbA1c and weight of diabetic patients and myometrial contractility.

3.4.2 The effect of oxytocin on myometrial contractions of non-diabetic and diabetic pregnant women

Although several researchers have reported that oxytocin increases contractility in normal myometrium at term (Kimura *et al.*, 1992; Fuchs *et al.*, 1995; Furuya *et al.*, 1995), to the best of

my knowledge no previous work investigated the effect of oxytocin on human diabetic myometrial contractility and calcium transients at late pregnancy. However, apart from human, one previous study showed that compared to control rats the response of the myometrium of diabetics to oxytocin was less at day 21 but similar at day 22 of pregnancy (Jawerbaum *et al.*, 1996). However, another study showed that oxytocin-induced myometrial contraction in diabetic rats was less as compared to control animals (McMurtrie *et al.*, 1985). In the present work I also found that 10 nM oxytocin equally increased myometrial force of contraction and calcium transients in non-diabetics and diabetics; about 3 fold compared to preceding spontaneous activity. These *in vitro* findings suggest that oxytocin will stimulate myometrial contraction in diabetics to a similar extent to that seen in non-diabetics. However, diabetics experience a higher rate of failed labour induction using a combination of prostaglandin and oxytocin than non-diabetics (Evers *et al.*, 2004). Thus it is possible that in diabetics *in vivo* there is an impairment of oxytocin-induced myometrial contraction. This may be to do with half-life or receptor occupancy, or other hormonal levels.

Uterine contractility at term is dependent on high oestrogen/progesterone ratio in the circulating blood in order to maintain high concentration of myometrial oxytocin receptor (Soloff, 1975; Thorburn & Challis, 1979). Labour is induced with the use of prostaglandins to initially shorten then start dilating the cervix (Rayburn, 1989). Once cervical dilation has started, then labour is augmented with oxytocin. In diabetic rats $PGF_{\alpha 2}$–induced contraction in the myometrium was not changed when compared to controls (Jawerbaum *et al.*, 1996). Since oestrogen is involved in the synthesis of oxytocin receptors, diminished diabetic uterine response to oestrogenic action may contribute to a reduced response to oxytocin and the *in vivo* finding of and increased failed induction rate in diabetic women. (Jawerbaum *et al.*, 1996).

3.4.3 The effect of insulin on uterine contraction

In the present studies, I have shown that insulin is capable of inducing muscle relaxation and decreasing calcium transient in the myometrium of both diabetic and non-diabetic pregnant women at late pregnancy. However, the effects on non-diabetics were more compared with diabetics. These findings are consistent with previous studies conducted on vascular smooth muscle that demonstrated decrease in muscle contractions in response to hyperinsulinaemia (Omatsu-Kanbe & Kitasato, 1990; Hundal *et al.*, 1992; Muniyappa *et al.*, 1997). The dose of insulin used in my studies was within the range of physiological hyperinsulinaemia (Mitrakou *et al.*, 1992; Tack *et al.*, 1996).

The possible mechanisms which may be involved in insulin-induced muscle relaxation in the myometrium will be discussed in detailed in chapter 6.

In summary, the current results indicate that insulin and hyperinsulinaemia, which may be present in blood of diabetics during labour, interferes with muscle contraction in the myometrium and could also contribute to the high CS rate seen in diabetics.

3.4.4 The effect of insulin on oxytocin-induced uterine contraction

No previous study has examined the effect of insulin on oxytocin-induced myometrial contraction in humans at late pregnancy. Hence, I investigated this in myometrium obtained from non-diabetic and diabetic pregnant women. I found that insulin in the presence of oxytocin decreased significantly and equally the amplitude and AUC of both myometrial contractions and calcium transients in non-diabetic and diabetic women, when compared to that of the preceding oxytocin-induced contractions. These results indicate that insulin is capable of interfering with oxytocin-stimulated contractions in the myometrium.

Many diabetic patients are exposed to high levels of insulin during pregnancy and labour to achieve glycaemic control (Balsells *et al.*, 2000; Lepercq *et al.*, 2008; Stenninger *et al.*, 2008). My findings suggest that this may contribute to the high rate of failed labour induction with oxytocin in diabetics during labour. The next question I asked was how can insulin interfere with oxytocin action?

In vascular smooth muscle, insulin inhibition of agonist-induced calcium transient is due to inhibition of receptor- and voltage-operated calcium channels (Standley *et al.*, 1991), inhibition of IP_3-stimulated calcium release from intracellular stores (Saito *et al.*, 1993), and activation of plasma membrane Ca^{2+}-ATPase–mediated Ca_i^{2+} efflux (Zemel *et al.*, 1992). Similar effects of insulin on oxytocin-induced calcium transient may take place in myometrium.

3.4.5 Histology

The differences in force output in the uterine biopsies from women with diabetic pregnancy do not appear to be due to structural abnormalities. No obvious differences in cellular abundance or orientation were seen at the light microscopic level, and there were no differences in the amount of muscle present in biopsies. There have been no other histological studies of diabetic human myometrium and only one electron microscopic study performed in rats (McMurtrie *et al.*, 1985). These authors saw no differences in nuclei or mitochondria between streptozotocin-treated animals and control non-pregnant rats but noted, with no quantification or elaboration, that the number and cellular orientation of myofilaments in euglycaemic animal appeared different. Changes in endometrial ultra-structure with diabetes in rats have been reported (Kirkland *et al.*, 1981). Clearly further studies on human myometrium from diabetic patients are called for but we find no obvious histological differences. As discussed next, our data point to

impairment of Ca signalling as being the mechanism underlying decreased myometrial force in diabetic women.

3.4.6 Summary

My work has shown that intrinsic myometrial activity and the underlying calcium transients are decreased in biopsies from pregnant diabetic women delivered by caesarean section. This was the case even when glucose was well-controlled. This may in turn explain the increased rate of CS in these women as impaired contractility will lead to failure to labour to progress adequately. Insulin was found to be a potent inhibitor of myometrial contractility and this may contribute to the high CS rate in diabetic pregnancies and the high failed induction of labour rate in these women.

Chapter 4

The effect of glucose on uterine contractility and the impact of diabetes on myometrial glycogen storage in pregnant women at term

4.1 Introduction

There is a cumulative body of evidence which indicate that hyperglycaemia is capable of inducing muscle relaxation in different types of smooth muscle (Horowitz *et al.*, 1991; Williams & Schrier, 1992, 1993; de Boer *et al.*, 1994; Byrne *et al.*, 1998; Wang *et al.*, 2000). Several studies have revealed that the effect of hyperglycaemia is due to a biochemical rather than physical effect (Mene *et al.*, 1993; Barbagallo *et al.*, 1995; Liu *et al.*, 2001; Arun *et al.*, 2004; Gomes *et al.*, 2004). These studies exposed tissues to different concentrations of mannitol and L-glucose (5 to 30 mM) for up to 48 hours. Failure of mannitol and L-glucose to duplicate the effect of high glucose indicates that the reduction in force is not simply related to changes in extracellular osmolality. Many reports have shown that hyperglycaemia induce muscle relaxation in VSM by inhibiting calcium entry through VDCC channels (Williams & Schrier, 1992; Kam *et al.*, 1993; Williams & Schrier, 1993; Wang *et al.*, 2000). As myometrial contractility is known to dependant on calcium signalling (Wray *et al.*, 2003), any hyperglycaemia alteration in calcium flux is likely to affect uterine contractility.

Excess glucose in blood is stored in many cell including muscles as glycogen, where it can provide energy for contraction (Bouche *et al.*, 2004). Previous studies have shown that the glycogen content in myometrial biopsies from pregnant animals and humans is higher than that found in biopsies from non-pregnant (Milwidsky & Gutman, 1983; Sakamoto *et al.*, 1987; Wedenberg *et al.*, 1990). One study conducted in rats reported that just prior to parturition,

myometrial glycogen increased to levels similar to those in skeletal muscle and depleted immediately postpartum (Chew & Rinard, 1979). Thus, glycogen may serve as an important energy source for the contracting uterus during labour especially under conditions in which strong uterine contractions markedly reduce uterine blood flow (Assali *et al.*, 1958; Greiss, 1965).

In this chapter, I investigated the effect of high glucose and glucose deprivation on uterine contractions and measured the glycogen store in the myometrium of non-diabetic and diabetic pregnant women at term.

4.2 Methods

Details of the tissue preparation and measurement of calcium have been explained in chapter 2. Muscle strips were allowed to generate spontaneous contractions before the exposure to 25 mM glucose, mannitol (osmotic control) or 0-glucose.

Myometrial glycogen storage was measured in biopsies taken from diabetic and non-diabetic pregnant women undergoing elective caesarean section. Details of the tissue preparation and measurement of glycogen have been explained in chapter 2.

Table 2.1 and Table 2.2 give the medical details of non-diabetic and diabetic groups involved in this chapter's experiments. The patients numbers involved in this chapter are: 1) In response to 25 mm glucose; non diabetics (50-61) vs diabetics (20-23, 25-30). 2) In response to 0-glucose with strips left to contract until contractions disappear; non-diabetics (41-67) vs diabetics (14-22). 3) Glycogen content in tissues left to contract in 0-glucsoe until contractions disappear vs glycogen stores in samples frozen immediately after delivery for same patients (non-diabetics; 41-46). 4) Glycogen content; non-diabetics (41-59) vs diabetics (1-13).

Data were analyzed using Mann-Whitney U, Wilcoxon or student *t* tests. Differences were taken as significant if p values were <0.05. Results are expressed as median (first quartile, third quartile) or mean ± SEM and n is the number of samples.

4.3 Results

4.3.1 The effect of time and mannitol on myometrial contraction

As seen in Figure 4.1, myometrium was able to produce spontaneous, stable and regular phasic contraction for at least 2 hours (n=3). Compared with previous spontaneous contraction, mannitol (osmotic control) did not change the contraction activity (n=3), Figure 4.1.

4.3.2 The effect of high glucose on myometrial contractions of non-diabetic and diabetic pregnant women at term

To investigate the effect of high glucose on myometrial contractility, tissue was perfused with PSS to establish spontaneous contractions then 25 mM glucose added. Both non-diabetic (n=12) and diabetic (n=10) groups showed an equal reduction in force contractions and calcium transients in response to high glucose, Figure 4.2.

Frequency

The frequency of contractions and calcium transients was significantly diminished in response to high glucose; in non diabetic myometrium, 67% (50, 83), and in diabetic samples, 58 % (33, 70), compared with preceding (8 mM glucose) control contractions in each group. These reductions were not significant between the two groups.

Amplitude

The application of 25 mM glucose solution to spontaneously contracting myometrium (100%) significantly decreased the amplitude of contractions in non-diabetics to 56 % (54, 82) and in diabetics (n=10) to 88 % (56, 93). Similar changes occurred in calcium transients. However, no significant difference between non-diabetic and diabetic samples was found.

Area under the curve

Both diabetic and non-diabetics showed similar response to high glucose. The AUC of contractions and calcium transients was reduced significantly in non-diabetics, 76 % (58, 84), and diabetics, 74 % (42, 83). This response to high glucose was not significant between the two groups.

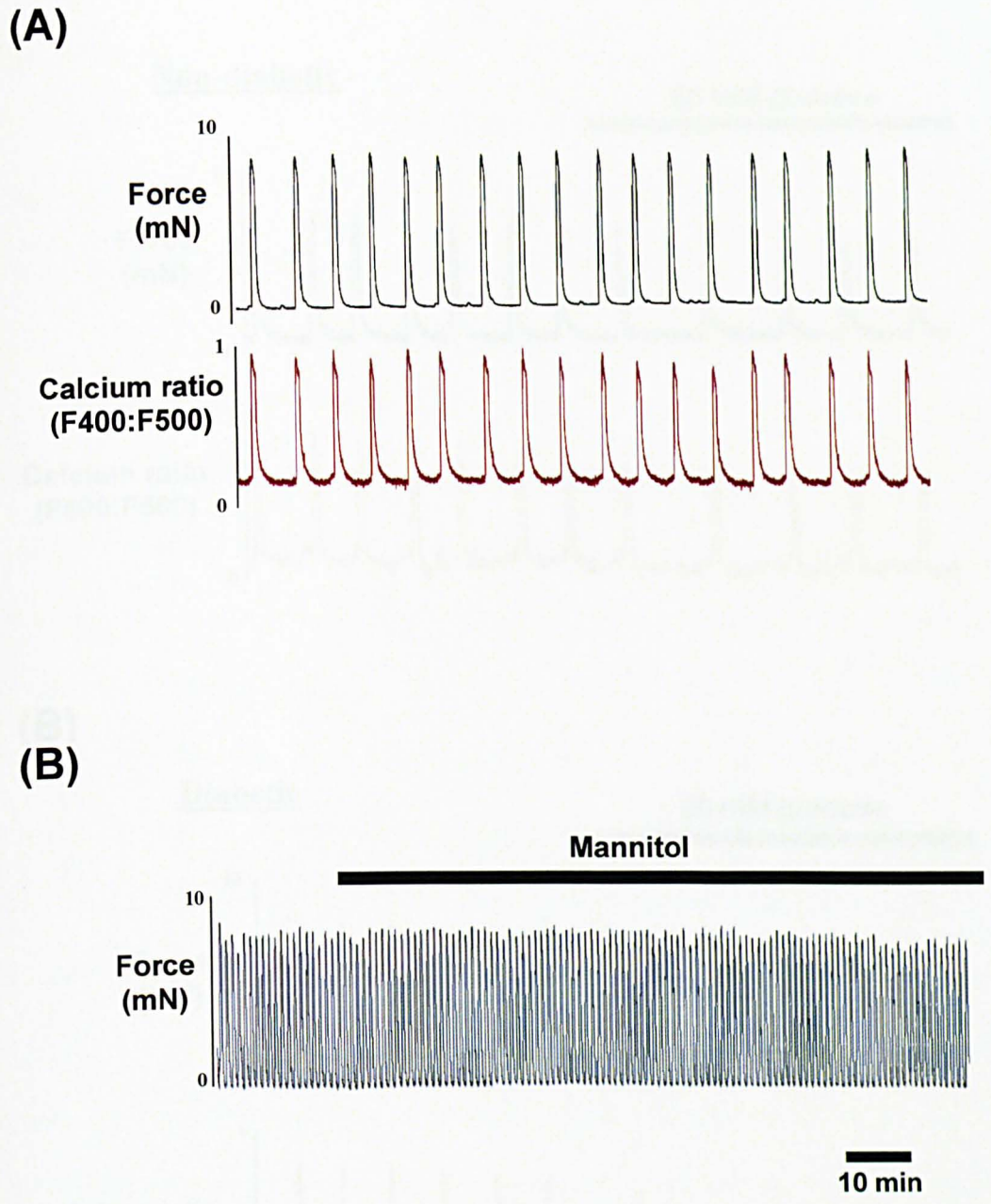


Figure 4.1: Representative control traces for spontaneous uterine contractility and in response to mannitol. A) A simultaneous recording of spontaneous contractions and calcium transients for 2 hours in response to 8 mM glucose PSS, n=3. B) The effect of mannitol on uterine contractility; n=3.

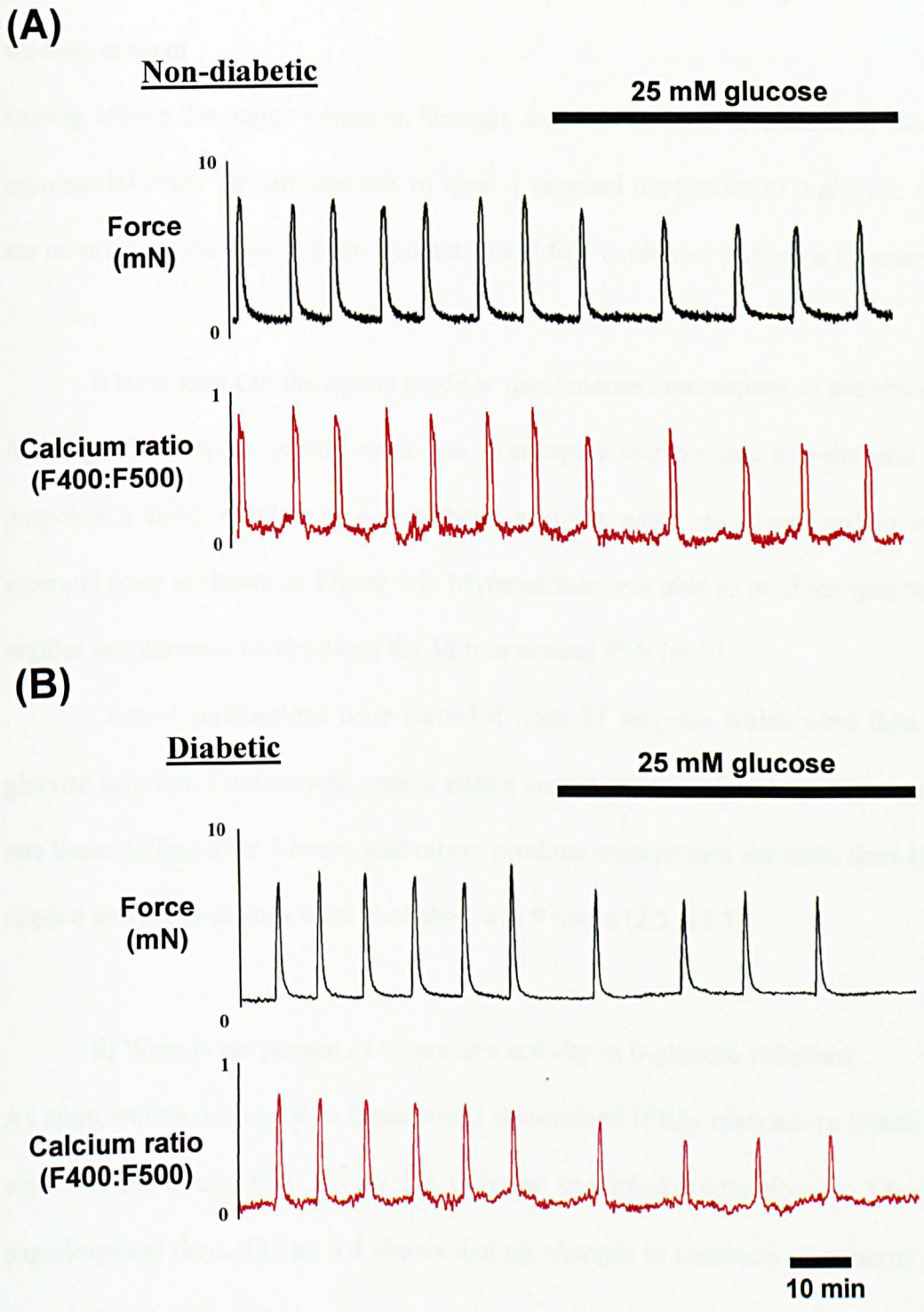


Figure 4.2: Representative traces for the effect of hyperglycaemia on uterine contractility. A simultaneous recording of force contractions and calcium transients in response to 25 mM glucose in (A) non-diabetics; n=12 and (B) diabetic; n=10 myometrium of pregnant women. The effect of 25 mM glucose was similar in both groups.

4.3.3 The effect of glucose deprivation on uterine contractility in non-diabetic pregnant women at term

During labour the uterus contracts strongly and this leads to occlusion of the blood supply to myometrial cells. To simulate this *in vitro*, I exposed the tissues to 0-glucose solution. As there are no previous data for human myometrium, I first examined protocols on non-diabetic samples

i) How long can the uterus produce spontaneous contractions in the absence of glucose?

Although the purpose of this work was to compare diabetic and non-diabetic uteri, for control purposes a small number of non-diabetic samples were also examined in control PSS. An example trace is shown in Figure 4.6. Myometrium was able to produce spontaneous, stable and regular spontaneous contractions for 48 h in normal PSS (n=3).

Control contractions were recorded from 27 biopsies which were then perfused with 0-glucose solution. Contractions ceased after a varied amount of time, as shown in Figure 4.3, with one tissue falling after 3 hours, and others produce contractions for more than 48 hours. The time needed until contractions were abolished was 9 hours (2.5, 15.3).

ii) What is the pattern of contractile activity in 0-glucose solution?

As contractions decreased in 0-glucose, I determined if this manoeuvre selectively affected any aspect of the contraction. To do this I choose specimen contractions at 3 hourly intervals and superimposed them. Figure 4.4 shows that no changes in contractions patterns as time passed in the selected contractions.

iii) Dose 0-glucose affect contractions amplitude and frequency?

Both amplitude and frequency carry on decreasing progressively in response to 0-glucose solution. Figure 4.5 illustrates the reductions in both amplitude and frequency in 3 hourly intervals compared to control contractions.

Having established how 0-glucose affects spontaneous contractions in human myometrium, I next compared diabetics and non-diabetics.

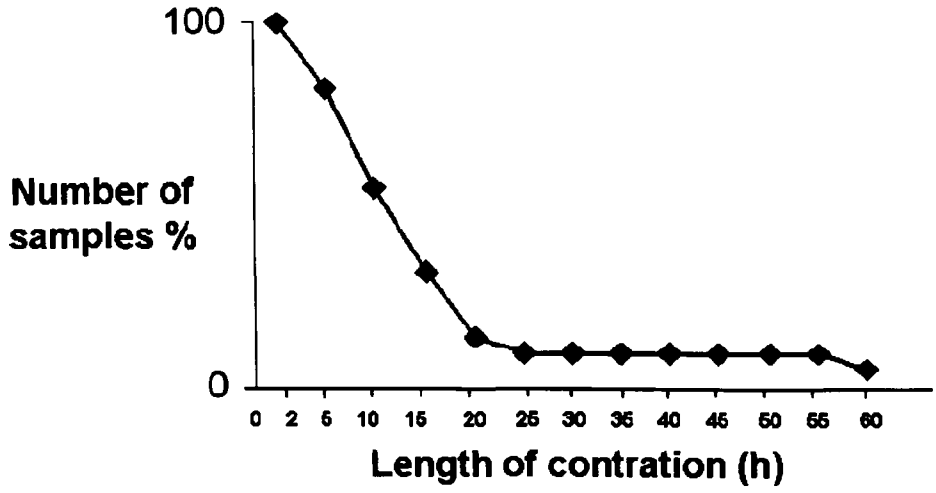
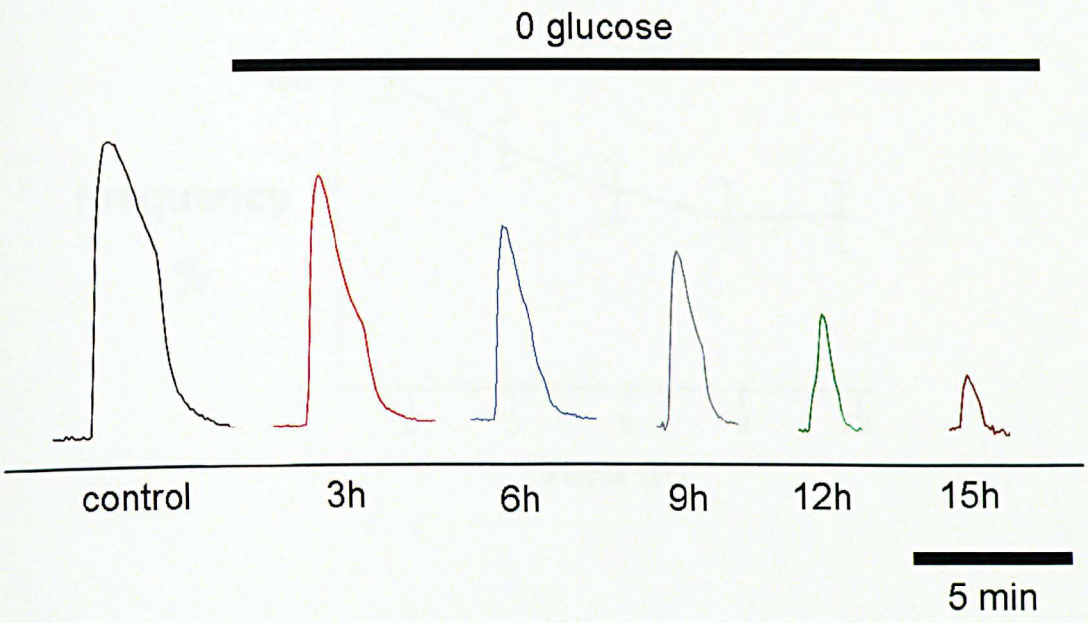


Figure 4.3: The length of myometrial contraction produced in hours in the absence of glucose. Control contractions were recorded from 27 non-diabetic samples which were then perfused with 0-glucose solution. Contractions ceased after a varied amount of time.

(A)



(B)

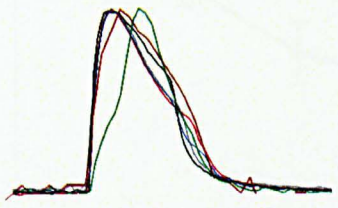
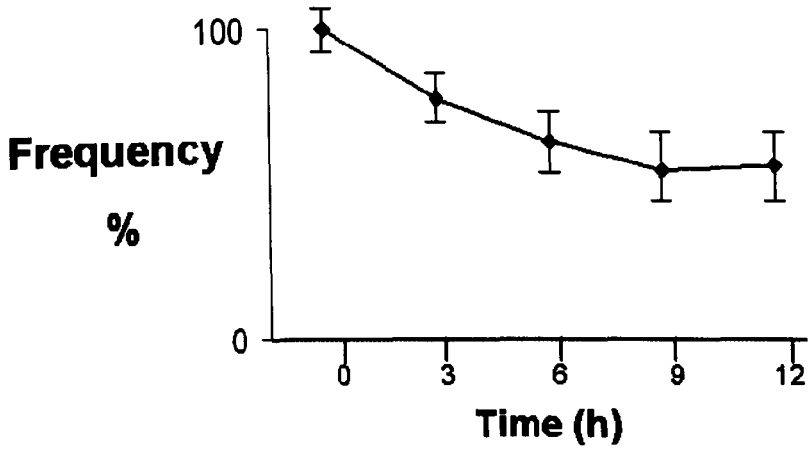


Figure 4.4: Representative traces for the pattern of contractile activity of myometrium of pregnant women (n=27) in 0-glucose solution. A) The shape of the contraction traces in 3 hours interval. B) The shape of these traces after normalization.

(A)



(B)

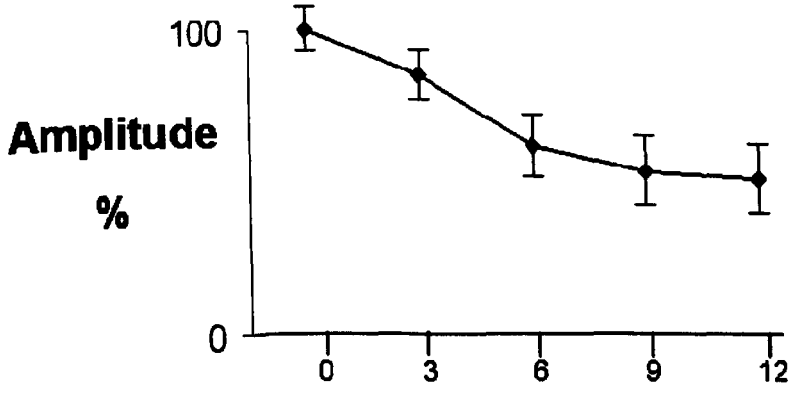


Figure 4.5: The changes in myometrial contraction's (A) frequency and (B) amplitude in 3 hour intervals, which was observed when the strips left to contract in 0-glucose solution. Both frequency and amplitude decrease as time proceeds; n=27.

4.3.4 Comparison of the effect of glucose deprivation on uterine contractility between non-diabetic and diabetic pregnant women at term

When the perfusate was changed from control (8 mM) to 0-glucose, as noted above, there was a marked variation in how long contractions were produced for between tissues in non-diabetic and diabetic groups. However, those from diabetics were abolished faster; 5.2 hours (2.3, 13.5), n=9, than non-diabetics; 9 hours (2.5, 15.3), n=27, Figure 4.6. Only 1 diabetic sample continued to contract after 12 hours in 0-glucose, whereas 10 controls did so, two of which continued to contract for over 2 days.

To find out more about the contractility in the absence of glucose supply, I studied the frequency, amplitude and duration of contractions in strips from non-diabetics and diabetics.

The frequency of contractions after 3 hours of glucose deprivation of non-diabetics and diabetics decreased significantly, 82 % (63, 90) and 60 % (37, 72) respectively, compared with the frequency of the control periods (100%). In addition, both non-diabetic and diabetic samples showed significant reduction in force amplitude (from 100% to 85 % (70, 95) and 45 % (30, 60). Force amplitude and frequency were significantly different between the groups, Figure 4.7. However, I found no significant changes in contraction duration in non-diabetics and diabetics, 95% and 95.2% respectively.

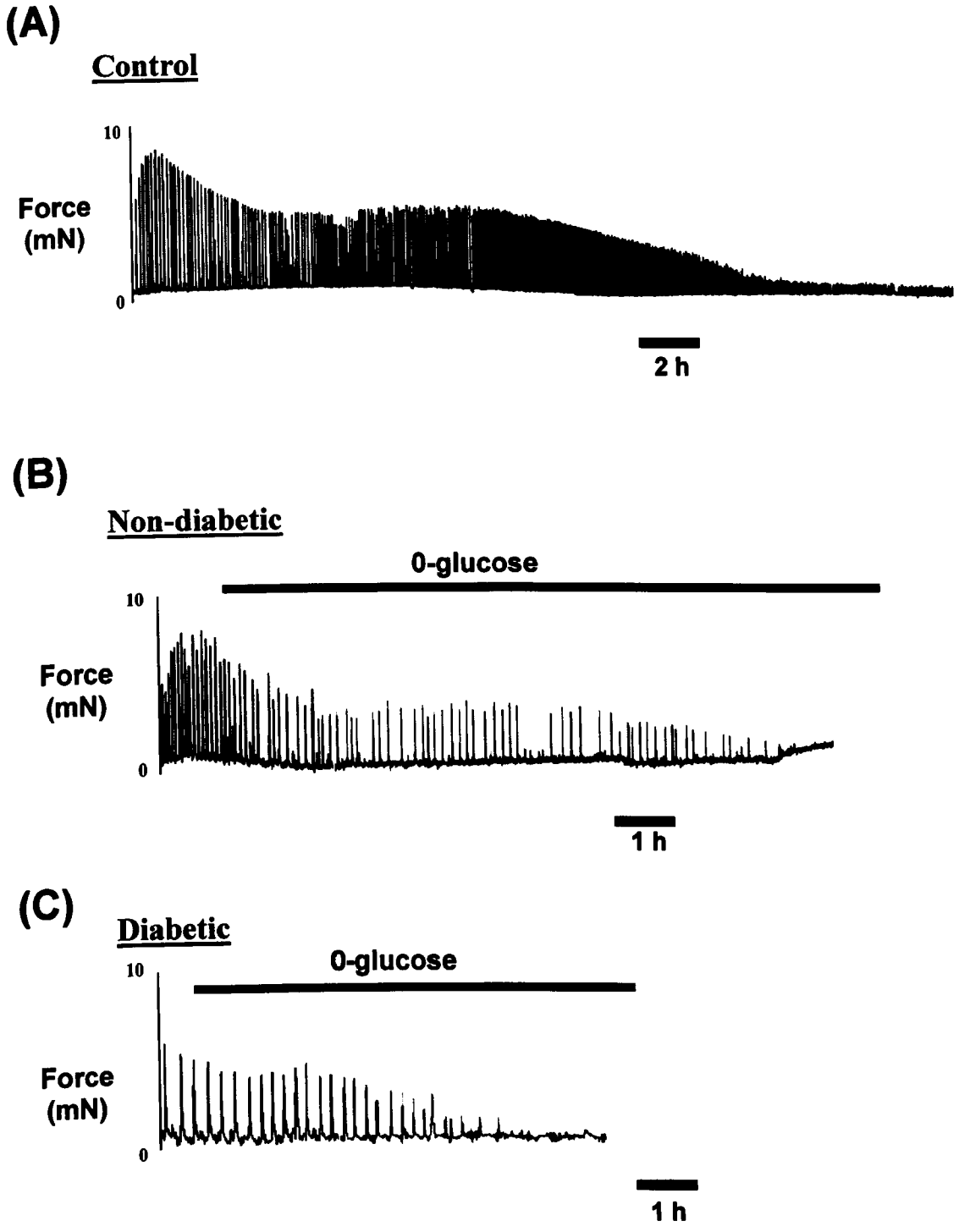


Figure 4.6: Comparison of the effect of (A) 8 mM glucose (control) and glucose deprivation on uterine contractility in (B) non-diabetic (n=27) and (C) diabetic (n=9) pregnant women. Control samples produced regular frequent contractions for 48 h in normal PSS (N=3). Notice that the time bars are different between A and other traces.

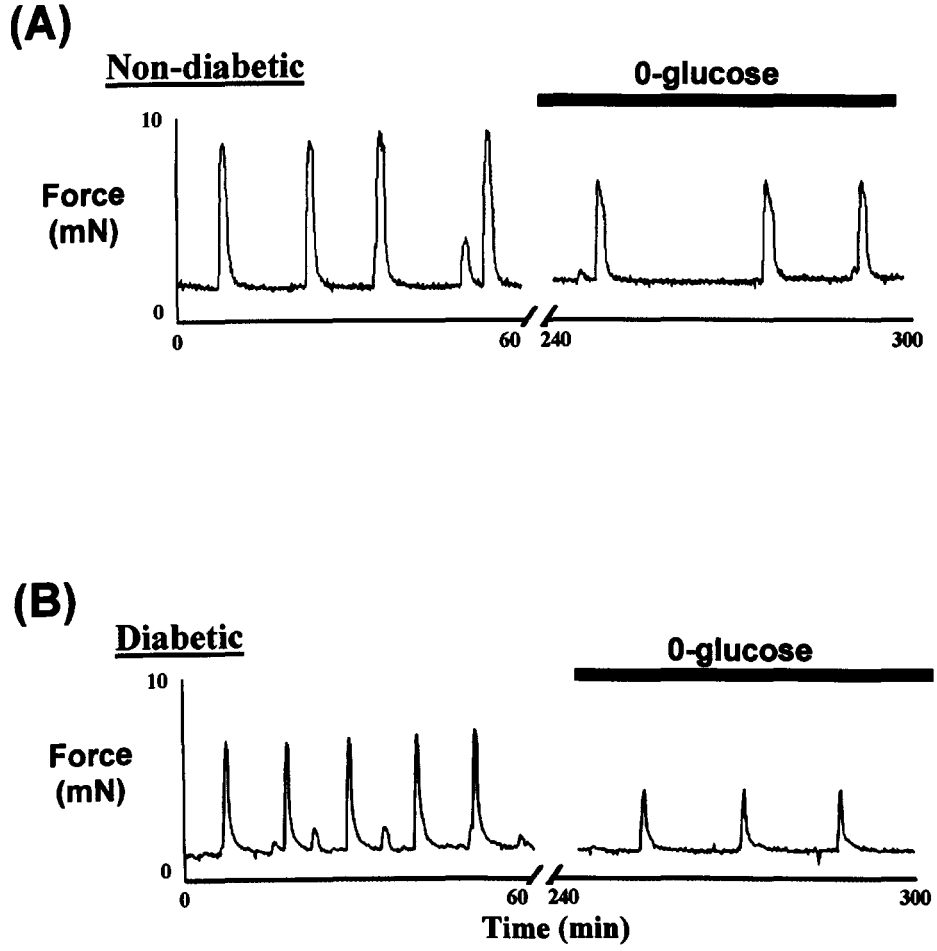


Figure 4.7: Comparison of the effect of glucose deprivation on uterine contractility in (A) non-diabetic (n=27) and (B) diabetic (n=9) pregnant women. Force amplitude and frequency were significantly different between the groups after 3 hours of glucose deprivation.

4.3.5 Glycogen stores in non-diabetic and diabetic pregnant women at term

Frozen biopsies obtained from non-diabetic (n=19) and diabetic (n=13) pregnant women undergoing elective CS were ground under liquid nitrogen, and then the glycogen content were measured.

Figure 4.8 shows that the amount of glycogen storage in diabetics (11.3 ± 1.3 mmol/Kg, n=13) was significantly lower than non-diabetics (16.6 + 2 mmol/Kg, n=19).

As I found above that myometrial tissues carry on contracting in the absence of glucose supply for many hours, I investigated if glycogen is a possible source of energy for driving the contractions. I measured the glycogen stores in six non-diabetic samples left to contract in the absence of glucose until contractions ceased. The amount of glycogen in these samples (0.5 ± 0.1 mmol/Kg) was significantly lower than control samples that frozen immediately after the caesarean sections (16.6 + 2 mmol/Kg), Figure 4.8.

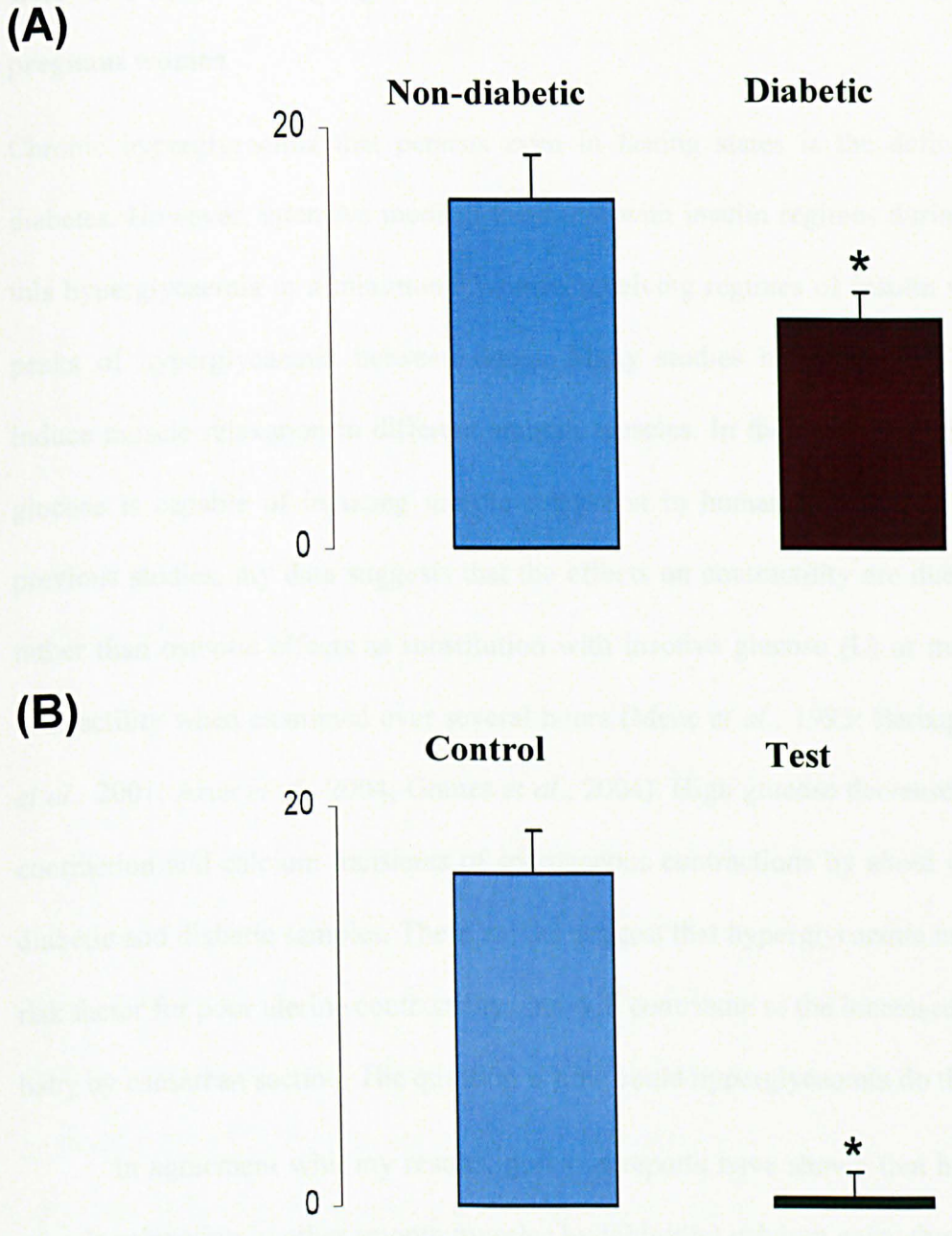


Figure 4.8: The glycogen content in the myometrium of pregnant women. (A) The amount of glycogen in non-diabetic (16.6 + 2 mmol/Kg, n=19, blue bar) compared to diabetic (11.3 ± 1.3 mmol/Kg, n=13, red bar) myometrial samples. (B) A comparison of glycogen content between control samples (16.6 + 2 mmol/Kg, n=19, blue bar) and test samples left to contract until contractions disappeared (0.5 ± 0.1 mmol/Kg, black bar); both sample groups obtained from non-diabetic pregnant women.

4.4 Discussion

4.4.1 The effect of high glucose on uterine contractility in non-diabetic and diabetic pregnant women

Chronic hyperglycaemia that persists even in fasting states is the defining characteristic of diabetes. However, intensive medical treatment with insulin regimes during pregnancy reduces this hyperglycaemia to a minimum. Women receiving regimes of insulin will experience some peaks of hyperglycaemia between doses. Many studies have reported that hyperglycaemia induce muscle relaxation in different smooth muscles. In the current studies, I found that high glucose is capable of inducing muscle relaxation in human myometrium. In agreement with previous studies, my data suggests that the effects on contractility are due to glucose elevation rather than osmotic effects as substitution with inactive glucose (L) or mannitol did not affect contractility when examined over several hours (Mene *et al.*, 1993; Barbagallo *et al.*, 1995; Liu *et al.*, 2001; Arun *et al.*, 2004; Gomes *et al.*, 2004). High glucose decreased equally the force of contraction and calcium transients of spontaneous contractions by about one fifth in both non-diabetic and diabetic samples. These results suggest that hyperglycaemia may be considered as a risk factor for poor uterine contractility, and will contribute to the increased rate of delivering the baby by caesarean section. The question is how could hyperglycaemia do this?

In agreement with my results, previous reports have shown that hyperglycaemia induce muscle relaxation in other smooth muscles by inhibiting calcium entry through calcium channels (Williams & Schrier, 1992; Kam *et al.*, 1993; Williams & Schrier, 1993). It has been reported that hyperglycaemia reduced current density of L-type VDCC in SMCs which may due to the alteration in these channels activity (Wang *et al.*, 2000). One study has shown that high glucose decreased L-type VDCC mRNA in rat pancreas, suggesting that hyperglycaemia may directly diminish channel expression (Iwashima *et al.*, 1993).

Calcium entry, which occurs mainly via the L-type VDCC, is crucial for initiation of myometrial contractions (Wray *et al.*, 2003). In my studies, I found that intracellular calcium transient diminished in both non-diabetic and diabetic groups in response to high glucose. Thus, hyperglycaemia may cause a defect in function or expression of the calcium channels in myometrial cells. However, other mechanisms that govern the intracellular calcium availability may be involved. It would be of interest to determine the magnitude and kinetics of the L-type calcium current in isolated voltage-clamped uterine myocyte, from diabetic and non diabetic women, to better understand the underlying mechanism.

4.4.2 Effect of glucose deprivation on uterine contractility and glycogen stores in non-diabetic and diabetic pregnant women

During pregnancy there are many metabolic alterations in the uterus including increases in nucleotide triphosphate concentration, [ATP], in uterine smooth cells (Dawson & Wray, 1985). It has been suggested that these changes occur to ensure adequate contractile activity at labour (Wray, 1990). However, intense contractions of the uterus during labour can narrow the blood vessels (Assali *et al.*, 1958; Greiss, 1965) with subsequent reduction in oxygen and glucose supply to myometrial cells. Many studies have shown *in vivo* a marked reduction in myometrial contractility, [ATP] and pH_i upon closure of the uterine blood vessels (Wray, 1990; Harrison *et al.*, 1994; Larcombe-McDouall *et al.*, 1998; Larcombe-McDouall *et al.*, 1999). Acidification *in vitro* is associated with muscle relaxation (Wray *et al.*, 1992). As ATP production is mainly by means of oxidative phosphorylation of glucose and ATP is required for muscle contraction, reduction of oxygen or glucose may impair contractility. To mimic the effect glucose deprivation which could occur during blood vessels occlusion due to myometrial contraction, I studied *in vitro* the contractility of uterus in the absence of glucose.

Myometrial tissues were able to contract for variable times in both control PSS and in response to glucose deprivation in both diabetic and non-diabetic groups. Although the *n* numbers are too small to enable a direct comparison between control PSS and 0-glucose, my preliminary data suggests that the uterus contracts for longer when glucose is present. However my main findings are that in the absence of glucose the contractions ceased faster in diabetic samples compare to non-diabetic controls. The amplitude and frequency of contractions of diabetic samples were significantly decreased more than non-diabetics. After three hours of contractions in 0-glucose solution, both amplitude and frequency reduced by 15% in non-diabetics and by 35% in diabetics. These finding indicates that: 1) there is another source of energy inside the myometrial cells providing energy to maintain the contraction in the absence of glucose which occur during strong uterine contractions throughout the labour, 2) the diabetic myometrium is more susceptible to glucose deprivation and differs in its metabolic resources.

It has been reported that glycogen is a considerable energy source for the contracting uterus during parturition in rats (Chew & Rinard, 1979; Sterin *et al.*, 1991). To find out if glycogen is used during contractions in the absence of glucose, I compared the glycogen stores in myometrial biopsies frozen immediately after elective caesarean sections (non-diabetic controls) with those frozen after they have left to contract *in vitro* until contractions disappeared (non-diabetic tests). I found that the amount of glycogen dropped in test biopsies to 5% of that in controls. This result suggests that the glycogen was consumed as energy for contracting myometrium in the absence of glucose. However, it would be better to have compared the glycogen store in the non-diabetic and diabetic (test) samples, left to contract in 0-glucose until contractions disappeared, with control samples from both groups left to contract in normal PSS for the same period of time.

My results showed, in addition, that diabetic myometrium stores less glycogen at term compared to non-diabetic. This finding may explain my previous results mentioned above in which uterine contractions were abolished faster in diabetics compared to non-diabetics. This reduction in glycogen stores may contribute to poor uterine contractility in diabetics. So, what are the possible causes for the lower glycogen stores in diabetic myometrium?

Cellular uptake of glucose is mediated thorough facilitative glucose transporters (GLUTs) or sodium-glucose co-transporters (SGLTs), described in more detailed in chapter 5 (Bell *et al.*, 1993; Scheepers *et al.*, 2004). After it has been up taken into cytoplasm, glucose quickly phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase enzyme, the first enzyme in the glycogen synthesis pathway (Hers, 1992; Braithwaite *et al.*, 1995). Glycogen is the primary storage form of glucose in human cells. The metabolism of the storage glycogen is linked with insulin action and blood glucose homeostasis. Insulin promotes glycogen synthesis by stimulating glucose uptake and by activating hexokinase and glycogen synthase, the rate limiting enzyme in glycogen synthesis, in skeletal muscle (Parker *et al.*, 1983; Lawrence & Roach, 1997; Pendergrass *et al.*, 1998; Halse *et al.*, 2003).

A key feature of diabetes is impairment in the stimulation of glycogen synthesis in skeletal muscle by insulin (Eriksson *et al.*, 1989; Shulman *et al.*, 1990). Diabetes is associated with insulin resistance which attributed to diminished insulin-stimulated glucose uptake and decreased hexokinase expression and glycogen synthase activities (Pendergrass *et al.*, 1998; Cline *et al.*, 1999; Halse *et al.*, 2001; Hojlund & Beck-Nielsen, 2006). The low glycogen stores in diabetic myometrium could be due to similar defects. It would be interesting to also measure Hexokinase expression and glycogen synthase activity in human myometrium.

Other possible energy source of energy for labouring uterus is triglycerides (TG). In normal pregnant rats TG increases toward the end of pregnancy (Argiles & Herrera, 1981) .

However, previous studies have shown that uterine TGs are not used as energy sources during pregnancy in diabetics and non-diabetics (Gonzalez *et al.*, 1993; Jawerbaum *et al.*, 1994; Jawerbaum *et al.*, 1996).

In summary, my findings suggest that there are differences in uterine metabolism associated with diabetes, which could underlie change in contractility, and perhaps lead to the uterus being less able to withstand the metabolic demands of labour, as blood flow and hence glucose falls with contraction. This may relate to differences in glycogen storage and utilization which is lower in diabetics.

Chapter 5

The expression of glucose transporter 1, 3 and 4 in the myometrium

5.1 Introduction

Glucose transporter-1 (GLUT-1) has a wide tissues distribution (Birnbaum *et al.*, 1986; Jansson *et al.*, 1993; Camps *et al.*, 1994; Dudek *et al.*, 1994; Younes *et al.*, 1996; Nie *et al.*, 2000). Glucose transporter-3 (GLUT-3) is a high-affinity glucose transporter which expressed in tissues with high glucose demands such as the placenta, testis and brain (Gould *et al.*, 1991; Haber *et al.*, 1993). Unlike GLUT-1 and GLUT-3, the glucose transporter-4 (GLUT-4) is insulin-dependent for uptake of glucose and is expressed mainly in striated muscle and adipose tissue (Birnbaum, 1989; Charron *et al.*, 1989; James *et al.*, 1989).

Many authors reported changes in GLUT expression with diabetes. In human endometrium, GLUT-3 mRNA diminishes in late pregnancy in the diabetic but not in the non-diabetic women (Sciullo *et al.*, 1997). Diabetes is associated with reduced expression of GLUT-4 in adipose tissue but not in muscle (Shepherd & Kahn, 1999; Astrup & Finer, 2000). Moreover, it has been revealed that GLUT-4 recruitment at the cell surface is impaired in the skeletal muscle and adipose tissue of diabetic mouse (Miura *et al.*, 2001).

In this chapter, I investigated whether GLUT-1, 3 and 4 are expressed in myometrium. In addition I compared the immunostaining of these transporters in the myometrium of non-diabetic and diabetic pregnant women. In order to obtain gestational data in a highly correlated manner, I also investigated the expression of these transporters in rats at defined stages of pregnancy.

5.2 Methods

Human and animal tissue microarrays contained formalin-fixed paraffin embedded myometrial samples were obtained from non-diabetic (n = 9), and diabetic (n = 9) pregnant women undergoing elective caesarean section. Table 2.1 and Table 2.2 give the medical details of non-diabetic and diabetic groups involved in this chapter's experiments. The patients numbers involved in this chapter are non-diabetics (40-48) vs diabetics (18-26).

Another five samples were taken from women undergoing hysterectomy. Their age range (34-44 years) and indication of hysterectomy are: uterine prolapse (1), menorrhagia (2), menorrhagia associated with severe pain (1), menorrhagia associated with fibroids (1). Animal samples were obtained from myometrium of pregnant rat at 12, 16, 20, 21 days and labouring uterus (n = 1 for each). Another sample was taken from non pregnant rat. Samples from rat's skeletal muscle and kidney were used as a control samples. The samples were kindly provided by Dr. R. Floyd. The immunohistochemical study was performed as described previously in Chapter 2.

5.3 Results

5.3.1 The expression of GLUT-1 in myometrium

Immunohistochemistry was performed on formalin-fixed-paraffin-embedded human myometrial tissue sections. The staining intensity was scored as described in methods.

The immunostaining was evident in the red blood cells (positive control sample) and absent in negative controls as seen in Figure 5.1.

Human myometrium

The staining was variable in the myometrium of hysterectomy samples n= 5, Figure 5.2. The immunostaining was low in two samples, high in two samples and very high in one sample.

The results of the staining intensity of GLUT-1 in the myometrium of non-diabetic and diabetic pregnant women at term is summarised in Table 5.1 and Table 5.2. The average staining intensity was low in diabetic samples, n=9 and high in non-diabetics, n=9, Figure 5.3.

Rat myometrium

To determine the expression of GLUT-1 in the myometrium at different gestations of pregnancy, immunohistochemistry was performed on TMA sections from rat myometrium at different gestations of pregnancy and from non-pregnant rat.

The staining intensity was low in non-pregnant rat, moderate in day 12 and day 16, high in day 21 of pregnancy and abundant in labouring uterus, n= 1 for each (Figure 5.4).

Table 5.1: The GLUT-1 immunostaining scoring in the myometrium of non-diabetic pregnant women at term. Three high power fields (a, b and c) were scored to calculate the mean intensity for GLUT-1. Same scoring system was done in the rest of the tables.

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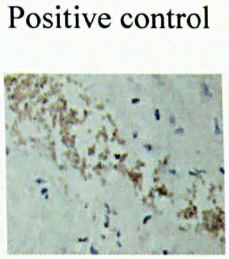
Non-diabetic samples	Staining intensity			Mean Intensity
	a	b	C	
1	+++	+++	+++	+++
2	+++	+++	+++	+++
3	+++	+++	+++	+++
4	+++	+++	+++	+++
5	+++	+++	+++	+++
6	+++	++	++	++
7	+++	+++	+++	+++
8	+++	++	++	++
9	+++	++	+++	+++
Mean	+++	+++	+++	+++

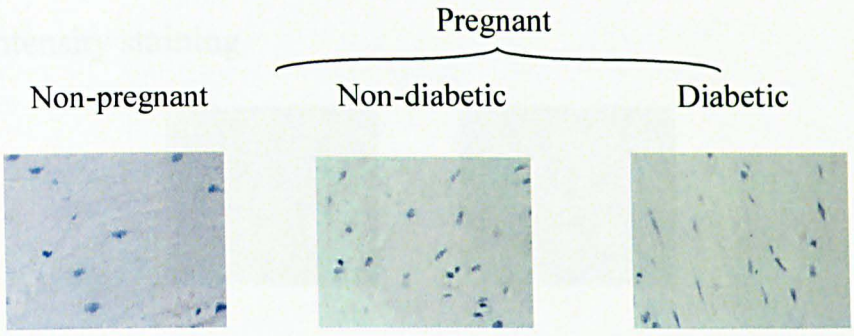
Table 5.2: Scoring the immunostained cells for GLUT-1 in the myometrium of diabetic pregnant women at term.

Diabetic samples	Staining intensity			Mean Intensity
	a	b	C	
1	+	+	+	+
2	++	+	++	++
3	+	+	+	+
4	+	+	+	+
5	++	+	++	++
6	+	+	+	+
7	+	+	+	+
8	+++	+++	+++	+++
9	+	+	++	+
Mean				+

(A) Red blood cells



(B) Human myometrium



(C) Rat myometrium

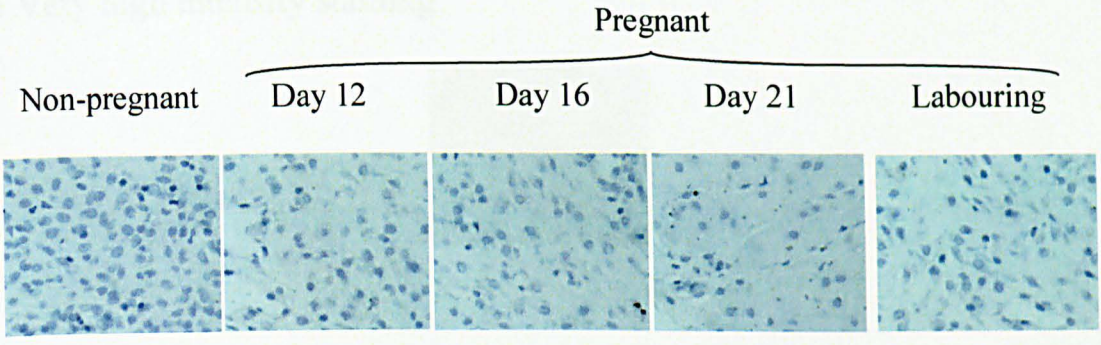
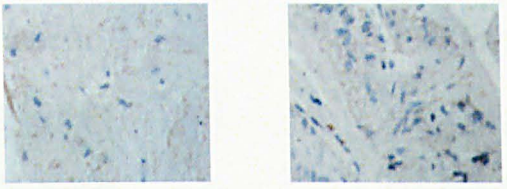
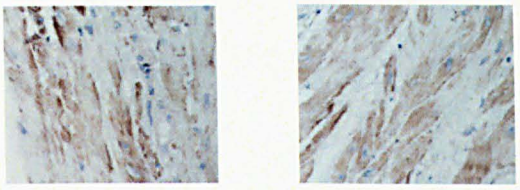


Figure 5.1: Immunohistochemical staining of GLUT-1 in the myometrium. The positive control (red blood cells) showed strong staining. The negative controls, (B) and (C), showed no staining. The scale is X100 magnification for this and the rest of the figures.

(A) Low intensity staining



(B) High intensity staining



(C) Very high intensity staining

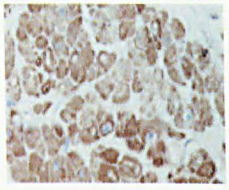


Figure 5.2: Immunohistochemical staining of GLUT-1 in the myometrium of non-pregnant women. The staining was variable; low in two samples (A), high in two samples (B) and very high in one sample (C).

Figure 5.3: Representative immunostaining of GLUT-1 in the myometrium of pregnant women. The staining of GLUT-1 in the myometrium of non-diabetic (n=9) pregnant women is stronger than the myometrium of diabetics (9).

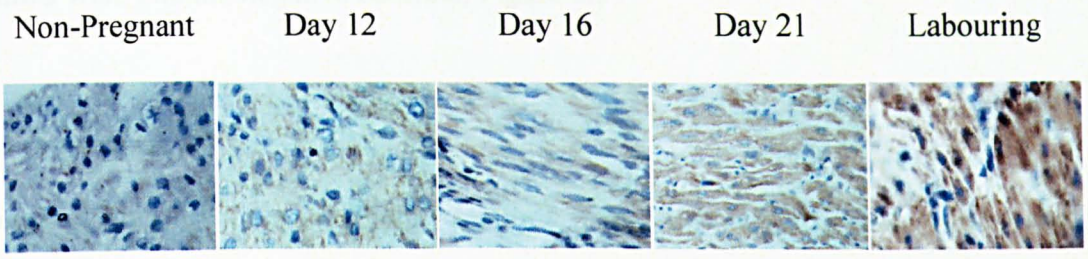


Figure 5.4: Immunohistochemical staining of GLUT-1 in the myometrium of rat. The staining of GLUT-1 increases in intensity as the pregnancy progress toward term.

5.3.2 The expression of GLUT-3 in myometrium

The immunostaining of GLUT-3 in most myometrial cells was perinuclear in both human and rat samples. Immunohistochemical staining included the positive control (endometrial tissue), which was stained well, and the negative controls, Figure 5.5.

Human myometrium

The staining intensity in hysterectomy sections, n=5, was variable. One section showed no staining while two sections have low and two with moderate staining intensity, Figure 5.6.

Table 5.3 and Table 5.4 illustrate the differences in the staining intensity of GLUT-3 in non-diabetic and diabetic human myometrium at term. The average staining intensity was higher in myometrium from non-diabetics compared with diabetics; very high in non-diabetic, n=8 and high in diabetic, n=8, sections (Figure 5.7).

Rat myometrium

Figure 5.8 shows the changes of GLUT-3 immunostaining during the progression of pregnancy and in non-pregnant rats. The non-pregnant rat myometrium showed no GLUT-3 staining. In addition, day 12 and 16 had also no positive staining for GLUT-3. However, day 21 had low GLUT-3 staining intensity while labouring uterus showed staining of moderate intensity.

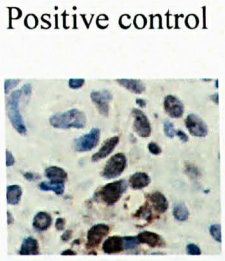
Table 5.3: The GLUT-3 immunostaining scoring in the myometrium of non-diabetic pregnant women at term.

Non-diabetic samples	Staining intensity			Mean Intensity
	a	b	c	
1	++++	++++	++++	++++
2	++++	++++	++++	++++
3	++++	++++	++++	++++
4	++++	++++	++++	++++
5	++++	+++	++++	++++
6	++++	+++	+++	+++
7	+++	+++	+++	+++
8	++++	++++	++++	++++
Mean				++++

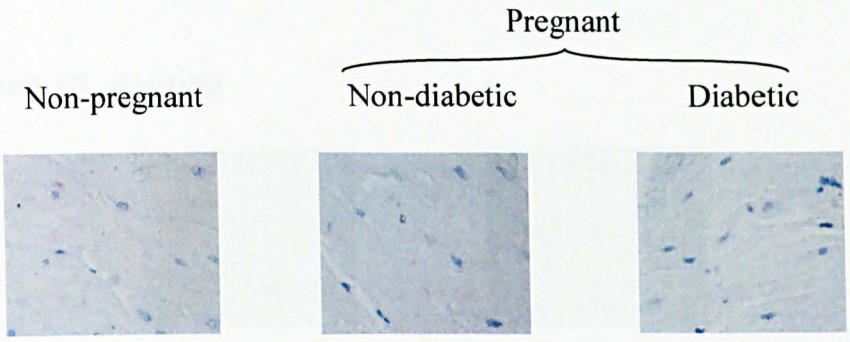
Table 5.4: Scoring the positively stained myometrial cells for GLUT-3 in the myometrium of diabetic pregnant women at term.

Diabetic samples	Staining intensity			Mean Intensity
	a	b	c	
1	++++	+++	++++	++++
2	++++	+++	+++	+++
3	++++	++++	++++	++++
4	++++	+++	+++	+++
5	++++	+++	++	+++
6	++++	+++	+++	+++
7	+++	+++	+++	+++
8	++++	++	++++	+++
Mean				+++

(A) Endometrium



(B) Human myometrium



(C) Rat myometrium

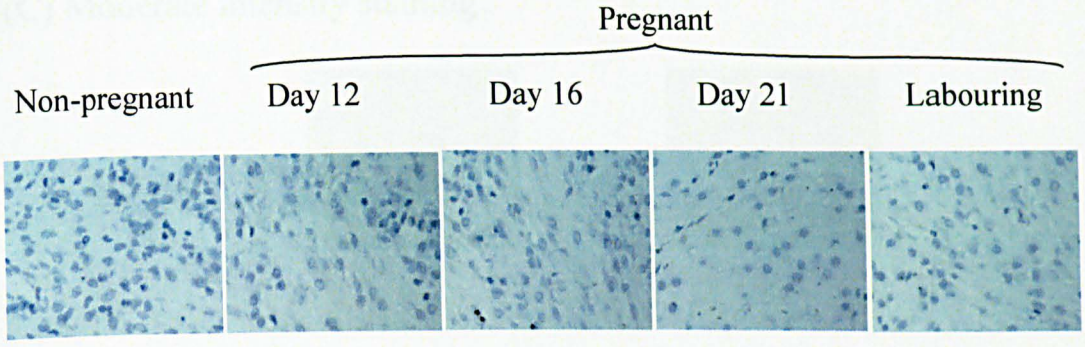
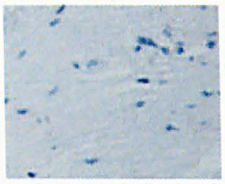
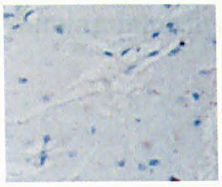


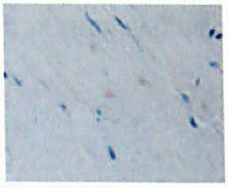
Figure 5.5: Immunohistochemical staining of GLUT-3 in the myometrium. The positive control (endometrial cells) showed strong staining. The negative controls, (B) and (C), showed no staining.

(A) No staining



(B) Low intensity staining





(C) Moderate intensity staining

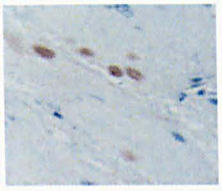


Figure 5.6: Immunohistochemical staining of GLUT-3 in the myometrium of non-pregnant women (n=5). The staining was variable; none in one sample (A), low in two samples (B) and moderate in two samples (C).

Figure 5.7: Representative immunohistochemical staining of GLUT-3 in the myometrium of pregnant women. GLUT-3 staining intensity was more in non-diabetic (n=9) than diabetic (n=9) pregnant women.

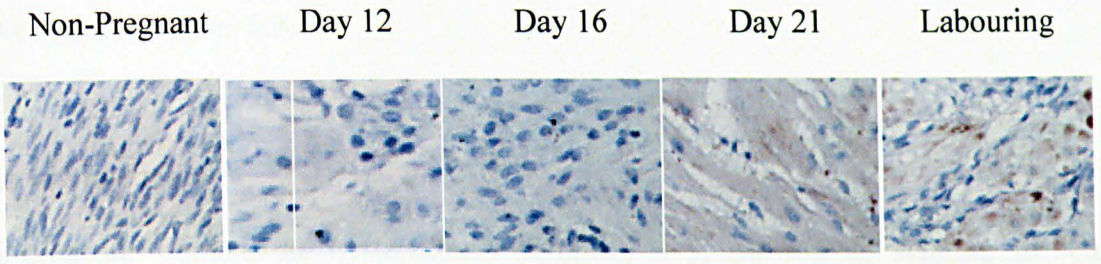


Figure 5.8: Immunohistochemical staining of GLUT-3 in the myometrium of rat. The staining of GLUT-3 was absent in non-pregnant, day 12 and day 16. Day 21 and labouring uterus stained with GLUT-3.

5.3.3 The expression of GLUT-4 myometrium

The immunostaining of GLUT-4 was perinuclear in the examined samples from human and rat. The staining was obvious in the positive control sample (skeletal muscle tissue) and absent in the negative controls, Figure 5.9.

Human myometrium

In non-pregnant women, the hysterectomy samples, n=5, showed no staining for GLUT-4 (Figure 5.10). On the other hand the number of positively stained cells in diabetic samples (37% ± 3, n=9), was significantly more compared to non-diabetic group (18 ± 5, n=9), Figure 5.11. The staining intensity scoring are summarised in table 5.5.

Rat myometrium

Rat myometrium showed no positive staining in non-pregnant sample. In addition, day 12, 16, 21 and labouring uterus had no positively stained cells in the myometrium. The n number is one for each, Figure 5.12.

Table 5.5: The GLUT-4 immunostaining scoring in the myometrium of non-diabetic (A) and diabetic (B) pregnant women at term. +ve = positive cells.

(A)

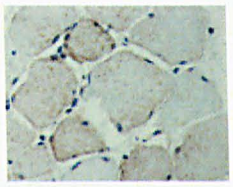
Non-diabetic samples	positive cells				Total cell number				Mean (+ve)
	a	b	c	Total	A	b	c	Total	
1	10	6	11	27	58	55	68	181	15%
2	3	2	0	5	43	47	54	144	3%
3	28	22	24	74	58	72	86	216	34%
4	10	11	6	27	89	83	60	232	12%
5	0	1	1	2	49	43	54	146	1%
6	22	17	17	56	80	83	104	267	21%
7	8	19	15	42	53	76	77	206	20%
8	35	47	49	131	83	108	100	291	45%
9	14	4	3	21	76	73	68	217	10%
Mean				**385**				**1900**	**20%**

(B)

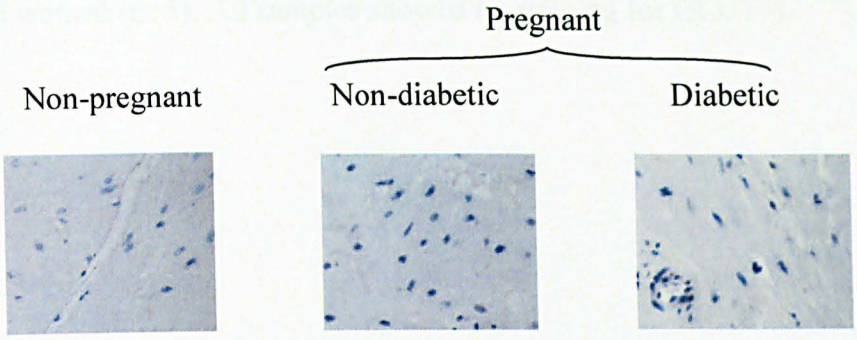
Diabetic samples	positive cells				Total cell number				Mean (+ve)
	a	b	c	Total	A	b	c	Total	
1	20	20	7	47	59	41	57	157	30%
2	30	32	20	82	59	66	70	195	42%
3	28	10	15	53	60	47	73	180	29%
4	44	24	22	90	101	87	78	266	34%
5	15	19	14	48	33	48	35	116	41%
6	40	37	29	106	82	69	68	219	48%
7	22	46	41	109	85	96	126	307	36%
8	15	18	19	52	32	35	43	110	47%
9	17	35	17	69	65	127	74	266	26%
Mean				**656**				**1816**	**36%**

(A) Skeletal muscle

Positive control



(B) Human myometrium



(C) Rat myometrium

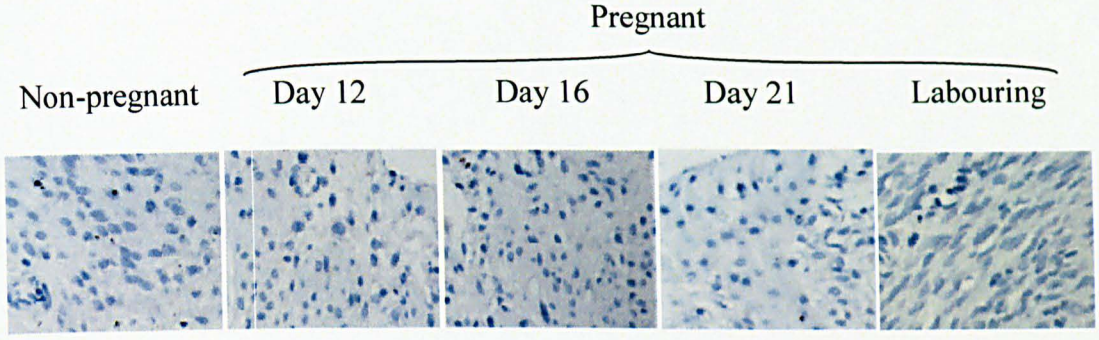


Figure 5.9: Immunohistochemical staining of GLUT-4 in the myometrium. The positive control (skeletal muscle) showed strong staining. The negative controls, (B) and (C), showed no staining.

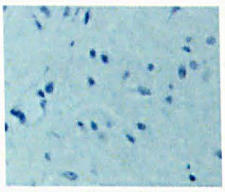
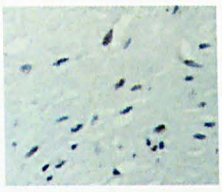


Figure 5.10: Representative immunohistochemical staining of GLUT-4 in the myometrium of non-pregnant women (n=5). All samples showed no staining for GLUT-4.

Non-diabetic



Diabetic

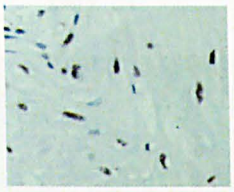


Figure 5.11: Representative immunohistochemical staining of GLUT-4 in the myometrium of pregnant women at term. The myometrium of diabetic (n=9) pregnant women expressed more GLUT-4 than non-diabetics (n=9).

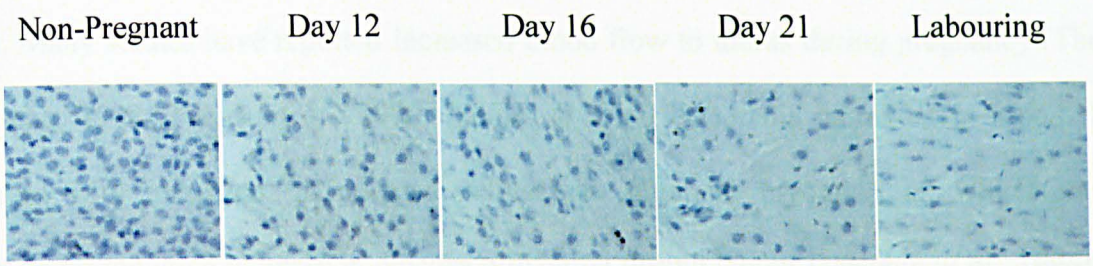


Figure 5.12: Immunohistochemical staining of GLUT-4 in the myometrium of rat. The staining of GLUT-4 was absent in non-pregnant and pregnant rat.

5.4 Discussion

Most mammalian cells depend on a continuous supply of glucose as an important source of energy. Many studies have reported increased blood flow to uterus during pregnancy (Thoresen & Wesche, 1988; Thaler *et al.*, 1990; Palmer *et al.*, 1992). This increased in blood flow is associated with increment in glucose delivery to pregnant myometrium, which could be contribute for the large increase in glucose utilization during labour (Maheux *et al.*, 1996). After it has been up taken into cytoplasm, excess glucose is stored in myometrium as glycogen (Hers, 1992; Braithwaite *et al.*, 1995). Several studies have shown that the glycogen amount in the myometrial of pregnant humans and animals is higher than that found in non-pregnant (Milwidsky & Gutman, 1983; Sakamoto *et al.*, 1987; Wedenberg *et al.*, 1990). This may indicate that there is increase in glucose uptake in the myometrium during pregnancy.

Uptake of glucose requires carrier proteins to mediate its transport into the cytosol, namely the glucose transporters. Glucose transporter 1, 3 and 4 are distributed in many tissues. It is not known if they are expressed in the myometrium and therefore, I examined their expression in myometrial cells.

I proposed that increased glucose transporter to the plasma membrane of the uterine muscle as well as skeletal muscle could be responsible for the large increase in glucose utilization during labor.

5.4.1 The expression of GLUT-1

The GLUT-1 transporter has a wide tissues distribution including smooth muscle of blood vessels and the endometrium of rats and humans (Nie *et al.*, 2000; Von Wolff *et al.*, 2003). In my current study I found that GLUT-1 is expressed in the myometrium of non-pregnant rat. Its expression is more prominent during pregnancy. The expression is increased gradually toward

the end of pregnancy. I found also that GLUT-1 is expressed more in myometrium of pregnant women at term compared to that seen in non-pregnant myometrium. These findings suggest that GLUT-1 is a major glucose transporter in rat and human myometrium. The increased intensity of GLUT-1 staining with progression of pregnancy may be explained by the increase in myometrial cell size, number or both with subsequent increase in glucose demand that necessitate more glucose transporters. In addition, the increased activity of individual myometrial cells during late pregnancy and labour may also stimulate increased expression of GLUT-1, to facilitate the uptake of more glucose to provide the needed energy.

I found also that GLUT-1 staining intensity is more in non-diabetic pregnant women at term compared to diabetic control. These results suggest that diabetes is interfering with GLUT-1 expression. The reduction of GLUT-1 expression may lead to inadequate glucose uptake, to supply the myometrial cells with the energy required for contractions. Therefore, the decrease of GLUT-1 expression may contribute to poor uterine contractions and an increase in caesarean sections seen in diabetic women.

5.4.2 The expression of GLUT-3 in the myometrium

The GLUT-3 transporter is expressed in tissues with high glucose demand such as brain and placenta (Gould *et al.*, 1991; Haber *et al.*, 1993). In this research I investigated the presence of this glucose transporter in another tissue with high glucose requirement namely myometrium as no previous studies have done so.

In rat myometrium I found that non-pregnant and day 12 and 16 did not express GLUT-3, while day 21 showed low and labouring uterus showed moderate staining intensity. These findings may indicate that GLUT-3 is not a major glucose transporter in myometrium of rats. As the

myometrium increase in size and number of its cells, it compensates by increasing GLUT-3 expression toward the end of pregnancy to meet the increased demand for glucose supply.

In non-pregnant women the staining intensity of GLUT-3 was variable with one sample showed no staining. The possible reasons for this variability were discussed above. In pregnant women at term the staining intensity of GLUT-3 was lower in diabetics compared with non-diabetics. This result is in agreement with previous studies which showed that GLUT-3 expression was lower in the placenta of diabetics compared to non-diabetics in late pregnancy (Sciullo *et al.*, 1997). My findings may suggest that GLUT-3 is not a major glucose transporter in human myometrium compared to GLUT-1 and its expression in pregnant women at term may occur to augment the delivery of glucose as a source of energy at this time. In addition, diabetes may interfere with GLUT-3 expression in myometrium.

5.4.3 The expression of GLUT-4 in myometrium

Insulin mediates the uptake of glucose in tissues expressing GLUT-4, such as skeletal muscle. Are myometrial cells insulin dependent for uptake of glucose? To answer this question I performed immunostaining of the myometrium. In my study I found that rat myometrium showed no staining of GLUT-4 neither in non-pregnant nor in different stages of pregnancy samples. Similarly, myometrial samples obtained from non-pregnant women showed no staining, too. However, samples from pregnant diabetic and non-diabetic women at term showed different levels of staining intensity. Diabetics expressed more GLUT-4 than non-diabetics.

These results indicate that GLUT-4 is not a major glucose transporter in the myometrium of non-pregnant rat and human. This suggests that insulin is not ordinarily required for glucose uptake into uterine myometrial cells, and that facilitative diffusion of GLUT-1 and GLUT-3 is sufficient to meet the metabolic requirement of the tissue. This is also consistent with *in vitro* data, where

physiological solution does not contain insulin, and contractile activity is supported. The exception to these conclusions is revealed by my data on term-pregnant diabetic women.

Insulin is known to induce GLUT-4 synthesis in insulin-sensitive cells such as adipocytes (Sargeant & Paquet, 1993). In many cases the myometrium of diabetics is exposed during pregnancy and labour to high levels of insulin to achieve glycaemic control (Balsells *et al.*, 2000; Lepercq *et al.*, 2008; Stenninger *et al.*, 2008). As more insulin is around in the blood of diabetics at term compared to non-diabetics, more GLUT-4 is expressed in response to insulin stimulation. In addition, insulin induces the translocation of GLUT-4 from intracellular stores to the plasma membrane resulting in increase in glucose transport (Shepherd & Kahn, 1999; Bryant *et al.*, 2002). However, it has been indicated that the uptake of glucose in skeletal muscle and adipose tissue via GLUT-4 is impaired in diabetics (Miura *et al.*, 2001). I found GLUT-4 immunostaining in myometrium of diabetics and to a lesser extent in non-diabetics pregnant women at term. It is simple to suggest that this is due to the high circulating (administered) insulin before delivery in diabetics (Kautzky-Willer *et al.*, 1997). Circulating insulin is also increased in non-diabetics during late pregnancy but in lower level than that in diabetics (Maheux *et al.*, 1996; Kautzky-Willer *et al.*, 1997). Physiological increase in insulin resistance arises in all women during the second half of pregnancy (Catalano *et al.*, 1991; Stanley *et al.*, 1998). However, pregnant diabetic women are more insulin resistant than pregnant non-diabetics and, hence, secreting (type 2) or are administered (type 1)more insulin (Kautzky-Willer *et al.*, 1997).

In summary, it seems that GLUT-1 is the major glucose transporter in myometrium of both rat and human. The reduction of GLUT-1 and GLUT-3 expression in pregnant diabetic women at term may participate in the poor uterine contractility which may contribute to high CS rate.

Although the expression of GLUT-4 was detected in diabetics, GLUT-4 appears not to be normally involved in glucose transportation in the myometrium.

Chapter 6

Preliminary investigation of the underlying mechanism of the changes in intracellular calcium seen with diabetics and in response to insulin

6.1 Introduction

Previous studies showed either impairment or no changes of the myometrial contractility in diabetic myometrium in pregnant and non-pregnant rats compared to non-diabetic controls (McMurtrie *et al.*, 1985; Franchi *et al.*, 1988; Jawerbaum *et al.*, 1996). There was no measurement of calcium transients in any of the studies. Other studies from several laboratories have demonstrated that other smooth muscles contractility is altered in experimental diabetes. However, the findings from these studies have not always been consistent (Owen & Carrier, 1980; Pfaffman *et al.*, 1982; MacLeod & McNeill, 1985; Sakai & Honda, 1987; Aihara & Sakai, 1989; Longhurst *et al.*, 1991).

It has been reported that insulin impairs smooth muscle contractions in rat uterus, trachea and human myometrium (Kuznetsova *et al.*, 2006). Insulin exerts an inhibitory effect on myometrial contractions of pregnant women, as I found in chapter 3. In contrary, Goldraij et al has previously demonstrated that insulin enhances the contractility of the myometrium in non-diabetic non-pregnant rats (1979, 1982). Insulin also induces muscle relaxation and interferes with agonist-induced calcium transients in VSM of human and animals (Kim & Zemel, 1993; Muniyappa *et al.*, 1997).

In this chapter, I investigated the effects of diabetes on L-type calcium channel activity using BAY K8644 and high KCl. In addition, I examined the effect of insulin on strips

stimulated with Bay K8644 to study the activity of L-type calcium channels. I also investigated the effects of insulin on potassium channels activity. Since membrane potassium conductance is largely responsible for determining excitability (Barany, 1996; Okawa *et al.*, 1999), I investigated weather insulin is capable of inhibiting the inhibitory effect of potassium channels blocker, TEA, and hence, induce muscle relaxation in strips stimulated by TEA. If insulin was acting through this mechanism then it can be suggested that insulin may induce myometrial relaxation via activating potassium channels, which lead to the hyperpolarisation of the myometrial cells with subsequent relaxation.

6.2 Methods

Details of the tissue preparation and measurement of calcium have been explained in chapter 2. Muscle strips were allowed to generate spontaneous contractions before the exposure to 40 mM KCl, 700 pM insulin, 10 mM TEA, 1 μM Bay K8644 or ethanol (0.01%). *In vitro*, voltage dependent L-type calcium channels can be activated either by direct stimulation with BAY K8644 or by membrane depolarization using high K^+ concentration. Table 2.1 and Table 2.2 give the medical details of non-diabetic and diabetic groups involved in this chapter's experiments. The patients numbers involved in this chapter are. 1) In response to Bay K8644; non-diabetics (45-49) vs diabetics (27-30). 2) The insulin plus Bay K8644 experiments; (non-diabetics; 45-49). 3) TEA experiments; (non-diabetics; 50-53). 4) In response to high KCl; non-diabetic (10-23) vs diabetics (3, 6, 7, 10, 11, 13, 14, 16, 17, 20-22, 25, 31).

In all experiments, results are expressed as mean ± SEM and *n* refers to the number of patients. Data were analyzed using the Student's *t* test and analysis of variance (ANOVA). Differences were taken as significant if *p* values were <0.05.

6.3 Results

6.3.1 Effect of diabetes on voltage dependent L-type calcium channels in the myometrium

The application of ethanol (the vehicle control of Bay K8644;) to spontaneously contracting myometrium (100%) has no significant effect on the amplitude of contractions; the mean of the amplitude was 91.3% ± 4, n=3, Figure 6.1. Application of 1μM Bay K8644 to both diabetic (n=4) and non-diabetic (n=4) samples induced marked increase in the total area under the curve (AUC) of tension and calcium transients. During the application of Bay K8644, a clear increase in baseline force and calcium was seen, indicating a substantial amount of calcium influx. However, diabetic samples showed smaller increments in AUC of force and calcium transients compared to non-diabetic samples. Compared with control force AUC (100%), the AUC were increased significantly to 353 ± 48% and 194 ± 36% in non-diabetic and diabetic groups respectively, after adding Bay K8644. The changes in calcium transients were significant and similar to changes in force contractions; 224 ± 36 and 124 ± 20 in non-diabetics and diabetics respectively. The difference in AUC of tension and calcium transients between the two groups was significant, Figure 6.2.

In response to 40 mM K^+, which applied for 2 minutes, strips from the myometrium of diabetic (n=14) women produced weaker contractions compared to non-diabetic (n=14) controls. The area under the curve (AUC) of contractions was significantly less in diabetics compared with non-diabetics; 3 a.u (1.2, 6) *vs* 5.4 a.u (3.2, 17.2). A parallel changes happened in calcium transients; 0.4 a.u (0.2, 0.9) vs 1.3 a.u (0.8, 4.2). The amplitude of contractions in diabetics and non-diabetics were 1.7 mN (0.7, 3.3) and 3 mN (1.2, 4), respectively, $p > 0.05$. The amplitude of calcium transients in diabetics; 0.3 mN (0.12, 0.7), was significantly smaller than that in non-diabetics; 0.9 mN (0.4, 1.3). The duration of contraction and calcium transients at 50% amplitude were 5.9 min (3.5, 10.3) and 7.7 min (5.3, 10.3) in non-diabetics and 3.5 min (2,3, 5.2)

and 3.5 min (2.4, 5.5) in diabetics; the differences were significant between the groups. Further, no apparent differences have been seen in the pattern of calcium transients traces between the groups. The time needed for calcium trace to reach the peak in diabetic patients was not significantly different compared with non-diabetics; 1.3 min (1.05, 2.25) *vs* 0.96 min (0.83, 2.02). However, the relaxation rate was significantly lower in diabetics, 2.5 min (1.8, 5.0) compared with non-diabetics, 7.0 min (4.9, 9.2), Figure 6.3.

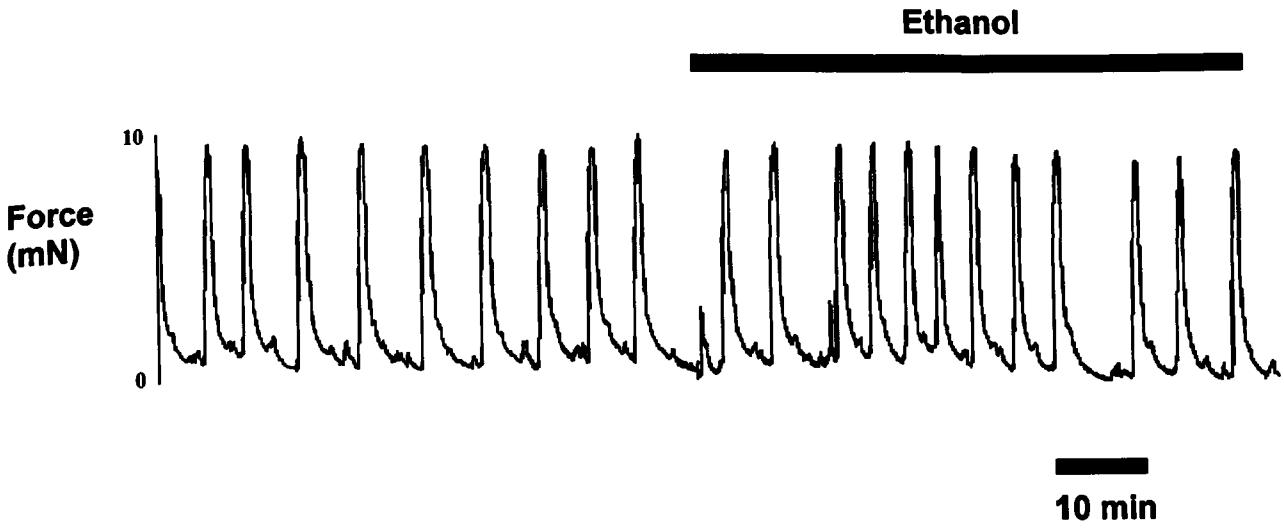


Figure 6.1: The effect of ethanol (0.01 %) on spontaneous uterine contraction. Ethanol has no significant effect on myometrial contraction.

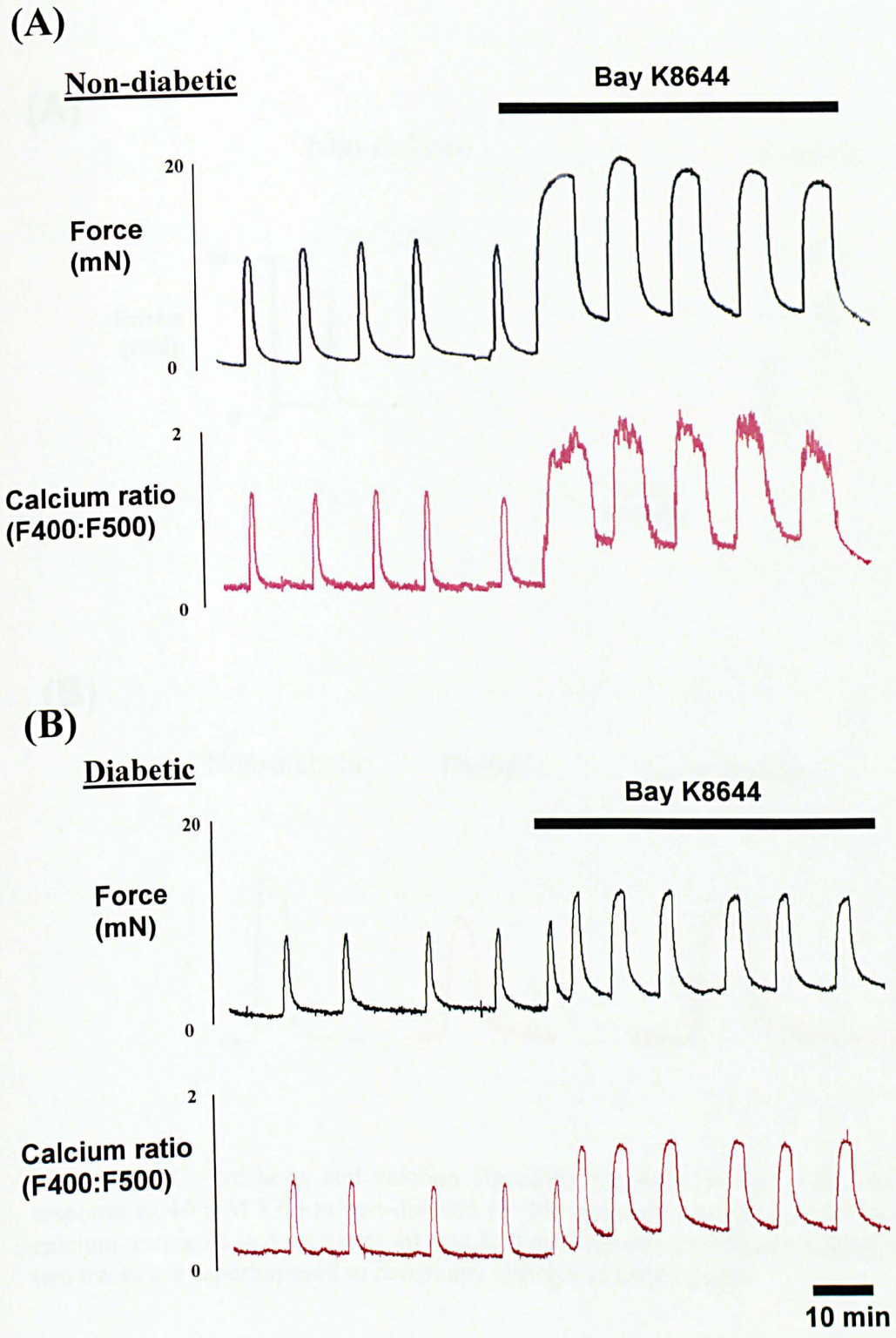
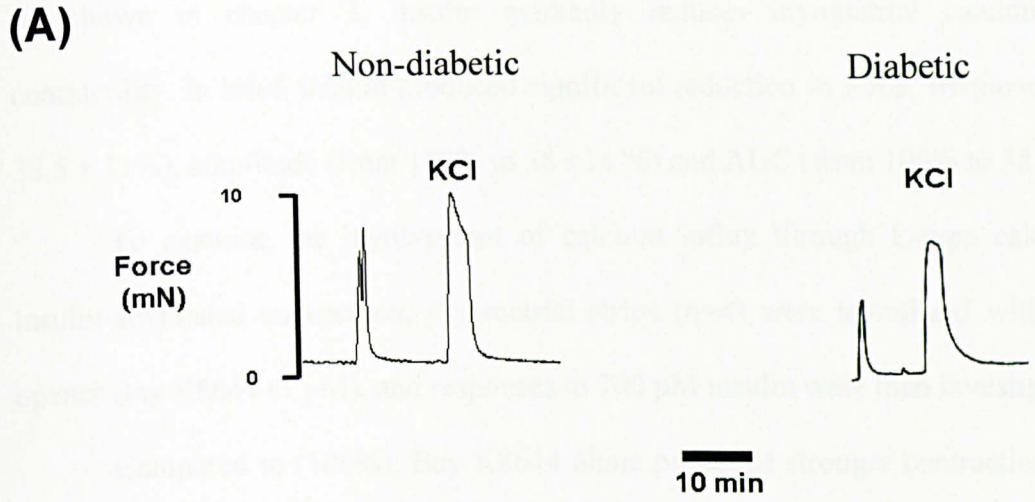


Figure 6.2: The effect of 1μM Bay K8644 on myometrial spontaneous contractions and calcium transients. 1μM Bay K8644 significantly increased the calcium transients and contractility in uterine muscle strips in both (A) non-diabetic (n=4) and (B) diabetic (n=4) samples. The effect, however, was significantly greater in non-diabetic strips.



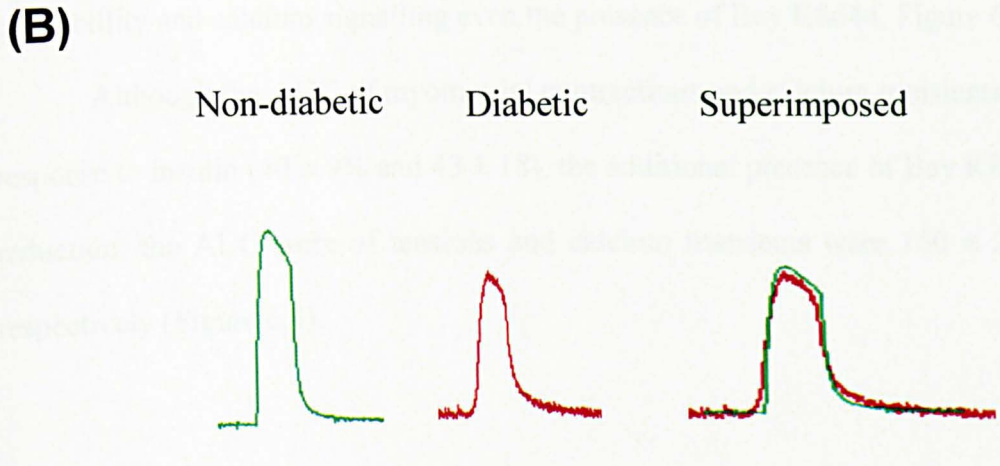


Figure 6.3: Contractions and calcium signalling response to high KCl. A): Contractions in response to 40 mM KCl in non-diabetic (n=14) and diabetic (15) samples. C): The changes in calcium transients in response to 40 mM KCl non-diabetic (n=14) and diabetic (15) samples. The two traces are superimposed to reveal any changes in traces shape.

6.3.2 Effect of insulin on voltage dependent L-type Calcium channels in the myometrium of pregnant women

As shown in chapter 3, insulin markedly reduces myometrial calcium signalling and contractility. In brief, insulin produced significant reduction in force; frequency, (from 100% to 38.5 + 11%), amplitude (from 100% to 38 +14 %) and AUC (from 100% to 38 ± 11), Figure 3.5.

To examine the involvement of calcium influx through L-type calcium channels on insulin-attenuated contraction, myometrial strips (n=4) were stimulated with calcium channel opener Bay K8644 (1 μM), and responses to 700 pM insulin were then investigated.

Compared to (100%), Bay K8644 alone produced stronger contraction than Bay K8644 in the presence of insulin; the AUC contractions and calcium transients were 382 ± 55% *vs* 156 ± 15% and 341 ± 65% *vs* 142 ± 10% respectively. Thus insulin is able to significantly decrease contractility and calcium signalling even the presence of Bay K8644, Figure 6.4.

Although the AUC of myometrial contractions and calcium transients (100%) reduced in response to insulin (40 ± 9% and 43 ± 18), the additional presence of Bay K8644 overcomes this reduction; the AUC were of tensions and calcium transients were 150 ± 20% and 140.9 ± 9 respectively (Figure 6.4).

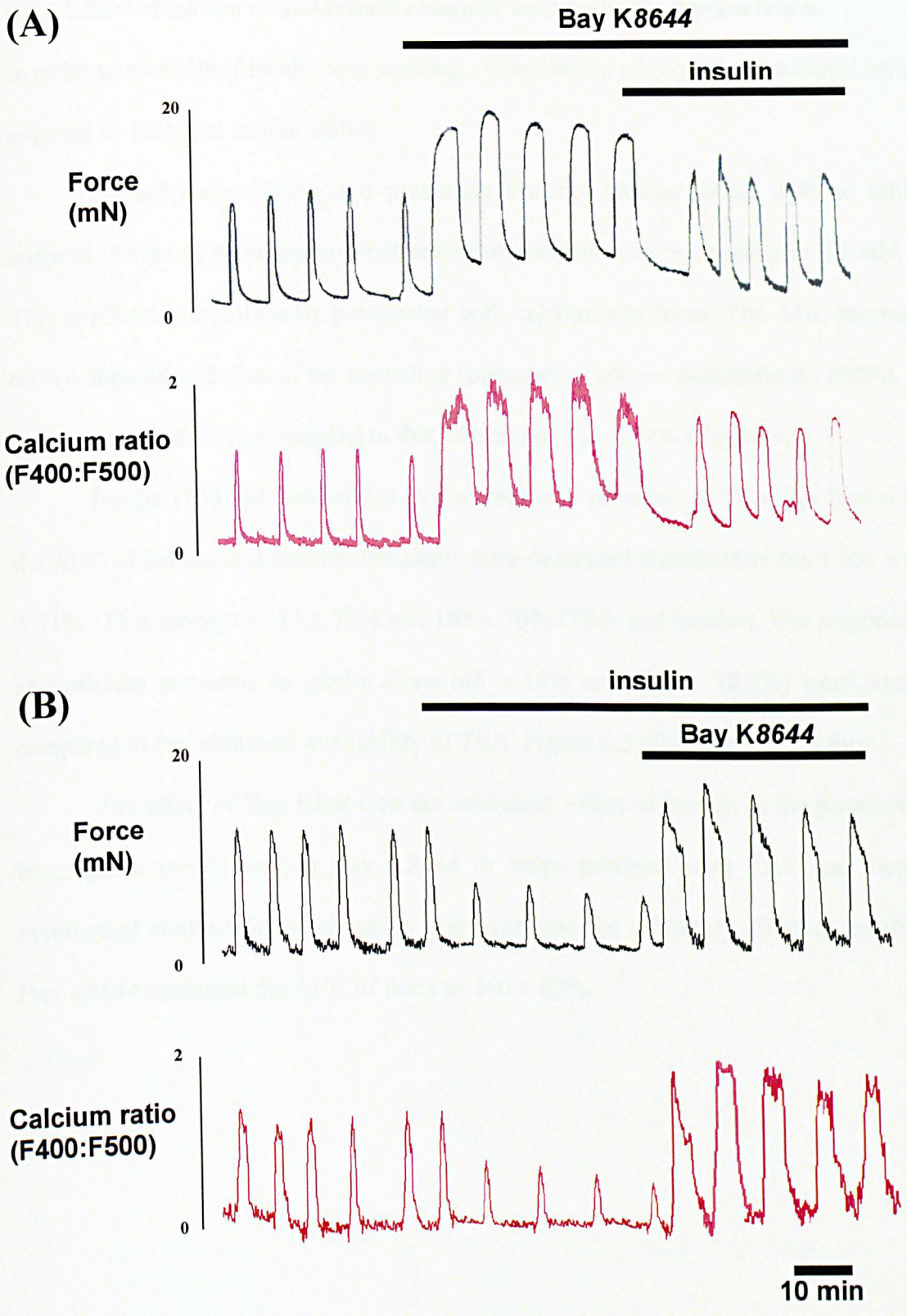


Figure 6.4: The effect of insulin on Bay K8644-induced myometrial contraction. A) Insulin (700 pM) significantly attenuated Bay K8644-induced myometrial contraction and diminished calcium transients. B) Calcium transients reduction and muscle relaxation in response to insulin were reversed by adding Bay K8644 (1 μM) to perfuse, (n = 4).

6.3.3 Effect of insulin on potassium channels activity in the myometrium

In order to establish if insulin was working via inhibition of potassium channels, strips were first exposed to TEA and insulin added.

Tetraethylammonium is a potassium channel blocker which able to inhibit outward currents. Strips of spontaneous contracting myometrium were exposed to 10 mM TEA (n=4). This application significantly potentiated both calcium and force. The AUC increased to 306 ± 60% compared with that of the preceding spontaneous control contractions (100%). The changes in calcium transient were parallel to that of tension; 331 ± 71% (Figure 6.5).

Insulin (700 pM) was added in the continued presence of TEA. As shown in figure 6.5, the AUC of tension and calcium transients were decreased significantly from 306 ± 60% and 331 ± 71% (TEA alone) to 157 ± 78% and 165 ± 76% (TEA and insulin). The responses of tensions and calcium transients to insulin alone (45 ± 10% and 49.6 ± 10.2%) were significantly less compared to the additional availability of TEA, Figure 6.5. The *n* number is four.

The effect of Bay K8644 on the inhibitory effect of insulin in the presence of TEA was investigated (n=4). Adding Bay K8644 to strips perfused with TEA and insulin increased myometrial contractility significantly and overcame the inhibitory effect of insulin, Figure 6.6. Bay K8644 increased the AUC of force to 240 ± 62%.

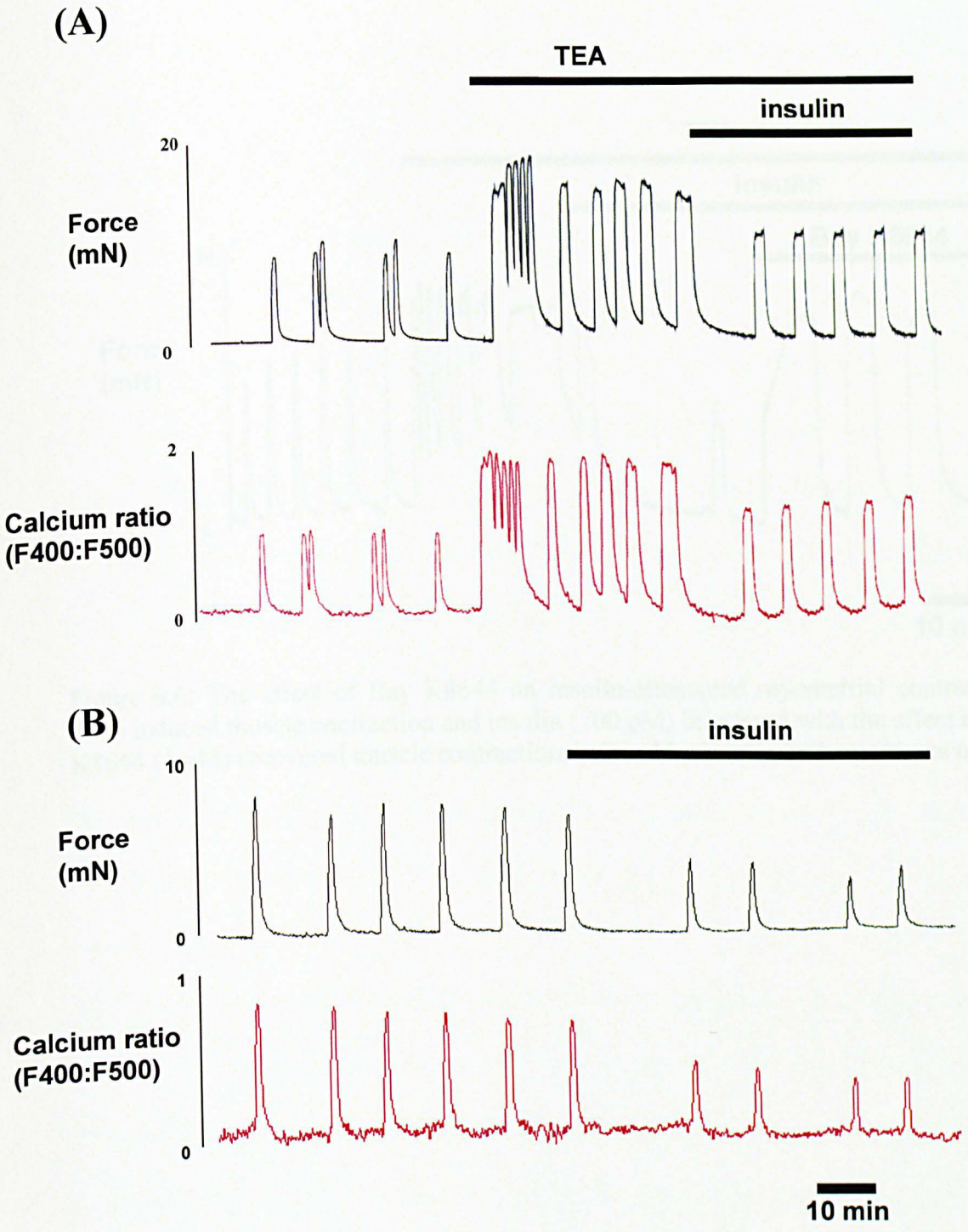


Figure 6.5: The effect of insulin in the presence of TEA (tetraethylammonium) on myometrium contraction. A) TEA (10 mM) induces calcium signalling and contractility in myometrium. In its continued presence, insulin (700 pM) decreased calcium transients and relaxes uterine muscle strips. B) Insulin induces muscle relaxation of the spontaneously contracting myometrium and reduces calcium transients. The strips used in both experiments were obtained from the same women (n = 4).

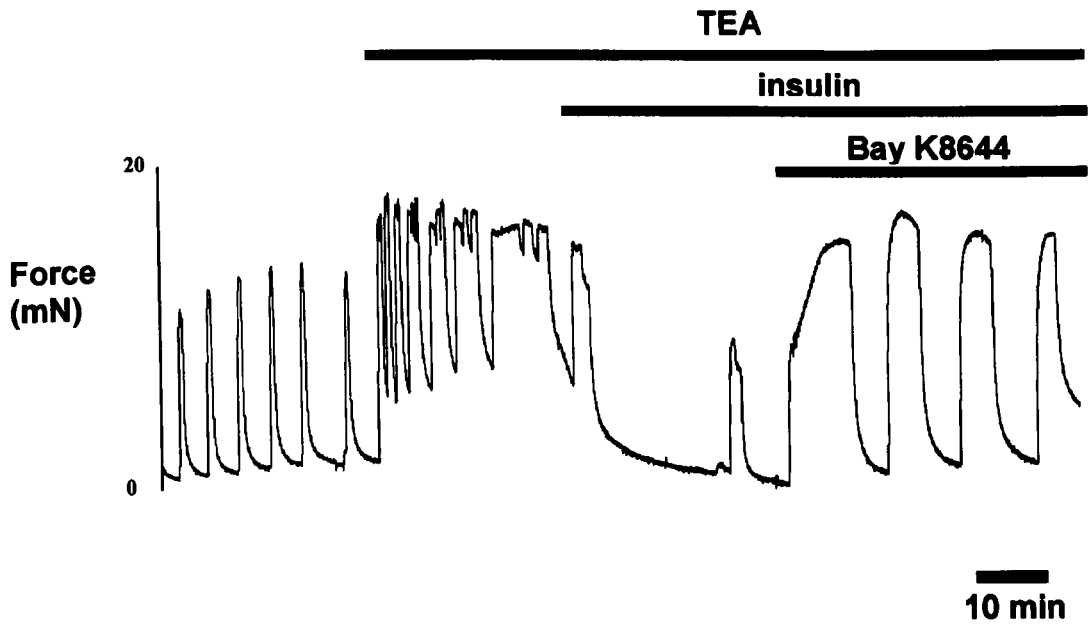


Figure 6.6: The effect of Bay K8644 on insulin-attenuated myometrial contraction. TEA (10 mM) induced muscle contraction and insulin (700 pM) interfered with the effect of TEA but Bay K8644 (1 μM) recovered muscle contractions relaxed by insulin in the presence of TEA; n=4.

6.4 Discussion

6.4.1 Effect of diabetes on voltage gated L-type calcium channel activity

Several studies worldwide have shown a higher CS rate in diabetic compared to non-diabetic women (Dunne *et al.*, 2003; Ehrenberg *et al.*, 2004; Jensen *et al.*, 2004). In chapter 3, I found that the spontaneous contractions were less in the uterus of diabetic pregnant women compared with non-diabetic controls and these effects were mirrored by changes in calcium transients. Poor uterine contractility in diabetics could contribute to a higher CS rate in diabetic women. Voltage gated L-type calcium channels provide a key route for calcium entry into the smooth muscle of the uterus, which is an important target for therapeutic intervention. As a first step in investigating the mechanisms leading to the decrement of force in diabetic myometrium, L-type calcium channels are an obvious starting point. To obtain an indication of the possible involvement of voltage gated L-type calcium channels, I compared the effect of Bay K8644 and KCl (40mM) on the spontaneous uterine contractility and calcium transients of diabetic and non-diabetic strips obtained from women delivered by caesarean section. If calcium channel opening in diabetics is impaired, then Bay K8644 might be expected to restore force to that seen in controls.

In agreement with previous studies, I have shown that the vehicle ethanol has no significant effect on uterine contractility (Burghardt *et al.*, 1999; Perusquia & Navarrete, 2005; Perusquia *et al.*, 2005; Kupittayanant *et al.*, 2009). In the present study I found, as expected, that the spontaneous uterine contractions and calcium transients were significantly increased in diabetics and non-diabetics in response to the application of Bay K8644. However, the effect was significantly lower in diabetics compared to non-diabetics. The force and calcium transients in diabetics were half that from non-diabetics. These results are consistent with studies showing that the contractile response to Bay K 8644 is diminished in smooth muscles of diabetic aortas in

rats (Carmines *et al.*, 1996; Hattori *et al.*, 1996), however, other studies showed that the responses were increased (White & Carrier, 1990; Inazu *et al.*, 1991). My current results suggest that the calcium entry through the calcium channels into the myometrial cells of diabetic women is impaired, and can not be restored by increasing channel open probability. This decreased Bay K8644 effects might caused by a reduction in conductance which determined by calcium inward current and/or number of L-type VDCC. It has been reported that the densities of L-type VDCC currents are reduced in VSM of diabetic rats using the patch-clamp technique (Wang *et al.*, 2000). Previous studies in the cardiomyocytes of diabetics also found calcium entry through L-type calcium channels is impaired (Wang *et al.*, 1995; Bracken *et al.*, 2004; Bracken *et al.*, 2006). Many other investigators have shown decreased intracellular calcium in diabetic cardiac cells (Lagadic-Gossmann *et al.*, 1996; Bracken *et al.*, 2006; Lu *et al.*, 2007). This could be due to defects in calcium channels function or expression (Bracken *et al.*, 2006; Pereira *et al.*, 2006; Lu *et al.*, 2007) leads to a decrement in L-type calcium current (Wang *et al.*, 1995; Bracken *et al.*, 2004; Pereira *et al.*, 2006). The reduction in calcium entry can explain the poor uterine contractility in diabetics. It seems that diabetes could significantly affect the activity or expression of L-type calcium channels in the myometrium.

As another way to examine the activity of L-type VDCC, diabetic and non-diabetic strips were exposed to high KCl to depolarize the membrane potential. I have shown in this Chapter that the response of contractions and calcium transients to High K^+ was significantly less in diabetics compared with non-diabetics. This finding comes in agreement with previous studies conducted in rats VSM (Carmines *et al.*, 1996; Hattori *et al.*, 1996). However, other studies have shown increased response to high KCl in VSM of diabetic rats (White & Carrier, 1990; Inazu *et al.*, 1991). My finding suggests that L-type VDCC response to depolarization and/or expression is reduced in the myometrium of diabetic women at late pregnancy. In addition, my results may

indicate that membrane potential is more hyperpolarized in diabetics. Further, changes in membrane excitability may take place due to changes in potassium channels activity or expression. Opening of potassium channels generates outward current that leads to membrane hyperpolarisation along with reduction in calcium entry through L-type VDCC, and hence, decrement of myometrial contractions (Barany, 1996; Okawa *et al.*, 1999; Wray *et al.*, 2003). Indeed, a previous study conducted in VSM has shown that the contractility and membrane depolarization by an elevation of the extracellular KCl concentration were diminished in diabetic rats (Kawasaki, 1997). However, other study has reported no changes in resting membrane potential and magnitude of KCl-induced membrane depolarization in VSM of diabetics compared to non-diabetic rats (Hattori *et al.*, 1996). Direct measurement of K^+ currents in VSM showed functional defect in voltage dependent potassium channels in diabetic rats (Bubolz *et al.*, 2005; Bubolz *et al.*, 2007).

Further studies are needed to clarify my current preliminary findings regarding the possible mechanisms of poor uterine contraction in the myometrium of diabetic pregnant women at term. These studies include the measurement of membrane potential, potassium outward current and L-type VDCC inward current using patch-clamp studies. The potassium channels blocker, TEA, can be used to compare the activity of potassium channels in non-diabetic and diabetic women. Polymerase change reaction (PCR) could also be used to examine the expression of calcium and potassium channels in diabetics.

6.4.2 Involvement of voltage gated L-type calcium channel in insulin-attenuated myometrial cells contractions

I showed in chapter 3 that insulin reduces muscle contraction in uterine smooth muscle and I pointed the possible involvement of voltage gated L-type calcium channels. As insulin affected

both diabetic and non-diabetic samples, I performed these experiments on non-diabetic samples. To figure that out, I exposed the myometrial strips obtained from pregnant women to calcium channels agonist, Bay K8644, then examined the effect of insulin on Bay K8644-induced muscle contraction.

My data have shown that insulin is able to reduce contractility even in the presence of Bay K8644-induced muscle contraction. When added to PSS containing Bay K8644, insulin decreased significantly the total area under the curve of the force and calcium transients induced by Bay K8644 by 50%. However, adding Bay K8644 to perfuse containing insulin reverses its inhibitory effect on contracting myometrium. My finding comes in agreement with a previous study that reported insulin exposure decreased the contractility of cavernous smooth muscle of rabbit that was elicited by Bay K8644 (Myung *et al.*, 2006). In VSM, insulin is able to inhibit calcium influx controlled by voltage-operated calcium channels in rats and dogs (Standley *et al.*, 1991; Kahn & Song, 1995; Muniyappa *et al.*, 1997). It has been shown that insulin increase (Maier *et al.*, 1999) , decreases (Nakipova *et al.*, 1987) or has no effect (Myung *et al.*, 2006) on L-type VDCC current in human atrial myocytes , dog myocardium and cavernous smooth muscle of rabbit, respectively. Further, KCl-induced smooth muscle contractions in human myometrium and rats uterus and trachea are decreased in response to insulin (Kuznetsova *et al.*, 2006). This may suggest that insulin hyperpolarizes the membrane potential, which leads to decreases of calcium entry through L-type VDCC. Indeed, several workers have demonstrated that in animal models that insulin hyperpolarizes the membrane potential in smooth muscle (Zemkova *et al.*, 1982), cardiac myocyte (Lantz *et al.*, 1980; Eckel & Reinauer, 1990) in skeletal muscle (Hazlewood & Zierler, 1967; Zierler & Rogus, 1981; Zierler & Moore, 1984; Iannaccone *et al.*, 1989; Li & Sperelakis, 1993).

Myometrial contractility is dependent mainly on calcium influx via the voltage-gated L-type calcium channels, and hence in turn, on membrane potential (Wray *et al.*, 2003). In the current study, I found that insulin attenuates the increased intracellular calcium transient by Bay K8644. Thus, my results suggest that insulin-attenuated myometrial contractions and calcium signalling may be as a result of the ability of insulin to induce hyperpolarisation of myometrial cell membrane or decreased the L-type VDCC calcium currents. These alterations in L-type calcium channels may contribute to poor uterine contractility and increase of CS in diabetic women who experienced elevated levels of insulin as part of their chronic disease. Further studies are needed to explain the current possible mechanisms; patch-clamping to assess the membrane potential and L-type VDCC current changes in response to insulin.

6.4.3 The role of potassium channels in insulin-attenuated myometrial cells contractions

As mentioned above, insulin reduces contraction in the smooth muscle of the myometrium. The possibility that potassium channels can modulate insulin-attenuated myometrial contractions was examined by determining strip contractile responses to insulin after incubating strips with the potassium channel blocker TEA. According to my data, TEA evoked significant rise in the tensions and calcium transients compared to preceding spontaneous contractions but insulin considerably attenuated the effect of TEA on myometrial contraction. However, the change in force and calcium transients in response to insulin alone is lower than that to TEA in the presence of insulin. The ability of insulin to decrease TEA-induced muscle contraction was reported previously in rabbit cavernous smooth muscle (Myung *et al.*, 2006). Furthermore, the potassium channel inhibitor TEA significantly attenuated the vasorelaxation response to insulin in porcine epicardial arteries (Hasdai *et al.*, 1998). Many researchers reported that insulin hyperpolarizes membrane potential of different types of muscles in animal models (Hazlewood

& Zierler, 1967; Zierler & Rogus, 1981; Zemkova *et al.*, 1982; Zierler & Moore, 1984; Iannaccone *et al.*, 1989; Li & Sperelakis, 1993), which may mediated by activation of potassium channels. Blocking of low-conductance Ca2+-activated potassium channels using apamin inhibited insulin-induced hyperpolarization in bovine retinal capillary pericytes (Berweck *et al.*, 1993). Dimitropoulou et al. gave evidence that potassium currents are reduced in smooth muscle cells from insulin-resistant rats (Dimitropoulou *et al.*, 2002). It has been reported that potassium channels outward current is decreased in cardiac myocyte of diabetic rats and this electrophysiological change was reversed by insulin (Xu *et al.*, 2002).

My finding suggests that insulin-attenuated myometrial contractions are mediated by activating potassium channels. In myometrium, potassium channels activation is associated with hyperpolarisation, which leads to a reduction in opening of L-type calcium channels and a drop of calcium entry, hence relaxation (Wray *et al.*, 2001; Wray, 2007). Thus, insulin-activated potassium channels may lead to a fall in calcium entry with subsequent reduction in muscle contraction. However, electrophysiological studies are needed to investigate the effect of insulin on the potassium channels current.

6.4.4 Other mechanisms may involved in insulin-attenuated myometrial contractions and calcium signalling in the myometrium

My data are preliminary and suggest future studies. It seems that insulin-attenuated uterine muscle contractions are mediated by decreasing of intracellular calcium through inhibiting L-type calcium channels and/or activating potassium channels. However, other mechanisms which govern intracellular availability may also mediate insulin-attenuated myometrial contractions.

In addition to its ability to inhibit potassium channels, TEA inhibits Na^+-K^+ pump and Na^+-K^+-ATPase activity in rat cardiac myocytes and mouse diaphragm muscle (Zemkova *et al.*,

1988; Peluffo *et al.*, 2004). Insulin is known to activate the Na^+-K^+-ATPase in VSM (Omatsu-Kanbe & Kitasato, 1990; Hundal *et al.*, 1992). In myometrium and VSM increased Na^+-K^+-ATPase activity will result in hyperpolarisation of the cell membrane, blocking of VDCC channels (Turi *et al.*, 1991; Kahn & Song, 1995). Thus, insulin could inhibit Na^+-K^+ pump in myometrium and this could be investigated.

In the myometrium plasma membrane Ca^{2+} ATPase activation leads to efflux of calcium (Wray, 2007). Insulin decreases intracellular calcium levels via stimulation of plasma membrane Ca^{2+}-ATPase–mediated Ca_i^{2+} efflux in rat VSM (Zemel *et al.*, 1992). Furthermore, it has been reported that insulin inhibits IP_3-induced Ca^{2+} release from intracellular stores (Saito *et al.*, 1993). Similar effects may take place in smooth muscle of uterus in response to insulin. So, insulin may decreases calcium influx or induces calcium efflux. However, Kahn & Song (1995) indicated that insulin-attenuated muscle contractions is mediated by calcium influx, but not by reducing of calcium release from internal stores or increasing calcium efflux .

In vascular smooth muscle cells (VSMC), insulin effects can be mediated by two different pathways of insulin signalling; the mitogen-activated protein kinase (MAPK) pathway and insulin receptor substrate-1 (IRS-1)-phosphatidyl inositol 3 kinase (PI3K)-Akt pathway (Hsueh & Law, 1999). In VSMC, insulin may inhibit signal transduction of vasoconstrictor agents acting on receptor-coupled G proteins and phospholipase C (Sowers, 1997). Moreover, it has been reported that insulin-induced VSMC relaxation is dependent on activation of Akt, also known as protein kinase B (PKB) or Rac (Lee & Ragolia, 2006). Akt protein is a downstream effector of PI3-kinase and its activation by insulin is mediated via tyrosine kinase activity of the insulin receptor, IRS-1, and IRS-2 (Sun *et al.*, 1991). Indeed, insulin activation of PI3-kinase results in phosphorylation and thereby activation of Akt. Several studies have shown that insulin induces relaxation of VSMC via stimulation of myosin phosphatase, decreasing the

phosphorylation of the myosin-bound regulatory subunit and inhibition of Rho kinase activity (Sun *et al.*, 1991; Kimura *et al.*, 1996; Begum *et al.*, 2000). These mechanisms lead mainly to calcium desensitization. In my thesis I found that the changes in calcium transients were parallel to those seen in force of contractions in response to insulin. Therefore, such mechanisms may not play a major role in insulin-induced myometrial relaxation. However, more in depth studies are needed to examine this.

6.4.5 Summary

In this chapter, I have shown that the reduction in calcium transients in myometrium of pregnant diabetic women may be due to the impairment of the activity and/or the expression of L-type calcium channels. This may contribute to poor uterine contractility seen with diabetic women at term, which may end pregnancy with caesarean section. My work has, also, shown that insulin-attenuated uterine muscle contractions and decreased calcium transients can be explained by modulation of L-type calcium channels and potassium channels activity. However, other mechanisms, which control calcium availability inside the cells, may be involved.

Chapter 7

Final discussion

7.1 Overview

In my thesis, my ultimate aim was to study the effects of diabetes on uterine contractility and to investigate the mechanisms by which it attenuated contractions. Understanding the metabolic changes that occur in the myometria of diabetic women at late pregnancy will help in the modulation of the labour and improve pregnancy outcomes. I have addressed my aims in this thesis. To the best of my knowledge no previous study has measured simultaneously the contractions and calcium transients in myometrium of diabetic pregnant women in pregnancy. My findings clearly demonstrated that diabetes decreased myometrial contractility of women at late pregnancy. However, I found that the myometrial contractions showed no differences in diabetics controlled with insulin or diet regardless of the achievement of targeted levels of glycaemic control. Calcium entry is the key for initiating myometrial contractions (Barany, 1996; Okawa *et al.*, 1999; Wray *et al.*, 2003). My data showed that calcium transients are diminished in diabetics, which may contribute to this poor diabetic contractility. As L-type calcium channels are the main source of calcium entry in the myometrium, defects in these channels functionality or expression may take place in the myometrium of diabetic women. Indeed, my work suggests that the open probability L-type calcium channels in response to Bay K8644 was less in diabetics which may give an indication that the conductance and/or number of L-type calcium channels are changed in response to diabetes. Diabetic myometrium showed a reduction in calcium transients and contractions in response to high KCl depolarization. This again suggests that L-type calcium channel entry is impaired in diabetics and that they are less

activated; however, defects in potassium channels can not be excluded. More work is needed to clarify if calcium and potassium currents and expression are impaired in diabetic myometrium, this could be done by using patch clamping and polymerase chain reaction techniques. Moreover, my data suggested that changes in the levels of glucose and insulin circulating in the blood of diabetics may also alter myometrial contractility.

Normally and during late pregnancy, insulin resistance and glucose intolerance increase, allowing more availability of glucose in circulation to go to the fetus (Ryan *et al.*, 1985; Buchanan *et al.*, 1990), hence, more insulin is secreted from the pancreas to overcome insulin resistance and facilitate the uptake of glucose into the cells. These changes are more prominent in diabetic women as more insulin resistance will lead to more increase of glucose in the circulation and more insulin is needed to reach the level of euglycaemia (Bowes *et al.*, 1996; Kautzky-Willer *et al.*, 1997). Therefore, I studied the effect of high insulin and glucose on uterine contractility. I found that both insulin and glucose are able to decrease calcium transients and myometrial contractions. As the calcium entry through L-type VDCC is mainly needed to initiate contractions, the reductions in calcium transients may due to the defect in L-type VDCC functions or/and expression. Further, I found that insulin interferes with oxytocin-induced myometrial contractions in both non-diabetic and diabetic samples.

To find out if insulin affects L-type VDCC, I investigated the effect of insulin with Bay K 8644 and TEA on myometrial contractions and calcium transients. I found that insulin decreased calcium transients and contractions even with the presence of Bay K 8644 and TEA. This may indicate that insulin directly or indirectly affects L-type VDCC. The ability of insulin to decreased myometrial contractions in the presence of TEA indicates that insulin hyperpolarizes the membrane potential and/or alters the potassium channel function and/or expression. These findings may also help explain the poor uterine contractility that is seen in

diabetics. However, these are preliminary data that need additional investigations to establish the involvements of L-type calcium and potassium channels in poor uterine contractility.

Both increase and decrease of glucose levels are affecting myometrial contractility in pregnant women at late pregnancy. My thesis data showed that high glucose decreased myometrial contractions and calcium transients equally in non-diabetic and diabetic samples. High glucose may interfere with calcium entry in the myometrium which takes place mainly through L-type VDCC. I reported that myometrium can carry on contracting in the absence of a direct glucose supply. However, diabetic myometrium contracted for a shorter time compared to non-diabetic. After three hours of contractions in 0-glucose solution, myometrium produced less amplitude and frequency compared to control contractions. Tissues from diabetic women were affected more than tissues from non-diabetics. These findings indicated that there is another source of energy driving the myometrial contractility in the absence of glucose; most likely glycogen, and this source is lower in diabetics. The glycogen levels in the strips left to contract until contraction disappeared in 0-glucose solution were lesser than control strips. Further, my data showed that glycogen stores are reduced in diabetics. Overall, these findings indicate that myometrium used glycogen as another source of energy in the absence of glucose supply and reduction of glycogen in diabetics may contribute to the weaker contractions seen in the absence of glucose. The reduction of glycogen stores in diabetics may due to the reduction of glucose uptake. I found that the staining intensity of GLUT1 and GLUT3 is increased with the progression of pregnancy; however, the intensity was less in diabetics at late pregnancy. Although, the staining intensity of GLU4 was more in diabetics, which may be in response to increased level of administered insulin, glucose uptake by GLUT4 may be defective.

The differences in force output in the uterine biopsies from women with diabetic pregnancy do not appear to be due to structural abnormalities. I found no obvious differences in

cellular abundance or orientation were seen at the light microscopic level, and there were no differences in the amount of muscle present in biopsies.

7.2 Clinical translations

According to my data, poor uterine contractility seen in diabetic pregnant women at late pregnancy may contribute to the high caesarean sections reported in diabetics (Dunne *et al.*, 2003; Ehrenberg *et al.*, 2004; Jensen *et al.*, 2004; Crowther *et al.*, 2005; Bell *et al.*, 2008). The reasons for these reductions in contractility may be mainly due to defects in calcium channels as a response to chronic exposure to high levels of insulin and glucose. During pregnancy, achieving euglycaemia in diabetic mothers is necessary to protect the fetus from the harmful effect of maternal hyperglycaemia (Schwartz & Teramo, 2000; Taylor *et al.*, 2002). If the mother experienced hyperglycemia during delivery, glucose crosses the placenta and reaches the fetus circulation where it responds by releasing more insulin in its own circulation (Melamed & Hod, 2009). After delivery, the baby will suffer of life threatening hypoglycaemia as the insulin level is extremely high compared to glucose (Schwartz & Teramo, 2000; Taylor *et al.*, 2002). For this reason insulin is administrated in a highly controlled manner peripartum to prevent fetal hyperinsulinaemia and neonatal hypoglycaemia. If more insulin is needed to reach the euglycaemia, the more inhibition of myometrial contractility may take place, and hence, a higher chance of dysfunctional labour and delivery of the baby by cesarean section. To avoid the effect of insulin and reach euglycaemia, using oral hypoglycaemic drugs may be an alternative solution. However, it is not known what the effect of these medications on myometrial contractility is.

As of 2008, metformin, from the biguanide class, and glyburide are two oral hypoglycaemic agents in the WHO Model List of Essential Medicines (WHO, 2007). Indeed, it is now widely acceptable to use oral hypoglycaemic agents as an alternative to insulin to control blood glucose in diabetics during pregnancy. Among these agents, glyburide (also known as glibenclamide), a second generation hypoglycemic sulfonylurea, is considered a safe drug to be

used to achieve euglycaemia during pregnancy in women with type 2 or gestational diabetes. Compared to metformin and other sulfonylureas, glyburide does not cross the placenta in significant amount (Elliott *et al.*, 1991; Langer *et al.*, 2000; Koren, 2001) and has no effect on the rate of fetal anomalies (Langer, 2002). It has been reported that using glyburide to achieve euglycaemia in patients with gestational diabetes is as effective as the use of insulin (Langer *et al.*, 2000).

Sulfonylureas act by inhibiting potassium ATP channels in the pancreas and other cells and this leads to depolarization of membrane potential and as a result, calcium channels open leading to an increase in calcium entry that stimulates insulin release (Groop *et al.*, 1987; Gedeon & Koren, 2006). Sulfonylureas work primary by enhancing insulin secretion, which suppresses hepatic glucose production; the main cause of fasting hyperglycemia commonly seen in diabetics (Groop *et al.*, 1987; Groop *et al.*, 1991). Glyburide also enhances insulin sensitivity in peripheral organs (DeFronzo & Simonson, 1984; Simonson *et al.*, 1984), therefore, less insulin will be needed for uptake of glucose and less glucose will be produced by liver. In the myometrium, glyburide bocks ATP-sensitive potassium channels (Cheuk *et al.*, 1993; Heaton *et al.*, 1993). It has been reported that *in vitro* glyburide has small effect on non-pregnant human myometrium (Cheuk *et al.*, 1993) or no effect on uterine contractility in non-pregnant or pregnant rats. So, this drug will not interfere with uterine quiescence usually seen until labour time (Heaton *et al.*, 1993). However, it is not known what the effect of glyburide on the contractility of laboring uterus in non-diabetic and diabetic women is. Glyburide may mediate the increase in calcium transients in diabetics and, hence, the contractility during labour. By this way, a drug like glyburide could maintain the glycaemic control and enhance myometrial contractility. If glyburide does not enhance contractility, it is at least not inhibiting it comparing to insulin, which I found to inhibit the contractility of uterus. However, several studies reported

that in women diagnosed with gestational diabetes there was no significant difference in the rate of cesarean sections in group treated with insulin compared with those treated with glyburide (Langer *et al.*, 2000; Bertini *et al.*, 2005; Jacobson *et al.*, 2005; Ramos *et al.*, 2007). Logically, in women diagnosed with gestational diabetes, this drug will be used in the third trimester but with type 2 diabetes it will be used from day one of pregnancy if not before. To avoid any possibility of fetal anomalies, which could occur mainly in first trimester; the organogenesis period, I think introducing glyburide after the first trimester in type 2 diabetic pregnant women would be safer. Insulin is an essential mode of treatment of type 1 diabetes during pregnancy.

The standard management of prolonged labour is augmentation with oxytocin in the form of syntocinon that is administered to further stimulate uterine contractions (Mozurkewich, 2006). I found that insulin interfered with oxytocin-induced myometrial contractions. Therefore, the recommended doses of oxytocin used during delivery in case of diabetic women may need to be revised to overcome the inhibitory effect of insulin.

7.3 Future work

My thesis findings showed that myometrial contractility and calcium transients are decreased in diabetic women at late pregnancy and in response to insulin and glucose. My work suggests further studies to understand the mechanisms better whereby diabetes affects myometrial contractions and calcium signalling. The preliminary investigations pointed out the involvement of calcium and potassium channels. The patch clamp technique would allow me to study the effects of diabetes on membrane potential and current of potassium and calcium channels in diabetic pregnant women at late pregnancy. In addition, the effect of insulin and glucose on membrane potential and current of potassium and calcium channels in non-diabetic and diabetic pregnant women at late pregnancy can be clarified by patch clamp studies. I would also undertake polymerase chain reaction (PCR) technique to study the changes in the number of calcium and potassium channels in diabetics and in response to insulin and glucose in pregnant women at late pregnancy. I would also suggest a study of the activities of PMCA and NCX in diabetics at late pregnancy and if their activities changes in response to insulin and glucose. I found also that insulin decreases oxytocin-induced myometrial contractions and calcium transients. Further studies are needed to investigate the potential mechanisms.

Further work will be needed to study the expression of GLUT1, 3 and 4 in the myometrium during different stage of pregnancy and in non-pregnant diabetic and non-diabetic women and rats using western blotting and PCR techniques.

Although blood glucose concentrations are elevated in diabetic, I found that glycogen storage is less in the myometrium of diabetics and this may be due to a defect in glycogen synthase enzyme which is involved in glycogen synthesis. It would be of an interest to examine if the expression or function of this enzyme is altered in diabetics.

Many other future studies can be conducted, for instance, the effect of diabetes on expression of caveolae, gap junctions, contractile proteins. I would also study the effect of estrogen and progesterone on myometrial contractility and calcium signalling in diabetic women at late pregnancy.

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Appendix

Copies of ethics letters, patient information sheet and consent forms

NHS Research Ethics Committee
APPLICATION FORM

This form should be completed by the Chief Investigator, after reading the Guidance Notes.
See Glossary for clarification of different terms in the application form.

Short title and version number (maximum 70 characters - this will be inserted as header on all forms):
Diabetes and myometrial contractility

Name of NHS research ethics committee to which application for ethical review is being made:
Liverpool Research Ethics Committee

Project Reference number from above REC:

Submission Date:

PART A

A1. Title of Research

Full title: Diabetes and myometrial contractility

Key words: Diabetes,

A2. Chief Investigator

Title:	Dr.	Forename/Initials:	Siobhan	Surname:	Quenby
Post:	Senior Lecturer/Honorary Consultant				
Qualifications	MBBS,Bsc, MD, MRCOG, CCST				
Organisation:	University of Liverpool				
Address:	Ist Floor Liverpool Women's Hospital				
	Crown Street				
	Liverpool				
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Email:	squenby@liv.ac.uk				
Telephone:	0151 702 4100				
Fax:	0151 702 4024				

A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with application.

A3. Proposed Study Dates and Duration

Start date: 01/09/2005

End date: 01/09/2010

Duration Years 5 Months

A4. Primary purpose of the research: *(Tick as appropriate)*

- ☐ Commercial product development and/or licensing
- ☒ Publicly funded trial or scientific investigation
- ☒ Educational qualification
- ☐ Establishing a database/data storage facility
- ☐ Other

A5. Tick the box if your research:

- ☐ involves testing a medicinal product
- ☐ involves investigating a medical device
- ☐ involves additional radiation above that required for clinical care
- ☒ involves using stored samples of human biological material (e.g. blood, tissue)
- ☒ involves taking new samples of human biological material
- ☐ involves only patient records or data, with no direct patient contact
- ☐ involves prisoners or others in custodial care
- ☐ Involves adults unable to consent for themselves through physical or mental incapacity
- ☒ Has the primary aim of being educational (e.g. a student project, or a project or research necessary for a postgraduate degree or diploma)

A6. Do you consider that this research falls within the category where there is no need to appoint a Principal Investigator at each site? YES ☐ NO ☑

Advice can be found in the Guidance Notes on this topic. Some studies do not require further consideration of site-specific issues by local research ethics committees, but will still require approval to proceed from the host organisation(s).

A7. What is the principal research question/objective? *(Must be in language comprehensible to a lay person.)*

Why do women with diabetes have labours that frequently end in Caesarean section?

A8. What are the secondary research questions/objectives? *(If applicable. Must be in language comprehensible to a lay person.)*

What is the CS rate in diabetic women in LWH? Why is the CS rate so high in these women? Is there evidence for poor myometrial contractility? "Does Ca signalling differ in the myometrium from diabetic women?Is the cholesterol and phospholipid content of cell membranes different in the myometrium of women with diabetes inn pregnancy?"Does hyperglycaemia an/or hyperinsulinaemia directly effect myometrial contractility?"Do diabetic women have a different response to myometrial hypoxia and lactic acidosis than normal women?

A9. What is the scientific justification for the research? What is the background? Why is this an area of importance? *(Must be in language comprehensible to a lay person.)*

The aim of this project is to apply advances in the understanding of the physiology of uterine muscle (myometrial) contractility to the problem of high Caesarean (CS) rates in women with diabetes mellitus.

Many studies have reported high CS rates in women with diabetes mellitus in pregnancy, rates of 10-56% have been quoted. CS in women with pregestational diabetes mellitus and gestational diabetes mellitus pregnancies are associated with significant maternal morbidity, wound infection, an increased risk of thrombosis and post-partum haemorrhage. Thus despite the fact that CS in diabetic women is a high risk procedure there is a lack of understanding as to how to prevent these CS.

In order to be able to develop strategies to prevent CS, it is important to understand factors that contribute to the high CS rate. The high rates of CS could be due to fetal distress because of associated utero-placental insufficiency, large babies or poor myometrial contractility. In a Dutch study 51% of emergency CS in diabetic women were performed for prolonged labour thus poor myometrial contractility may contribute to the high CS rate in diabetic women. Further evidence to implicate poor myometrial contractility is that post partum haemorrhage was six times more common in diabetic women.

Recently, we have found a reason for the high rate of Caesarean section in obese women. The myometrium from obese women contracts poorly and this poor myometrial contractility was mediated by a direct effect of cholesterol. However, other factors may affect myometrial contractility in diabetes. During labour the myometrium is subjected to intermittent hypoxia during contractions that are strong enough to decrease myometrial blood supply. We have found that in women contacting poorly and having a CS there is a myometrial acidosis sufficient to decrease uterine contractility. Furthermore we have found that the myometrium from some women is more susceptible to myometrial acidosis than others. Thus the next hypothesis we will investigate is whether the myometrium from women with diabetes is more susceptible to the effects of lactic acidosis. We will also used tests to see why any change in susceptibility to acidosis occurs.

A10. Give a brief synopsis/summary of methods and overview of the planned research, including a brief explanation of the theoretical framework which informs it. It should include a brief description of how prospective research participants and concerned communities (not necessarily geographical) from which they are drawn have been consulted over the design and details of the research.
(Where appropriate a flow chart or diagram should be submitted separately. It should be clear exactly what will happen to the research participant, how many times and in what order.)

This section MUST be completed in language comprehensible to a lay person. Do NOT simply reproduce the protocol.

Clinical study
What is the CS rate in diabetic women in LWH? Why is the CS rate so high in these women? Is there evidence for poor myometrial contractility?
We wish to study the computerized and paper records on the labours of all women with diabetes mellitus who have delivered in the Liverpool Women's Hospital over the last ten years. We will note the following details:
Regarding the labour; The type of delivery, the records of the uterine contractions, the amount of blood lost, the length of labour
Regarding the fetus; weight, apgar score, cord blood gases
Regarding the mother; Diabetes severity -insulin requirement, HBA1C levels, serum lipid levels, body mass index.
The results will then be analysed statistically.

Laboratory study
Biopsies (1x0.5x0.5cm) will be taken from the usual incision in the uterus after delivery of the baby.
Women with Diabetes Mellitus in pregnancy will be approached in clinic and ask if they would consent to myometrial biopsy should the require CS.
Controls; Similar projects are already occurring in non-diabetic women having both elective and emergency CS these will constitute control groups.

Experiments
Does Ca signalling differ in the myometrium from diabetic women?
Pieces of myometrium will be taken to the physiological laboratories dissected, loaded with indicators that determine calcium and pH changes. Strips of myometrium are then allowed to contract and the force of the contractions and calcium changes are simultaneously measured. We will compare the characteristics of the contractility of the myometrium in women with diabetes to that we have already studied in women without diabetes.
Is the cholesterol and phospholipid content of cell membranes different in the myometrium of women with diabetes in pregnancy?
We will use the methods we have already established fro obese women to measure these.
Does hyperglycaemia an/or hyperinsulinaemia directly effect myometrial contractility?
The myometrium will be bathed in hyperglycaemia and hyperinsulinaemic Krebs solution and the effect on contractility and calcium signalling will me assessed as described above.
Do diabetic women have a different response to myometrial hypoxia and lactic acidosis than normal women?
Myometrial force and contraction frequency will be studied in samples from women with diabetes whilst their samples are continuously superfused at different myometrial pH. Intracellular pH will be measured in this group and in this way we will also calculate buffering power, as well as responses to pH alteration.
Does the lactate dehydrogenase isoenzyme differ in diabetic women having CS compared to normally labouring women with a the same BMI?
Tissue samples will be homogenised and LDH isoenzyme quantified by electrophoresis using a commercially available Titan Gel LD isoenzyme system
Is there a difference in glycogen and triglyceride storage in diabetic women having CS compared to normal women?
Stored glycogen and lipids will be assessed in biopsies from each group of women using standard biochemical methodology.
Is there and difference in Na, K-ATPase isoforms in women with diabetes in pregnancy?
RT-PCR will be performed using human , and specific primers. Isoform-specific antibodies (1-3, 1-3 and) will be used for immunohistochemistry.

A11. Will any intervention or procedure, which would normally be considered a part of routine care, be withheld from the research participants? YES ☑ NO ☐

If Yes, give details and justification:

Patients will be asked if they would donate a small part of their myometrium during their Caesarean section. We have done this on more than 700 women in the Liverpool Women's Hospital (with LREC approval) over the last 10 years. There have been no adverse effects and nearly all women have agreed to this and are happy to help with the research.

A12. Will the research participants receive any clinical intervention(s) or procedure(s) including taking samples of human biological material over and above that which would normally be considered a part of routine clinical care? YES ☑ NO ☐

Additional intervention	Average number per patient		Average time taken (mins/hrs /days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.
	Routine Care	Research		
Other tissue/bodily sample		1	1 minute	myometrial biopsy(1x0.5x0.5cm) from the upper edge of the incision made to deliver the baby taken after the baby is born. This is undertaken by the SPR/consultant performing Caesarean section. All SPR's are trained to do this during elective Caesarean section lists.

Do you need another page? YES ☐ NO ☐

A13. Will the research participant be subject to any non-clinical research-related intervention(s) or procedure(s)?
(These include interviews, non-clinical observations and use of questionnaires.)
YES ☐ NO ☑

A14. Will individual or group interviews/questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews/group discussions, or use of screening tests for drugs)?
YES ☐ NO ☑

The Information Sheet should make it clear under what circumstances action may be taken.

A15. What is the expected total duration of participation in the study for each participant?

The biopsy only takes one minute but women will be consented several months before their Caesarean section in antenatal clinic.

A16. What are the potential adverse effects, risks or hazards for research participants either from giving or withholding medications, devices, ionising radiation, or from other interventions (including non-clinical)?

There is a theoretical risk that taking the sample will lead to increased intra-operative blood loss however, we have taken about 700 such biopsies over 10 years for various projects (all with LREC approval) and no adverse effects have occurred.

A17. What is the potential for pain, discomfort, distress, inconvenience or changes to lifestyle for research participants?

The patients are all under local (spinal/epidural) or general anaesthetic thus they do not experience pain.

A18. What is the potential for benefit for research participants?

I find that the women enjoy being involved in this type of research and are impressed that some doctors are keen to do research aimed at understanding their pathology.

A19. What is the potential for adverse effects, risks or hazards, pain, discomfort, distress or inconvenience to the researchers themselves? *(if any)*

none

A20. How will potential research participants in the study be *(i)* identified, *(ii)* approached and *(iii)* recruited?
Give details for cases and controls separately if appropriate:

Clinical study
Patients will be identified from the computerized hospital (Meditech) database and data will be taken from both the Meditech system and from the labour and delivery case notes.

Laboratory study
All women attending the medical disorders clinic with a diagnosis of diabetes mellitus will be approached and ask if they would consent to myometrial biopsy should they require CS. We aim to obtain samples from 50 women with diabetes, 60 such women are seen per year and 50% have CS therefore this should be easily achievable over three years.

A21. Where research participants will be recruited via advertisement, give specific details.

Not Applicable ☑

A22. What are the principal inclusion criteria? *(Please justify.)*

Women with diagnosed by the endocrinologist as having diabetes mellitus in pregnancy.

A23. What are the principal exclusion criteria? *(Please justify.)*

A24. Will the participants be from any of the following groups? *(Tick as appropriate.)*

- ☐ Children under 16
- ☐ Adults with learning disabilities
- ☐ Adults who are unconscious or very severely ill
- ☐ Adults who have a terminal illness
- ☒ Adults in emergency situations
- ☐ Adults with mental illness (particularly if detained under Mental Health Legislation)
- ☐ Adults suffering from dementia
- ☐ Prisoners
- ☐ Young Offenders
- ☐ Adults in Scotland who are unable to consent for themselves
- ☐ Healthy volunteers
- ☐ Those who could be considered to have a particularly dependent relationship with the investigator, e.g. those in care homes, medical students
- ☐ Other vulnerable groups

Justify their inclusion:

Some women will require an emergency Caesarean Section however, these will have been consented in the non-emergency environment of the antenatal clinic

A25. Will any research participants be recruited who are involved in existing research or have recently been involved in any research prior to recruitment?

What steps will you take to find out? YES ☐ NO ☐ Not Known ☑

The Liverpool Women's Hospital research committee continuously monitors how many women are recruited to each project to protect the women from being in too many projects. This is a dynamic situation and is kept under continuous review.

A26. Will informed consent be obtained from the research participants?

YES ☑ NO ☐

Give details of who will take consent and how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material.

If participants are to be recruited from any of the potentially vulnerable groups listed in A24, give details of extra steps taken to assure their protection. Describe the arrangements to be made for obtaining consent from a legal representative.

The consent will be done by Dr. Siobhan Quenby (Consultant Obstetrician), or a PHD student trained by her to take consent. The student will be closely supervised by both Siobhan Quenby and Steve Walkingshaw.

Copies of the written information and all other explanatory material should accompany this application.

A27. Will a signed record of consent be obtained?

YES ☐ NO ☐

Attach a copy of the information sheet to be used, with a version number and date.

If NO, please justify:

A28. How long will the participant have to decide whether to take part in the research?

Up to 20 weeks

A29. What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

Interpreter will be available

A30. What arrangements are in place to ensure participants receive any information that becomes available during the course of the research that may be relevant to their continued participation?

not applicable

A31. Does this study have or require approval of the Patient Information Advisory Group (PIAG) or other bodies with a similar remit? *(see Guidance Notes)*

YES ☐ NO ☑

A32a. Will the research participants' General Practitioner be informed that they are taking part in the study?

YES ☐ NO ☑

Explain why not:

This will be of no relevance to the General Practitioner

A33. Will individual research participants receive any payments for taking part in this research?

YES ☐ NO ☑

A34. Will individual research participants receive *reimbursement of expenses* or any other *incentives or benefits* for taking part in this research?

YES ☐ NO ☑

A35. What arrangements have been made to provide indemnity and/or compensation in the event of a claim by, or on behalf of, participants for *negligent* harm?

Liverpool Women's Hospital Trust Clinical Negligence Scheme

Please forward copies of the relevant documents.

A36. What arrangements have been made to provide indemnity and/or compensation in the event of a claim by, or on behalf of, participants for *non-negligent* harm?

Not applicable

Please forward copies of the relevant documents.

A37. How is it intended the results of the study will be reported and disseminated? *(Tick as appropriate)*

- ☒ Peer reviewed scientific journals
- ☒ Internal report
- ☒ Conference presentation
- ☐ Other publication
- ☐ Submission to regulatory authorities
- ☐ Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- ☐ Written feedback to research participants
- ☐ Presentation to participants or relevant community groups
- ☐ Other/none e.g. Cochrane Review, University Library

A38. How will the results of the research be made available to research participants and communities from which they are drawn?

A patient information sheet with the result on will be made available on request

A39. Will the research involve any of the following activities at any stage (including identification of potential research participants)? *(Tick as appropriate)*

- ☒ Examination of medical records by those outside the NHS, or within the NHS by those who would not normally have access
- ☐ Electronic transfer by magnetic or optical media, email or computer networks
- ☐ Sharing of data with other organisations
- ☐ Export of data outside the European Union
- ☐ Use of personal addresses, postcodes, faxes, emails or telephone numbers
- ☐ Publication of direct quotations from respondents
- ☐ Publication of data that might allow identification of individuals
- ☐ Use of audio/visual recording devices
- ☐ Storage of personal data on any of the following:
  - ☐ Manual files including X-rays
  - ☐ NHS computers
  - ☐ Home or other personal computers
  - ☐ University computers
  - ☐ Private company computers
  - ☐ Laptop computer

Further details:

For the clinical study we wish to examine the medical records of women with diabetes mellitus, only section of the notes that record labour details will be studied.

A40. What measures will be put in place to ensure confidentiality of personal data? Give details of whether any encryption or other anonymisation procedures will be used and at what stage:

The details of the labours will be extracted from the computer data base and from case records in such away as to not include patients names or addresses.

All biopsies will be labeled with a study code when used in the laboratory and will hence be anonymous. The consent forms recording patients names will be stored in a locked draw. A

A41. Where will the analysis of the data from the study take place and by whom will it be undertaken?

Analysis will take place in the departments of physiology, reproductive and developmental medicine and veterinary science in the university of Liverpool. This will be undertaken by Dr. Siobhan Quenby, a PHD student, Professor Susan Wray
y.

A42. Who will have control of and act as the custodian for the data generated by the study?

Siobhan Quenby

A43. Who will have access to the data generated by the study?

Siobhan Quenby, A PHD student, Professor Susan Wray, Steve Walkingshaw.

A44. For how long will data from the study be stored? 10 Years [ ] Months

Give details of where they will be stored, who will have access and of the custodial arrangements for the data:

Data will be stored in the department of Reproductive and Developmental Medicine, Liverpool Womens Hospital. Siobhan Quenby will have access to the data.

A45. How has the scientific quality of the research been assessed? *(Tick as appropriate)*

- ☐ Independent external review
- ☐ Review within a company
- ☐ Review within a multi-centre research group
- ☒ Internal review (e.g. involving colleagues, academic supervisor)
- ☐ None external to the investigator
- ☐ Other, e.g. methodological guidelines

If you are not in possession of any referees or other scientific critique reports relevant to your proposed study, justify and describe the review process and outcome. If review has been undertaken but not seen by the researcher, give the details of the body which has undertaken the review:

A copy of any referees' comments or other scientific critique reports relevant to the proposed research must be enclosed with the application form.

A46. Has similar research on this topic been done before?

YES ☐ NO ☑

A47. Have all existing sources of evidence, especially systematic reviews, been fully considered?

Please give details of search strategy used: YES ☑ NO ☐

A48. What is the primary outcome measure for the study?

The contribution of poor myometrial contractility to the Caesarean section rate of diabetic women in labour in Liverpool Women's Hospital.

A49. What are the secondary outcome measures? *(If any)*

The reason for this poor myometrial contractility as determined by a series of laboratory experiments

A50. How many participants will be recruited? How many of these participants will be in a control group?

180 women will be asked if they would consent to the study in order to provide at least 50 myometrial samples. With an estimated 50% CS rate only 90 will be eligible for biopsies then the obstetrician will forget to take many of these despite consent.

A51. Has the size of the study been informed by a formal statistical power calculation?

YES ☐ NO ☑

A52. Has a statistician given an opinion about the statistical aspects of the research?

YES ☐ NO ☑

A53. Describe the statistical methods and/or other relevant methodological approaches (e.g. for qualitative research) to be used in the analysis of the results. Give details of the methods of randomisation process to be used if applicable:

Clinical study
Crude odds ratios (ORs) and 95% confidence intervals (CIs) will be calculated initially to determine which factors predict the need for emergency CS. Then a multiple logistic regression analysis will be used to evaluate the influence of risk factors and $P < 0.05$ two-sided will be taken as significant.

Laboratory
Each laboratory experiment will be repeated on myometrium from at least ten different women. Professor Wray's laboratory are world leaders in the techniques that will be used. From experience with similar projects ten samples are enough to show large differences in all the parameters listed.

A54. Where will the research take place? *(Tick as appropriate)*

- ☑ UK
- ☐ Other States in the European Union
- ☐ Other States in the European Economic Area
- ☐ Other

A55. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK, the European Union or in the European Economic Area?

YES ☐ NO ☑

A56. In how many and what type of host organisations (NHS or other) in the UK is it intended that the proposed study will take place?

Indicate the type of organisation by ticking the box and give approximate numbers if known:

		Number of organisations
☒	Acute teaching NHS Trusts	1
☐	Acute NHS Trusts	
☐	NHS Community and/or Primary Care Trusts	
☐	NHS Trusts providing Mental Healthcare	
☐	NHS Care Trusts	
☐	Social Care Organisations	
☐	Prisons	
☐	Independent hospitals	
☐	Educational establishments	
☐	Independent research units	
☐	Other (give details)	

A57. What arrangements are in place for monitoring and auditing the conduct of the research?

All investigators meet on a monthly basis to assess progress in the project.

Will a data monitoring committee be convened? YES ☐ NO ☑

What are the criteria for electively stopping the trial or other research prematurely?

This project involve s series of laboratory experiments that will be modified as the project progresses

A58. Has funding for the research been secured? YES ☑ NO ☐

Give details of funding organisation(s), amount secured and duration:

i
Organisation: PHD Studentship Egyptian ministry of health
Address:
Postcode:
UK Contact:
Telephone: Fax:
Email:
Amount: £ £10,000.00 Duration: 36 months

ii
Organisation:
Address:
Postcode:
UK Contact:
Telephone: Fax:
Email:
Amount: £ Duration: months

iii
Organisation:
Address:
Postcode:
UK Contact:
Telephone: Fax:
Email:
Amount: £ Duration: months

A59. Has the funder of the research agreed to act as sponsor as set out in the Research Governance Framework?
YES ☐ NO ☑ Not Known ☐

Has the employer of the Chief Investigator agreed to act as sponsor of the research?
YES ☑ NO ☐ Not Known ☐

Give details of the organisation who will act as the sponsor of the research:

UK Contact:
Title: Forename/Initials: Surname:
Organisation:
Address:
Postcode:
Telephone: Fax:
Email:

A copy of documentation indicating that the organisation has accepted the role of sponsor should be enclosed if the sponsor is not the main funder, the Chief Investigator's employer, or an NHS body hosting the research.

A60. Has any responsibility for the research been delegated to a subcontractor?

YES ☐ NO ☑

A61. Will individual *researchers* receive any personal payment over and above normal salary for undertaking this research?

YES ☐ NO ☑

A62. Will individual *researchers* receive any other benefits or incentives for taking part in this research?

YES ☐ NO ☑

A63. Will the host organisation or the researchers' department(s) or institution(s) receive any payment or benefits in excess of the costs of undertaking the research?

YES ☐ NO ☑

A64. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share-holding, personal relationship etc.) in the organisation sponsoring or funding the research that may give rise to a possible conflict of interest?

YES ☐ NO ☑

A65. Other relevant reference numbers if known *(give details and version numbers as appropriate):*

Applicant's/organisation's own reference number, e.g. R&D (if available):
Sponsor's/protocol number:
Funder's reference number:
International Standard Randomized Controlled Trial Number (ISRCTN):
European Clinical Trials Database (EUDRACT) Number:
Project website:

A66. Other key investigators/collaborators *(all grant co-applicants should be listed)*

i
Title: Prof Forename/Initials: SW Surname: Wray
Post: Head of Department of Physiology
Qualifications: PHD
Organisation: University of Liverpool
Address: Department of Physiology
University of Liverpool
Telephone: 0151 7945329
Fax:
Postcode: Email: s.Wray@liv.ac.uk

ii
Title: MR Forename/Initials: S A Surname: Walkingshaw.
Post: Consultant Obstetrician
Qualifications: MD
Organisation: Liverpool Women's Hospital Trust
Address: Crown street
Liverpool
Telephone: 0151 7089988
Fax:
Postcode: L8 7SS Email: squenby@liv.ac.uk

iii
Title: Forename/Initials: Surame:
Post:
Qualifications:
Organisation:
Address:
Telephone:
Fax:
Postcode: Email:

iv
Title: Forename/Initials: Surname:
Post:
Qualifications:
Organisation:
Address:
Telephone:
Fax:
Postcode: Email:

v
Title: Forename/Initials: Surname:
Post:
Qualifications:
Organisation:
Address:
Telephone:
Fax:
Postcode: Email:

If there are more than 5 collaborators, please enter at end of section or attach a further sheet.

A67. If the research involves a specific intervention, (e.g. a drug, medical device, dietary manipulation, lifestyle change, etc.), what arrangements are being made for continued provision of this for the participant (if appropriate) once the research has finished?

Not Applicable ☒

Summary of Ethical Issues

A68. What do you consider to be the main ethical issues or problems which may arise with the proposed study, and what steps will be taken to address these?

Their are no ethical issues in this case

A69. Do you need to add further information about certain questions in Part A?

YES ☐ NO ☑

Student Page

A70. Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/degree:	PHD
Name of educational establishment:	University of Liverpool
Name and contact details of education supervisor:	Prof Sue Wray email s.wray@liv.ac.uk Siobhan Quenby squenby@liv.ac.uk

A71. Declaration of Supervisor

I have read and approved both the research proposal and this application for ethical review. I undertake to fulfil the responsibilities of a supervisor as set out in the Research Governance Framework for Health and Social Care. I can confirm on behalf of my academic institution that any necessary indemnity or insurance arrangements are in place.

Signature: .....

Date:

Print Name: Siobhan Quenby

A one-page summary of the supervisor's CV should be submitted with the application

PART B: Section 4 - Use of Existing Stored Samples

1. What samples and how many will be included in the study?

2. What tests/techniques will be carried out on the samples?

3. How will samples be labelled/identified?

Indicate if samples can be considered to be "identified", "coded", "de-identified", "anonymised" or "anonymous", and at what stage identifiers are removed. (See Guidance Notes for definitions):

4. Has specific consent been obtained previously to use stored samples for this purpose?

YES ☐ NO ☐

Give details or explain why consent has not, or is not being, obtained:

5. Does the research involve the analysis or use of genetic material from human biological materials?

YES ☐ NO ☑

6. Would it be possible to link the results of any genetic analysis back to individuals?

YES ☐ NO ☑

7. Is it intended to link the results of any genetic analysis back to individuals?

YES ☐ NO ☑

8. If the samples are from an established tissue bank, give the name and location of the tissue bank, the organisation responsible for it and the name of its manager.

9. Signature of the tissue bank manager.

I confirm that I have read this research proposal. I agree to the use of samples from this tissue bank for the purposes stated in this application.

Signature: .....

Date:

Print name:

PART B: Section 5 - Use of newly obtained Human Biological Materials

1. What samples will be collected and/or analysed, and by whom will they be collected?

Myometrial samples from 50 women with diabetes in pregnancy who require a Caesarean section.

2. Are samples taken solely for research purposes (or are they a by-product of those taken primarily for clinical purposes i.e. surplus to clinical requirements)?

Yes they are solely for research purposes.

3. How will samples be labelled/identified?

Indicate if samples can be considered to be "identified", "coded", "de-identified", "anonymised" or "anonymous", and at what stage identifiers are removed. (See Guidance Notes for definitions.)

Samples will be coded as soon as they reach the physiology laboratory. The consent forms with patient names on them will be stored in a locked draw in a secure room in the department of physiology. A key that enables samples to be decoded will also be stored in these secure facilities.

4. Give details of where the sample(s) will be stored, for how long, who will have access and of the custodial arrangements.

The samples will be stored in the physiology department of the University of Liverpool for a maximum of 5 years Siobhan Quenby will have access to these samples an be responsible for them

5. Will the research participant retain any rights to the sample(s)?

YES ☑ NO ☐

Give details. If the sample is a gift, this must be clear in the information sheet. What will happen to samples if a participant withdraws from the study?

If the patients withdraws from the study the sample will be destroyed

6. Is it known how the samples will be used in the future?

YES ☐ NO ☑

7. Does the research involve the analysis or use of genetic material from human biological materials?

YES ☑ NO ☐

8. Would it be possible to link the results of any genetic analysis back to individuals?

YES ☐ NO ☑

9. Is it intended to link the results of any genetic analysis back to individuals?

YES ☐ NO ☑

PART B: Section 7 - Declaration

- The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
- I undertake to abide by the ethical principles underlying the Declaration of Helsinki, and Good Practice Guidelines on the proper conduct of research.
- If the research is approved I undertake to adhere without unagreed deviation to the study protocol, the terms of the full application of which the main REC has given a favourable and any conditions set out by the main REC in giving its favourable opinion.
- I undertake to inform the main REC of any changes in the protocol, and to submit annual reports setting out the progress of the research.
- I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
- I understand that research records/data may be subject to inspection for audit purposes if required in future.
- I understand that personal data about me as a researcher in this application will be held by the relevant RECs and their operational managers and that this will be managed according to the principles established in the Data Protection Act.

Signature: .....

Date:

Print Name: Siobhan Quenby

1. Do you need to add further information about certain questions in Part B?

YES ☐ NO ☑

ENSURE THAT YOU COMPLETE AND SIGN THE FORM, AND ENCLOSE ALL RELEVANT ADDITIONAL DOCUMENTS.

NHS Research Ethics Committee
APPLICATION FORM
PART C: SITE-SPECIFIC ASSESSMENT

This form should be completed by the Principal Investigator for each site (see glossary)

Part C should be completed and sent with relevant enclosures to each NHS Research Ethics Committee or Research & Development (R&D) department which needs to consider site-specific issues. Consult the application procedure on the COREC website.

The data in this box is populated from Part A.

Name of NHS Research Ethics Committee to which application for ethical review is being made:

Liverpool Research Ethics Committee

Project Reference number from above REC:

Name of site NHS REC responsible for SSA:

SSA reference (for REC office use only):

Questions C1, C4, C5, C6, C7, C8 and C13a correspond to questions A1, A2, A65, A10, A12, A13 and A29 on the main application form respectively and will populate automatically:

C1. Title of Research. *(Populated from A1)*

Full title: Diabetes and myometrial contractility

Key words: Diabetes,

C2. Who is the Principal Investigator for this study at this site?

Title: Dr. Forename/Initials: SM Surname: Quenby

Post: Senior Lecturer/Honorary Consultant

Qualifications: MD MBBS BSC CCST

Organisation: University of Liverpool

Address: Ist Floor
Liverpool Womens Hospital
Crown Street

Postcode: L8 7SS

Email: squenby@liv.ac.uk

Telephone: 0151 708 9988

Fax: 0151 702 4024

A copy of a current CV (maximum 2 pages of A4) for the Principal Investigator(s) must be submitted with application.

C3. Indicate the number of trials/projects within the organisation that the local Principal Investigator has been involved with in the previous 12 months: 10

How many are still current (active or recruiting)? 10

Give details of other members of the local research team responsible to the local Principal Investigator

i Title: Forename/Initials Surname:
Position:
Qualifications:
Role in the research team:

ii Title: Forename/Initials Surname:
Position:
Qualifications:
Role in the research team:

iii Title: Forename/Initials Surname:
Position:
Qualifications:
Role in the research team:

If there are more members of the local research team, details should be provided at question C18 or on an attached sheet.

C4. Chief Investigator. *(Populated from A2)*

Title: Dr. Forename/Initials: Siobhan Surname: Quenby
Post: Senior Lecturer/Honorary Consultant
Qualifications: MBBS,Bsc, MD, MRCOG, CCST
Organisation: University of Liverpool
Address: 1st Floor Liverpool Women's Hospital
Crown Street
Liverpool
Postcode: L8 7SS
Email: squenby@liv.ac.uk
Telephone: 0151 702 4100
Fax: 0151 702 4024

C5. Other relevant reference numbers if known: *(Populated from A65)*

Applicant's/organisation's own reference number, e.g. R&D (*if available*):
Sponsor's/protocol number:
Funder's reference number:
International Standard Randomized Controlled Trial Number:(ISRCTN):
European Clinical Trials Database (EUDRACT) Number:
Project website:

C6. Give a brief synopsis/summary of methods and overview of the planned research. This should include a brief description of how prospective research participants and concerned communities (not necessarily geographical) from which they are drawn have been consulted over the design and details of the research. *(Where appropriate a flow chart or diagram should be submitted separately. It should be clear exactly what will happen to the research participant, how many times and in what order.) (Populated from A10.)*

Clinical study
What is the CS rate in diabetic women in LWH? Why is the CS rate so high in these women? Is there evidence for poor myometrial contractility?
We wish to study the computerized and paper records on the labours of all women with diabetes mellitus who have delivered in the Liverpool Women's Hospital over the last ten years. We will note the following details:
Regarding the labour; The type of delivery, the records of the uterine contractions, the amount of blood lost, the length of labour
Regarding the fetus; weight, apgar score, cord blood gases
Regarding the mother; Diabetes severity -insulin requirement, HBA1C levels, serum lipid levels, body mass index.
The results will then be analysed statistically.

Laboratory study
Biopsies (1x0.5x0.5cm) will be taken from the usual incision in the uterus after delivery of the baby.
Women with Diabetes Mellitus in pregnancy will be approached in clinic and ask if they would consent to myometrial biopsy should the require CS.
Controls; Similar projects are already occurring in non-diabetic women having both elective and emergency CS these will constitute control groups.

Experiments
Does Ca signalling differ in the myometrium from diabetic women?
Pieces of myometrium will be taken to the physiological laboratories dissected, loaded with indicators that determine calcium and pH changes. Strips of myometrium are then allowed to contract and the force of the contractions and calcium changes are simultaneously measured. We will compare the characteristics of the contractility of the myometrium in women with diabetes to that we have already studied in women without diabetes.
Is the cholesterol and phospholipid content of cell membranes different in the myometrium of women with diabetes in pregnancy?
We will use the methods we have already established fro obese women to measure these.
Does hyperglycaemia an/or hyperinsulinaemia directly effect myometrial contractility?
The myometrium will be bathed in hyperglycaemia and hyperinsulinaemic Krebs solution and the effect on contractility and calcium signalling will me assessed as described above.
Do diabetic women have a different response to myometrial hypoxia and lactic acidosis than normal women?
Myometrial force and contraction frequency will be studied in samples from women with diabetes whilst their samples are continuously superfused at different myometrial pH. Intracellular pH will be measured in this group and in this way we will also calculate buffering power, as well as responses to pH alteration.
Does the lactate dehydrogenase isoenzyme differ in diabetic women having CS compared to normally labouring women with a the same BMI?
Tissue samples will be homogenised and LDH isoenzyme quantified by electrophoresis using a commercially available Titan Gel LD isoenzyme system
Is there a difference in glycogen and triglyceride storage in diabetic women having CS compared to normal women?
Stored glycogen and lipids will be assessed in biopsies from each group of women using standard biochemical methodology.
Is there and difference in Na, K-ATPase isoforms in women with diabetes in pregnancy?
RT-PCR will be performed using human , and specific primers. Isoform-specific antibodies (1-3, 1-3 and) will be used for immunohistochemistry.

C7. Will the research participants receive any clinical intervention(s) or procedure(s) including taking samples of human biological material over and above that which would normally be considered a part of routine clinical care? *(Populated from A12)* YES ☑ NO ☐

Additional intervention	Average number per patient		Average time taken (mins/hrs /days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.
	Routine Care	Research		
Other tissue/bodily sample		1	1 minute	myometrial biopsy(1x0.5x0.5cm) from the upper edge of the incision made to deliver the baby taken after the baby is born. This is undertaken by the SPR/consultant performing Caesarean section. All SPR's are trained to do this during elective Caesarean section lists.

C8. Will the research participant be subject to any non-clinical research-related intervention(s) or procedure(s)?
(These include interviews, non-clinical observations and use of questionnaires.) (Populated from A13)

YES ☐ NO ☑

C9a. Give the name of the NHS or other organisation with which the PI holds the necessary contract (substantive or honorary) to undertake the research at this site:

Liverpool Women's Hospital

C9b. Give the name of the research site for which the PI is responsible, if different from the above. (The site may be a whole organisation, an individual unit, or a consortium):

University of Liverpool

C9c. Give the name and contact details for the Research Governance lead for the research site:

Title:	Dr.	Forename/Initials:	L	Surname:	Webster
Address:	First Floor				
	Liverpool Womens Hospital			Telephone:	0151 7089988
	Liverpool			Fax:	0151 702 4024
Postcode:	L8 7SS	Email:			

C10. Specify the location(s)/department(s) within the NHS or other organisation where the research will take place.

Department of Reproductive and Developmental Medicine
Department of Physiology

C11. How many research participants/samples is it anticipated will be recruited/obtained from this organisation in total?

180

C12a. Give details of who will be responsible for obtaining informed consent locally, their qualifications and relevant expertise and training in obtaining consent for research purposes:

Dr. Siobhan Quenby and MR Steve Walkingshaw. consultant obstetricians

C12b. Explain what local arrangements will be made for obtaining the consent of a "legal representative" on behalf of a participant with physical or mental incapacity who is unable to consent for himself:

NOT APPLICABLE

C13a. What local arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English? *(e.g. translation, use of interpreters etc.)*(Populated from A29)

Interpreter will be available

C13b.What local arrangements have been made to meet these requirements (where applicable)?

The trust provides interpreters where necessary

C14. In addition to informing the GP (if required), what arrangements have been made to inform those responsible for the care of the research participants in the host care organisation of their involvement in the research?

All consultant involve in the care of women with diabetes in pregnancy within the trust will be informed

C15. Are the facilities and staffing available locally adequate to perform any necessary procedures or interventions required for the study, and to deal with any unforeseen consequences of these? *(This should include consideration of procedures and interventions in both control and intervention arms of a study.)*

YES ☑ NO ☐

Give the information necessary to justify your answer:

There is consultant cover to delivery suite at all times in the case of any complications arising from Caesarean section

C16a. Give brief details of a contact point where participants may obtain further information about the study.

Siobhan Quenby email squenby@liv.ac.uk
telephone number 0151 7089988

C16b. What is the contact point for potential complaints by research participants?

Liverpool Women's Hospital complaints procedure.

C16c. Is there a local source where potential participants can obtain independent information about being involved in a research study?

yes from midwives.

C16d. Please specify the headed paper to be used for the participant information sheet.

Liverpool Womens Hospital trust

C17. If any extra support might be required by research participants as a result of their participation, what local arrangements are being made to provide this?

not applicable

C18. Do you need to add further information about certain questions in Part C?

YES ☐ NO ☑

Part C: Declaration

- The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
- I undertake to abide by the ethical principles underpinning the Declaration of Helsinki, and Good Practice Guidelines on current proper conduct of research.
- If the research is approved I undertake to adhere without unagreed deviation to the study protocol, the terms of the full application of which the main REC has given a favourable and any conditions set out by the main REC in giving its favourable opinion.
- I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Controller.
- I understand that research records/data may be subject to inspection for audit purposes if required in future.
- I understand that personal data about me as a researcher in this application will be held by the relevant RECs and their operational managers and that this will be managed according to the principles established in the Data Protection Act.

Signature of the local Principal Investigator* **Signature** .....

Date: (dd/mm/yyyy)

Print Name: Siobhan Quenby

** The Chief Investigator should sign where there is no local Principal Investigator for the research locality.*

PART C IS NOW COMPLETE AND SHOULD BE SUBMITTED to the NHS Research Ethics Committee or NHS organisation conducting site-specific assessment

9 November 2005

Dr S Quenby
Consultant Obstetrician
Liverpool Women's Hospital
Crown Street
Liverpool
L8 7SS

Dear Dr Quenby

Full title of study: Diabetes and myometrial contractility in pregnancy
REC reference number: 05/Q1505/114

Thank you for your letter responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		22 August 2005
Investigator CV	Dr S Quenby	22 August 2005
Protocol		22 August 2005
Participant Information Sheet	2	17 October 2005
Participant Consent Form	2	17 October 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

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.

05/Q1505/114 **Please quote this number on all correspondence**

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

Yours sincerely

Dr T S Purewal
Chair

E-mail: jenny.cross@centralliverpoolpct.nhs.uk

Enclosures *Standard approval conditions*

Copy to: Dr L Webster, R&D Department, Liverpool Women's Hospital

Friday 11th November 2005

Dear Dr Quenby,

Re: Application to Trust's R&D Committee to Conduct a Research Project.

Following your recent submission to the Trust's R&D committee, I am pleased to inform you that the R&D Committee Chairman approved your application on 11th November 2005. The title of your study will appear on the agenda of the next R&D Committee Meeting for their information only.

The project entitled **"Diabetes and myometrial contractibility in pregnancy"** is now registered on the Trust's R&D database under the local project reference LWH0609 which I would be grateful if you could quote in all future correspondence regarding the project.

The co-sponsors of this project under the Research Governance Framework for Health and Social Care (RGF) are Liverpool Women's NHS Foundation Trust and the University of Liverpool. You will be expected to undertake your responsibilities as Chief Investigator in accordance with the RGF and comply with any other regulations relevant to this study throughout its lifetime.

I would like to take this opportunity to wish you the best of luck with the study and to request a copy of the final report and any subsequent publications.

Yours sincerely,

Pp. N. J. Birch

Dr. N.J. Shaw,
Chair, Research & Development Committee

APPLICANT'S CHECKLIST

REC Ref:

Short Title of Study: Diabetes and labour Protocol
CI Name:
Sponsor:

Please complete this checklist and send it with your application

- *Send ONE copy of each document (except where stated)*
- *ALL accompanying documents must bear version numbers and dates (except where stated)*
- *Delete 'yes/no' as applicable if completing the form electronically; circle the appropriate option if completing the form by hand*
- *When collating please do NOT staple documents as they will need to be photocopied.*

Document	Enclosed?	Date	Version	Office use
Covering letter on headed paper	Yes No			
NHS REC Application Form, Parts A&B	Mandatory			
NHS REC Application Form, Part C (SSA)	(Yes) No			
Research protocol (6 copies) or project proposal	Mandatory			
Summary C.V. for Chief Investigator (CI)	Mandatory			
Summary C.V. for supervisor (student research)	(Yes) No			
Research participant information sheet (PIS)	(Yes) No			
Research participant consent form	(Yes) No			
Letters of invitation to participants	Yes (No)			
GP/Consultant information sheets or letters	Yes (No)			
Statement of indemnity arrangements	Yes (No)			
Letter from sponsor	Yes (No)			
Letter from statistician	Yes (No)			
Letter from funder	Yes (No)			
Referees' or other scientific critique report	Yes No			
Summary, synopsis or diagram (flowchart) of protocol in non-technical language	Yes No			
Interview schedules or topic guides for participants	Yes (No)			
Validated questionnaire	Yes (No)			
Non-validated questionnaire	Yes (No)			
Copies of advertisement material for research participants, e.g. posters, newspaper adverts, website. For video or audio cassettes, please also provide the printed script.	Yes (No)			
Other (please specify and continue on a separate sheet if necessary)	Yes (No)			

Liverpool Women's Hospital NHS
NHS Trust

Crown Street
Liverpool L8 7SS
Tel: 0151 708 9988

If telephoning please ask for:

.....

Direct Line:

www.lwh.org.uk

Patient Information Sheet **Version 2, Date 17/10/05**

Diabetes and Labour

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and support partner if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part.

Thank you for reading this.

Purpose of the study
It is become increasingly common to give birth by caesarean section particularly if you also have diabetes mellitus in pregnancy. One of the most common reasons for the caesarean section is because the womb does not contract properly during labour.

Little is known about how the muscle of the womb contracts. A study is being conducted at Liverpool University to try to understand what produces these contractions. It is hoped that this will eventually lead to a better understanding of labour.

You have been chosen because you have diabetes mellitûs and are pregnant. 180 other women will be chosen.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive. If you withdraw from the study after the biopsy is taken it will be disposed of immediately.

What will happen to me if I take part?
We are asking permission to take a small piece of the muscle from your womb (uterus) during your caesarean section. This piece is very small (1X0.5cm) and is known as a biopsy. The biopsy will be taken from the cut in the womb that the doctors make in order to deliver the baby. This biopsy will be done after the baby is born. The biopsy will not make any difference to how long the operation takes.

Experiments that study contractions will be done on the biopsy the day it is taken. Part of the biopsy will be stored so that further experiments can be done at a later date.

What are the possible benefits of taking part?
The information we get from this study may help us to design treatments to make the uterus contract better in labour. If these treatments were more effective than at present fewer women would require emergency caesarean sections.

Will my taking part in this study be kept confidential?
All information, which is collected, about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?
The results will be presented at international medical conferences and published in scientific journals.

Who is funding the research?
This research is funded by a studentship for a doctor studying for his PHD.

Who has reviewed the study?
The Liverpool Research Ethics Committee.

Contact for Further Information
Dr S. Quenby
Liverpool Women's Hospital
Crown Street
Liverpool L8 7SS
Tel: 0151 708 9988

Thank you for reading this

You will be given a copy of the information sheet and a signed consent form to keep.

Liverpool Women's Hospital NHS
NHS Trust

Crown Street
Liverpool L8 7SS
Tel: 0151 708 9988

If telephoning please ask for:

.....

Direct Line:

www.lwh.org.uk

CONSENT FORM

Title of Project: **Diabetes and labour**

Name of Researcher: Dr Siobhan Quenby

Please initial box

1. I confirm that I have read and understand the information sheet dated 17/10/05. (Version 2) for the above study. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Liverpool Women's Hospital. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐
5. I agree for my myometrial sample to be stored for further research ☐

Name of Patient	Date	Signature
Name of Person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 copy for research, 1 copy to be kept with hospital notes