Current Research in Physiology 6 (2023) 100105

Contents lists available at ScienceDirect

Current Research in Physiology

journal homepage: www.sciencedirect.com/journal/current-research-in-physiology

Multiple pregnancies, the myometrium and the role of mechanical factors in the timing of labour

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ARTICLE INFO

Keywords:
Myometrium
Uterus
Stretch
Twin pregnancy
Mechanosensitive channels
Preterm birth

ABSTRACT

Multiple pregnancy remains a relatively common occurrence, but it is associated with increased risks of adverse outcomes for the mother and her babies and presents unique challenges to healthcare providers. This review will briefly discuss multiple pregnancies, their aetiology and their problems, including preterm birth, before reviewing the processes leading to normal labour onset and how they may be different in a multiple pregnancy. The mechanisms by which mechanical factors i.e., uterine distension or 'stretch' contribute to uterine excitability and the timing of labour onset will be the major focus, and how over distention may pre-dispose multiple pregnancies to preterm birth. This includes current thinking around the role of mechano (stretch) sensitive ion channels in the myometrium and changes to other important regulators of excitability and contraction which have been identified from studies using *in vitro* and *in vivo* models of uterine stretch. Physiological stimuli arising from the fetus(es) and placenta(s) will also be discussed. In reviewing what we know about the myometrium in multiple pregnancy in humans, the focus will be on twin pregnancy as it is the most common type of multiple pregnancy and has been the most studied.

1. Introduction

A pregnancy with more than one fetus is a multiple pregnancy. Current (2021) rates of multiple birth in England and Wales are 13.7 per 1000 births (ONS, 2021) but higher in the US (Hamilton et al., 2015). Twin pregnancies are the most common multiple pregnancy, accounting for over 98%, with triplet and other higher order multiples accounting for less than 1.3%. Multiple pregnancies carry specific risks and complications for the babies and mother; the higher order the pregnancy, the greater the risks. Rates of multiple pregnancy across the globe have been increasing partly due to increasing maternal age and obesity as well as the use of assisted reproductive technologies (ART) such as ovulation induction medications and the clinical practice of transferring multiple embryos to maximise a live birth. Restrictions on the number embryos to be transferred as well as a push towards elective single embryo transfers has significantly reduced the multiple birth rate from IVF treatment (ASRM, 2021; HFEA, 2022). Nevertheless, multiple pregnancies still present unique challenges to healthcare providers, including the management of these pregnancies and the prediction and prevention of adverse maternal and neonatal outcomes.

2. Types of twin pregnancies

Twin pregnancies are classified according to their zygosity; monozygotic or dizygotic. Monozygotic twins form from one embryo which divides within the first two weeks of development. As both embryos are derived from one oocyte and one sperm, they are genetically identical. The timing of division after fertilisation determines the number of placentas (chorionicity) and amniotic sacs (amnionicity) and whether they are shared between the fetuses. If division occurs before implantation, this will result in twins with separate placentas and amnions. These twins are known as dichorionic diamniotic twins (DCDA) as they will implant in different sites within the uterus. If splitting occurs post-implantation at the blastocyst stage of development (approximately day 5 post fertilisation) the twins will share the chorion (and placenta) but have separate amnions and be monochorionic diamniotic (MCDA). If splitting occurs later than two weeks, the twins will share an amnion as well as the placenta and are referred to as monochorionic monoamniotic (MCMA) twins. This form of splitting is rare; however, it carries the most risks to fetal health. Dizygotic twins occurs when two oocytes are fertilised by two separate sperm resulting in DCDA twins, but they are non-identical, and sometimes referred to as fraternal twins.

In triplet pregnancies and other higher-order multiples, several

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https://doi.org/10.1016/j.crphys.2023.100105
Received 8 May 2023; Received in revised form 17 July 2023; Accepted 23 August 2023
Available online 24 August 2023

different combinations of shared and separate placentas can occur: Triplets can each have a separate placenta (trichorionic) and amnion (triamniotic), share a single placenta (monochorionic), or two fetuses can share one placenta whilst the other has a separate placenta (dichorionic).

3. Problems in multiple pregnancy

Multiple pregnancy is considered a 'high risk' pregnancy with increased risks to both mother and fetuses. Adverse maternal outcomes include higher rates of gestational diabetes, pre-eclampsia and other hypertensive disorders of pregnancy, maternal anaemia and venous thromboembolic disease such as deep vein thrombosis and pulmonary embolism. Maternal mortality for multiple births is also more than double that of singleton pregnancies. The incidence of nausea and vomiting is also greater in women with multiple pregnancies compared to singletons (NICE, 2011).

Newborn infants of multiple pregnancies are also at increased risk of adverse outcomes including congenital abnormalities, cerebral palsy, intrauterine growth restriction and low birth weight, stillbirth and early neonatal death as well as greater admissions to neonatal intensive care units (Rizwan et al., 2010; Qazi, 2011). In twin compared to singleton pregnancies, the rate of perinatal mortality in the UK is around three times higher (MBRRACE-UK et al., 2021) and even greater (up to 7 times) in low-and middle income countries (Bellizzi et al., 2018). In the case of twin pregnancies, several studies have also shown that perinatal outcomes are significantly worse for the second twin (Smith et al., 2007; Rossi et al., 2011; Santana et al., 2018). Both maternal and fetal risk increase further with the multiplicity of pregnancy (Matthews et al., 2015).

This degree of risk is also in part dependenton the chronicity and amnionicity of the pregnancy: All monochorionic fetuses share a common blood supply in which the connecting blood vessels of the placenta allow for blood to pass between the siblings. Blood flow however can become unbalanced resulting in decreased transfusion to one of the siblings, known as feto-fetal transfusion syndrome (FFTS) or twin-twin transfusion syndrome (TTTS), which impedes their growth and development (Rao et al., 2004). Additional complications also arise where the amnion is shared e.g., in MCMA twins. This can result in umbilical cord entanglement due to the lack of membranes separating the fetuses. The most notable cause of perinatal mortality in multiple pregnancy, however, is prematurity resulting from preterm delivery.

3.1. Preterm birth

Fifteen million births occur preterm each year which equate to approximately 1 in 10 infants (Blencowe et al., 2012). Traditionally preterm birth is defined as birth before 37 weeks however it has been further subcategorised according to gestation as: extreme preterm (<28 completed weeks); very preterm (28–32 weeks) and moderate to late preterm birth (32 and 36 completed weeks) (WHO, 2018).

Complications from early preterm delivery arise from immature organ systems which are not prepared to support life outside of the uterine environment. In the short term these include respiratory distress syndrome and chronic lung disease, intestinal injury such as necrotizing enterocolitis, intraventricular haemorrhage and brain injury, cardiovascular disorders from non-closure of the ductus arteriosus, and hearing and visual problems including retinopathy of prematurity. These conditions can all have lifelong consequence for the health of the infant (Behrman RE & Butler AS, 2007). Longer-term morbidities arise predominantly through neurological injury and neurodevelopmental delay and include motor disorders, reduced cognitive and academic performance as well as difficulties with mental health and social interactions (Morgan et al., 2022).

Survival and morbidity rates for preterm infants vary, most notably by gestational age at delivery. It is not difficult to see why infants born at the earliest gestation have the highest rates of mortality and complications, whilst those delivered at a more advanced age have greater chances of survival and better neurologic prognosis. Most preterm births occur between 32 and 37 weeks of pregnancy with many of these babies surviving with essential neonatal care.

Whilst multiple pregnancies represent a small fraction of all births, they disproportionately contribute to the rate of preterm birth. For example, 3.2% of all births are twin pregnancies but around 20% of all preterm births are twins. In the US, around 60% of twin pregnancies will deliver before 37 weeks and 19.5% at <34 weeks and 10% will deliver before 32 weeks (Roman et al., 2022). This contrasts markedly to singleton pregnancies in which around 8% deliver at <37 weeks, 2% < 34 weeks and 1.2% at <32 weeks (Hamilton et al., 2015). Similar trends are observed for UK (NHS Scotland, 2009) and elsewhere. On average, the rate of preterm birth is nearly ten times greater than in singletons. Twin pregnancies have 5 times higher risk of early neonatal and infant death related to prematurity compared to singletons.

3.1.1. Aetiology of preterm birth

Preterm birth can arise from spontaneous labour onset (with or without spontaneous premature rupture of membranes) or can be medically indicated and hence result from obstetric interventions designed to protect the mother and/or fetus(es) from harm from an ongoing pregnancy. Epidemiological studies have identified younger maternal age (less than 17 years) or older than 35 years, underweight or being overweight pre-pregnancy and short stature as risk factors for preterm birth. Additional risk factors include stress and smoking, and women from low- and middle-income countries have consistently been shown to have higher rates of preterm birth (Simmons et al., 2010). Pregnancies conceived through assisted reproduction technologies (IVF or ICSI) are also at greater risk of preterm birth (Pandey et al., 2012). Having a history of previous spontaneous preterm birth or short cervix (<25 mm), either naturally short or following cervical surgery puts the mother at risk of preterm birth (Iams et al., 1996; Kazemier et al., 2014). Most of the clinical trials for using an intervention to prevent preterm birth are targeted at these pregnancies.

Around one third of preterm deliveries in multiple pregnancy are medically indicated (Fuchs and Senat, 2016) but around half are spontaneous in onset. Like singleton pregnancy, the aetiology of spontaneous preterm birth in multiple pregnancy is probably multifactorial involving maternal, fetal and placental factors, however the risk factors pre-disposing to preterm birth may be different. For example, infection or short cervix are both common risk factors whilst in twins and other higher order multiples there may be other factors such as uterine (over) distention i.e. 'stretch,' (discussed later) as well as increased circulating pro-labour factors from increased placental mass and the maturing fetal lungs (see below) (Fig. 1).

Much of what we understand about the pathophysiology of preterm birth in multiple pregnancy has arisen from studies in animal models or from twin pregnancies, as they are the most common multiple pregnancy in humans. The remainder of this review will focus on what we understand about the changes within the myometrium (smooth muscle of the uterus) towards the end of pregnancy and factors which promote normal (i.e., term) labour onset. This includes those arising from the fetus or placenta, but the focus will be on mechanical factors such as uterine stretch and how it may contribute to the timing of labour. In doing so, what we do and do not know, yet, about differences in singleton and twin pregnancy including uterine overdistention, and how this may contribute to earlier labour onset in multiple pregnancy will be highlighted.

4. Normal labour onset

For labour to progress and the baby to be delivered, uterine and gestational tissues must undergo several changes including, but not necessarily in this order: weakening and rupture of the fetal membranes,

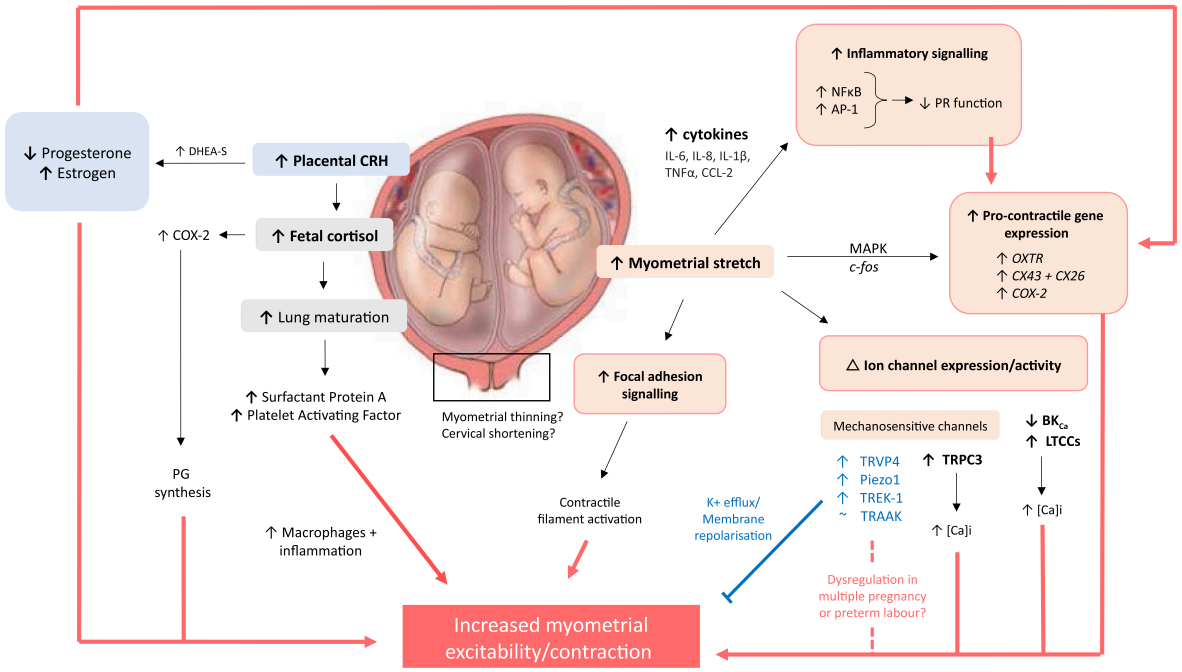


Fig. 1. Proposed mechanisms leading to increased myometrial excitability and preterm birth in multiple pregnancies
Greater placental mass and fetal number lead to increased placental corticotrophin releasing hormone (CRH), fetal cortisol and fetal lung maturation which in turn reduces progesterone and increases estrogen, surfactant protein A and platelet activating factors promoting myometrial and fetal membrane (not shown) activation. Increased myometrial stretch promotes inflammatory signalling, focal adhesion signalling and pro-contractile gene expression as well as changes to expression of ion channels such as Ca-activated, voltage-sensitive K^+ channels (BK_{Ca}) and L-type calcium channels (LTCCs) which affect intracellular calcium concentration and promote contraction. Increased expression of mechanosensitive channels acts to suppresses myometrial excitability by membrane hyperpolarisation. Dysregulation in their expression or activity could contribute to premature myometrial activation.

softening and effacement (opening) of the cervix and initiation of myometrial contractions. The myometrium must transition from a quiescent to an actively contracting organ with rhymical contractions powerful enough to dilate the cervix and then, in effect, power the progression of the baby through the birth canal. This transition is thought to be mediated at the level of gene expression in which the excitatory and contractile potential of the smooth muscle cells is increased. This is through increased expression of proteins associated with contraction e.g., ion channels, gap junction proteins and cyclo-oxygenase enzymes which synthesise prostaglandins, and reduced expression of genes within pathways associated with muscle relaxation e.g., progesterone and human chorionic gonadotropin (hCG) signalling, and cyclic adenosine monophosphate (cAMP). Together these changes promote increased excitation and connectivity between myocytes, increased calcium signalling and phosphorylation of myosin, cross bridge cycling and therefore, contraction. A short overview of uterine excitability and excitation-contraction coupling in smooth muscle is provided later but for a more detailed account see (Wray and Prendergast, 2019).

Several large-scale gene studies using a variety of gene expression platforms have identified many transcriptional differences between term-labouring and non-labouring myometrium in both human (Mittal et al., 2010; Chan et al., 2014; Sharp et al., 2016) and animals e.g. (Salomonis et al., 2005). Transcriptional signatures also identify that labour is an inflammatory event involving actions of interleukins, cytokines and immune responses, as well as upregulation of contractile signalling and cell remodelling. Indeed, independent studies have shown labour is associated with mass migration of macrophages and neutrophils to the uterine tissues, including myometrium and cervix (Osman et al., 2003; Bollapragada et al., 2009), with release of chemokines and cytokines and activation of inflammatory signalling. Throughout pregnancy these pathways are largely repressed through progesterone-receptor mediated inhibition of inflammation and concomitant repression of contraction-associated genes (Wu and DeMayo, 2017; Mendelson et al., 2019). Functional withdrawal of progesterone through progesterone metabolism or changes in expression of progesterone receptor isoforms or its co-regulators removes this inhibitory brake on contraction (Wilson and Messiano, 2020). Non labour pathways are more closely aligned with changes in smooth muscle-related processes, cell adhesion and those which favour muscle relaxation (Stanfield et al., 2019).

4.1. *Signals arising from the fetus*

Some of these labour-promoting factors may arise from the maturing fetus and placenta and are summarised in Fig. 1. These include increased production of placental corticotrophin releasing hormone (CRH) and fetal cortisol, and increased secretion of surfactant proteins and lipids from the fetal lungs. Maternal plasma CRH concentrations increase exponentially with advancing gestation before decreasing significantly and correlating with the timing of birth (Sasaki et al., 1987; McLean et al., 1995). The exact mechanism through which CRH contributes to labour onset is not certain but may include maturation of the fetus and stimulation of fetal adrenals to produce dehydroepiandrosterone (DHEA) and DHEA-sulfate to support production of placental estrogens and inhibition of placental progesterone, as well as regulating myometrial contractions directly (Tyson et al., 2009).

The maturing fetal adrenal glands also secrete cortisol which can signal for increased secretion of surfactants in the lung as well as promote expression of placental cyclooxygenase (COX-2) leading to prostaglandin synthesis and activation of steroidogenesis in the placenta to increase estrogen. Surfactant Protein A (SP-A) is a C-type lectin (collectin). It acts postnatally within the lung alveoli to enhance macrophage function to remove microorganisms. SP-A from human amniotic fluid has been shown to stimulate prostaglandin synthesis in amniotic membranes *in vitro* (López Bernal et al., 1988). Presumably the

surfactant phospholipids provide a source of arachidonic acid for this. Additionally, SP-A in mice was shown to increase the expression of IL-1β in macrophages within the amniotic fluid, promote their migration to the uterus and activation of NF-κB signalling (Condon et al., 2004). Platelet activating factor (PAF) is another potent inflammatory phospholipid secreted into the amniotic fluid alongside SP-A and may also contribute to the initiation of myometrial contractions.

In multiple pregnancy, it is conceivable that the increased number of fetuses as well as the enlarged placental mass may stimulate these labour-promoting physiological processes at an earlier timepoint in gestation. It is known that maturation of the fetal lung occurs earlier twin gestation compared to singletons (Leveno et al., 1984). Whether this correlates to enhanced secretion of SP-A in multiple pregnancy however, is not known but levels of CRH have been reported to be greater in twin gestations (Warren et al., 1990).

4.2. Mechanical factors (stretch) and the timing of labour

Human pregnancy, and indeed pregnancy in other mammal species, is accompanied by dramatic changes in blood flow to the uterus as well as significant hypertrophy and hyperplasia of the uterine myocytes. By the end of pregnancy, the overall capacity of the uterus increases from ~25 mL to 5000 mL (~200-fold) (Cunningham et al., 2005) and greater in multiple pregnancy. The ability of the uterus to adapt appropriately to a continued increase in volume is important, with stretch shown to induce several biochemical changes in the myometrial cells, stimulate growth (Lye et al., 2001; Shynlova et al., 2010) and continual remodelling. In accommodating the developing fetus(es), placenta(s) and amniotic fluid and membranes, the mechanical stress or 'stretch' on the uterus significantly increases as pregnancy advances. This is most marked in the final 10 weeks of pregnancy during the period of greatest fetal growth.

The idea that mechanical strain on the uterine wall is also responsible for, or at least contributes to labour onset has been proposed for many years. Evidence has come from several *in vivo* and *in vitro* model systems including the use of balloon catheters in humans and animals. In humans, extra amniotic placement of a balloon can trigger second trimester abortion, induce labour at term or induce contractions postpartum (Manabe et al., 1982). Interestingly, women randomised to a greater balloon volume had significantly shorter time to delivery (Schoen et al., 2018). In non-human primates, the use of a balloon catheter to raise intrauterine pressure induced preterm labour in 50% of animals (Adams Waldorf et al., 2015). Consistent with the theory of stretch promoting labour onset, clinical conditions such as polyhydramnios (excessive amniotic fluid), fetal macrosomia (large fetus) and multiple pregnancy which would contribute to increased (or excessive) mechanical stretch of the uterine wall, are also associated with higher rates of preterm labour (Hua et al., 2013). Studies involving unilaterally pregnant rats in which only one horn is distended (Shynlova et al., 2010) and prolonged stretching of primary human cells (Loudon et al., 2004; Sooranna et al., 2004; Terzidou et al., 2005; Lei et al., 2011) and tissue strips *in vitro* (Tattersall et al., 2012; Moraitis et al., 2015) which increases contractility, have attempted to shed light on the molecular mechanisms responsible which will be reviewed next.

5. The molecular mechanisms by which distention or stretch may contribute to labour onset

5.1. Changes to myometrial excitability

The myometrium is myogenic; it contracts spontaneously without the need for hormonal or nervous innervation. Its excitability is governed by ion channels expressed on the myocyte membranes (Wray and Arrowsmith, 2021). At rest, the myometrial membrane potential is negative; −60 mV in the non-pregnant state rising to −45mV near term (Parkington and Coleman, 1990; Parkington et al., 1999) and is maintained by the unequal distribution of Na^+, K^+, Cl^- and Ca^{2+} between the cytoplasm and the extracellular milieu, with K^+ conductance being the main determinant of the resting membrane potential. Contractions are critically dependent on Ca^{2+}; spontaneous contractions arise when the membrane potential becomes sufficiently depolarised (~−40 mV) to activate voltage-gated channels and the firing of action potentials and a large influx of Ca^{2+} through voltage-gated L-type Ca^{2+} channels (Mironneau, 1973; Wang et al., 1998). The rise in intracellular Ca^{2+} leads to the formation of the Ca-calmodulin complex, activation of myosin light chain kinase which in turn phosphorylates myosin and leads to cross-bridge formation with actin, and contraction ensues. This process is referred to as excitation-contraction coupling and has been extensively reviewed elsewhere; see (Aguilar and Mitchell, 2010; Wray and Prendergast, 2019). Relaxation results from inactivation of L-type Ca^{2+} channels, and repolarisation of the membrane towards resting levels by K^+ efflux, a fall in intracellular Ca^{2+} concentration and dephosphorylation of myosin light chains.

Other mechanisms that increase intracellular Ca concentration also exist, including agonist-mediated release of Ca from the sarcoplasmic reticulum (SR) into the cytosol via IP_3 signalling e.g., by hormones such as oxytocin and prostaglandin F2α which are important for labour, and store-operated calcium entry (SOCE) which occurs in response to a reduction in SR luminal Ca. For further discussion of these processes and their contribution to myometrial contraction the reader is directed to (Noble et al., 2009; Wray and Burdyga, 2010).

During gestation, K^+ efflux from the cells limits excitation by ensuring membrane hyperpolarisation and a reduction in action potential firing. Hence K^+ channel activity is generally associated with maintenance of myometrial quiescence (Brainard et al., 2007). Myometrial myocytes express a wide range of K^+ channels which are gated by ions (Ca^{2+}, Na^+), voltage and physiological stimuli including metabolites (ATP, pH) as well as stretch (discussed later) and hormones. Any activities that affect their conductance will therefore impact membrane potential and excitability. Generally speaking, their activation results in relaxation whilst their inactivation favours contraction.

Changes in expression of several K^+ channels and their regulatory subunits have been noted in pregnancy (see (Brainard et al., 2007; Wray and Arrowsmith, 2021). Consistent with the uterus transitioning from a quiescent to contracting phenotype for labour, their expression and/or activity is often reduced as gestation progresses towards term. The large Ca-activated K+ channel (BK_{Ca}) is one of the most abundant and well-studied K^+ channels in myometrium. It is both Ca- and voltage-sensitive. It's physiological role in many smooth muscles is to provide negative feedback on excitability, to counteract the depolarisation from Ca entry and thereby induce relaxation. In myometrium, the BK_{Ca} channel has a greater relaxant effect in mid compared to late gestation (Wang et al., 1998) hence, its contribution towards regulating excitability and contractility is thought to be more important in early pregnancy. Recently, stretching of non-pregnant cultured rat myometrial cells was shown to down regulate BK_{Ca} α- and β1 subunit expression in a time- and dose- (degree of stretch) dependent manner (Jia et al., 2022) which is consistent with the reduced expression of BK_{Ca} channels observed in late pregnancy and labour where the uterus would be more distended. Additionally, stretching of the myocytes also induced upregulation of the L-type Ca^{2+} channel α1c expression. Together, these findings support a role for stretch-mediated increases intracellular Ca concentration and myometrial contraction.

Several smooth muscles, including myometrium, also express a variety of mechano-gated and mechano-sensitive ion channels whose activation such as in response to stretching of membranes can affect membrane potential. When activated, inwardly rectifying channels provide a net inward current (net positive charge into the cell, often carried by Ca^{2+} or Na^+) which leads to membrane depolarisation, Ca entry and (potentially) favouring contraction. In contrast, activation of outwardly rectifying channels such as those which conduct K^+, result in net positive charge out of the cell, membrane hyperpolarisation and

favour relaxation. The following section reviews the different mechanosensitive channels and proteins which have been identified in myometrium, changes to their expression with gestation and the likely consequence of their activation towards excitability and myometrial contraction.

5.2. Mechanosensitive ion channels and proteins in myometrium

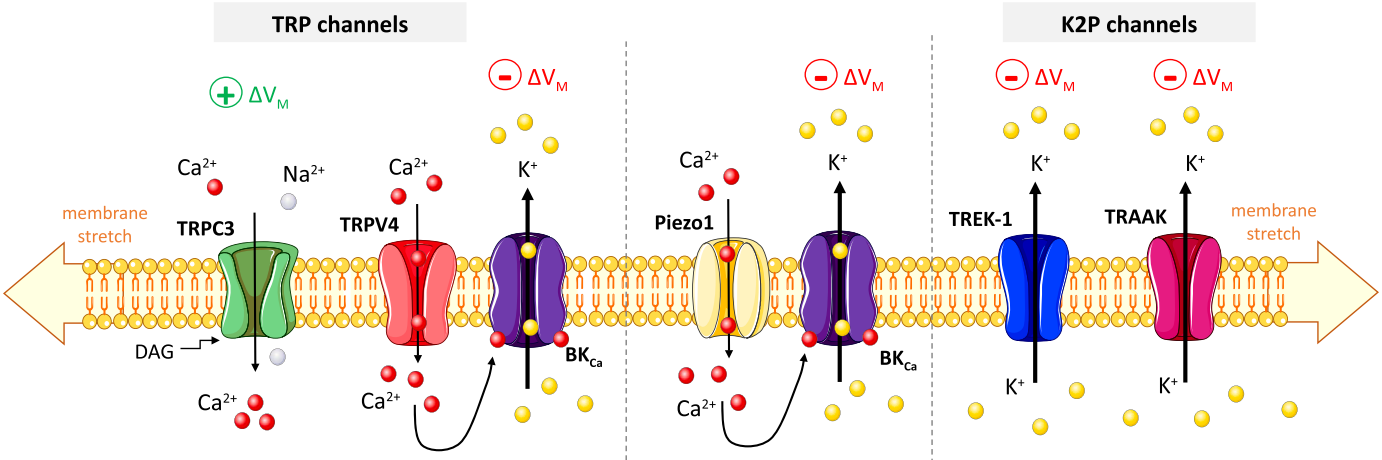
Myometrial cells are endowed with several mechanosensitive channels and receptors that can stimulate or supress contractility (Fig. 2). These include members of the group 1 Transient receptor potential (TRP) channels, TRPC and TRPV, Piezo-1, and stretch-sensitive K^+ channels of the two pore K^+ (K2P) channel family. Questions have arisen over the role of these mechanosensitive channels when activated by stretch: Do they promote contraction and support stretch-mediated labour onset? Or, instead, do they function to stabilise the membrane in the face of increased stretch from the developing fetus and help maintain myometrial quiescence?

5.2.1. Transient receptor potential (TRP) channels

The TRP channel family is large, comprising of 28 members and is subdivided into five classes: TRPC, TRPV, TRPA, TRPP, and TRPM (Earley and Brayden, 2015). They are nonselective cation channels permeable to Na^{2+}, Ca^{2+}, and Mg^{2+} and gated by various stimuli including chemicals, temperature, and mechanical force such as membrane stretch. Hence, they are important sensors of biochemical and physiological stimuli. Among the different subgroups, the Canonical (TRPC) and Vanilloid (TRPV) transient receptor potential families have been most closely associated with mechanotransduction in myometrial smooth muscle cells.

Transient receptor potential canonical (TRPC) channels have been implicated in transduction of stretch signals. Activation of TRPC preferentially leads to Ca^{2+} influx which directly increases the intracellular Ca^{2+} concentration and would therefore promote contraction (Fig. 2). It may also lead to Na^+ influx, membrane depolarisation and raise intracellular Ca concentration indirectly (Cheng et al., 2013). TRPC1, TRPC3, TRPC4, and TRPC6 proteins are expressed in uterine myometrial smooth muscle (Dalrymple et al., 2002) and their expression is regulated during pregnancy. Activation of TRPC channels in response to membrane stretch is not uniform across the family, with some requiring activation of receptors coupled to Phospholipase C or Diacylglycerol (Chen et al., 2020). Mechanical stretch of uterine smooth muscle *ex vivo* however, has been shown to upregulate expression and activate the TRPC3 channel. TRPC3 was suggested to form components of store-operated calcium entry (Dalrymple et al., 2007) however, STIM and Orai proteins are likely to be involved instead/as well (Chin-Smith et al., 2014). When SR Ca levels decrease, STIM and Orai interact to form a Ca-selective pore and together are suggested to stimulate the opening of TRPC channels. TRPC3 channels may also play a role in mediating oxytocin-induced contractions (Sharma et al., 2017) following IP_3-mediated SR Ca release. In supporting a role for stretch-induced labour onset, TRPC3 expression in uterine smooth muscle tissue was shown to be significantly higher in preterm labouring than in full-term labouring human myometrium (Jing et al., 2018). In the same study, TRPC3 knockout mice also show delayed labour onset in inflammation-induced preterm labour models, and muscle tension of *ex vivo* uterine strips were significantly lower in TRPC3−/− mice compared to wild-type (Jing et al., 2018).

The TRPV4 channel is most studied of the TRPV channels in myometrium. It is an inwardly rectifying Ca channel which is activated in response to cell swelling, shear stress or stretch, changes to physiological temperatures and hypotonic stress - all factors which are associated with late gestation and labour. TRPV4 activation in vascular endothelial and smooth muscle cells results in Ca entry, but with subsequent activation



	TRPC3	TRPV4	Piezo1	TREK-1	TRAAK
Δ V_m	Depolarisation	Hyperpolarisation	Hyperpolarisation	Hyperpolarisation	Hyperpolarisation
Function	Contraction	Relaxation	Relaxation	Relaxation	Relaxation
Δ Expression	↑ with stretch ↑ with gestation ↑ preterm vs. term labouring	↑ with gestation (mouse)	↑ in term labouring vs. non pregnant ↓ in preterm vs. term labouring	↑ with stretch ↑ with gestation	No change

Fig. 2. Mechanosensitive channels in human myometrium: Their mechanism of action and changes in expression with stretch and pregnancy
Human myometrium expresses the mechanosensitive canonical (TRPC3) and vanilloid (TRPV4) members of the Transient receptor potential (TRP) channel family, Piezo-1, and stretch-sensitive K^+ channels (TREK-1 and TRAAK) of the two pore K^+ (K2P) channel family. Stretch-mediated activation of TRPC3 results in Ca (or Na) entry and depolarisation. Activation may also require agonist-mediated generation of diacylglycerol (DAG). In human myometrium, Ca entry arising from activation of TRPV4 and Piezo1 results in BK_{Ca} channel activation, K^+ efflux and hyperpolarisation. TREK-1 and TRAAK channels mediate K^+ efflux which helps maintain the resting potential of the myometrium and contributes to myometrial quiescence during pregnancy. Table summarises the functional effect of channel activation and the reported changes in channel expression with stretch and gestation to date. Unless otherwise stated, data refers to human myometrium.

of the large conductance Ca^{2+}-activated (BK_{Ca}) channels, hyperpolarisation and vascular relaxation (Earley et al., 2005). Hence, TRPV4 activation is hypothesized to play a major role in controlling vascular tone under different physiological conditions (Filosa et al., 2013). A role for TRPV4 channels in regulating *myometrial* contraction or tone in response to stretch is therefore also plausible. TRPV4 is expressed in murine (Singh et al., 2015; Ying et al., 2015) and human (Villegas et al., 2021) uterus. Its expression increases markedly during pregnancy in the mouse (Ying et al., 2015). This increase is most pronounced in cell membrane fractions indicating increased trafficking of the channel to the plasma membrane. Their expression is also suggested to be regulated by β-arrestin (Ying et al., 2015) as shown in other smooth muscles (Shukla et al., 2010).

In mouse myometrium, activation of TRPV4 causes stimulation of contraction and their blockade relaxes it (Singh et al., 2015; Ying et al., 2015). In models of preterm labour using either RU486 (an anti-progestogen) or lipopolysaccharide (LPS) to mimic inflammation-induced labour onset, the administration of a TRVP4 channel antagonist significantly delayed time to labour onset. Pharmacological inhibition of TRPV4 channels either via siRNA approaches in cultured cells or using uterine strips from TRVP-null mice decreased oxytocin-induced contractions, whilst pharmacological activation enhanced it (Ying et al., 2015), supporting that stretch-mediated activation of TRPV4 *in vivo* may facilitate labour onset. TRPV4 channels were also shown to have a significant role in mediating contractions to PGF2α (Singh et al., 2015), which is an important mediator inducing labour, including preterm labour.

In organ bath studies of *human* myometrial contraction however, the opposite was true: Stimulation of TRPV4 caused a consistent tocolytic effect (Villegas et al., 2021). One that was reversed by ruthenium red, a non-specific blocker of non-selective channels (and hence blocker of TRPV4 channels in this instance) and the selective blocker of BK_{Ca} channel, iberiotoxin. In humans, it is proposed that the activation of TRPV4 channels and resulting Ca entry, leads to activation of nearby BK_{Ca} channels on the surface membrane (Fig. 2). Activation of BK_{Ca} channels results in K^+ efflux, membrane hyperpolarisation and concomitant closure of L-type calcium channels, and hence relaxation.

An explanation for differences in outcomes between studies may be due to different tissue species (murine and human) and the different approaches to assaying contraction i.e. myography of fresh tissue strips and collagen gel contraction cell assays, as well as the presence of oxytocin in some assays. Oxytocin is known to have a multitude of effects on membrane potential and calcium dynamics (Arrowsmith, 2020), which may impact on findings.

5.2.2. Piezo channels

More recently a second inwardly rectifying mechano-gated channel, Piezo1, which is preferential to Ca under physiological conditions was identified in myometrium (Barnett et al., 2022) and endometrium (Hennes et al., 2019). Stimulation of Piezo1 in human myometrial strips *ex vivo* using a Piezo 1 agonist, Yoda1, caused relaxation of oxytocin-induced contractions (Barnett et al., 2022). The effect was reversible with co-treatment of a Piezo1 antagonist. In addition, Piezo1 was also found to be expressed on microvascular endothelial cells of the myometrium with expression exceeding that in uterine myocytes by almost three times. Paradoxically however, in cultured myometrial myocytes and endothelial cells activation of Piezo1 was found to be accompanied by significant calcium uptake. Hence, similarly to activation of TRPV4 channels in human myometrium (Villegas et al., 2021), it is proposed that Ca entry resulting from Piezo1 activation results in relaxation via local BK_{Ca} activation and membrane hyperpolarisation (Fig. 2).

These results are interesting however, since we know that despite their large conductance, inhibition of BK_{Ca} channels does little to uterine contractility or calcium signalling (Aaronson et al., 2006). Hence, other mechanisms are likely to be involved. Barnett proposes that, in addition to stretch mediated activation of BK_{Ca} channels in myometrial myocytes, uterine stretch also stimulates nitric oxide (NO) production in myometrial endothelial cells which then migrates to the myometrial myocytes and further promotes relaxation (Barnett et al., 2022). This is an interesting concept as NO is a known relaxant of myometrial smooth muscle having activities such as S-nitrosylation of key contraction proteins including ion channels, pumps and actin and myosin myofilaments which modifies their activity (Danylovych and Danylovych, 2021). Different nitrosylation signatures have also been found in myometrium from preterm women (Ulrich et al., 2013) and the ability of NO to relax preterm tissues is reduced (Barnett et al., 2018). Whether NO signalling is different in the myometrium from multiple pregnancies however is not known.

Piezo1 expression was found to be upregulated in term labouring human myometrium compared to both non-pregnant and non-labouring tissues (at term or preterm). Given that the degree of uterine distension is likely to be similar in term non-labouring and term labouring patients, Piezo1 expression may be associated with labouring state rather than uterine stretch *per se*. Interestingly however, Piezo1 expression was significantly downregulated in myometrium from women with preterm labour, suggesting dysregulation in channel expression in these patients.

5.2.3. Stretch-activated K^+ channels

Another class of mechanosensitive channels expressed in the myometrium are stretch-activated K^+ channels. They are encoded by members of the two-pore K^+ (K2P) superfamily of K^+ selective ion channels (KCNK; (Patel et al., 1998). They are relatively voltage-insensitive and are thought to contribute to background or leak currents in excitable and non-excitable tissues (Enyedi and Czirják, 2010). They are suggested to maintain relaxation of myocytes within several hollow organs by hyperpolarising the membrane. Like other mechanosensitive channels they are activated by several chemical and physical stimuli including extracellular pH (Duprat et al., 1997), membrane stretch, arachidonic acid (Bang et al., 2000; Miller et al., 2003) and oxygen tension (hypoxia) (Lewis et al., 2001; Miller et al., 2003).

K2P channels are expressed in human (Bai et al., 2005; Tichenor et al., 2005; Buxton et al., 2010; Wu et al., 2012; Heyman et al., 2013) and mouse myometrium (Monaghan et al., 2011). Molecular candidates that mediate stretch dependent K^+ currents include the TWIK- (**T**wo--pore domain **W**eak **I**nward rectifying **K**+ channel) related K channels, TREK-1 and TREK-2 (*KNCK2* and *KCNK10*) and TWIK-related arachidonic acid-stimulated K channel, TRAAK (*KCNK4*). Several studies have shown that TREK-1 but not TREK-2 is present and up-regulated in the human myometrium during pregnancy (Bai et al., 2005; Tichenor et al., 2005; Buxton et al., 2010; Yin et al., 2018). Alternative splicing of TREK-1 at the N terminus has given rise to three known isoforms of TREK-1; TREK-1a, 1b and 1c. All have been identified in human myometrium (Wu et al., 2012). TRAAK channels are expressed in both pregnant and non-pregnant human myometrium but unlike TREK-1, TRAAK channel expression does not change in pregnancy (Buxton et al., 2010). In organ bath experiments, selective inhibition of TREK-1 channels augmented the frequency of oxytocin-induced and spontaneous contractions, consistent with notion that TREK-1 currents act during pregnancy to maintain a negative membrane potential and myometrial quiescence until term (Fig. 2). In line with this theory, a significant decrease in mRNA levels of TREK-1 in term and preterm labouring samples has been observed (Buxton et al., 2010). Prolonged stretch of human myometrial strips from singleton pregnancies *ex vivo* also increased TREK-1 expression, indicating that the increase in TREK-1 expression with gestation is partly mediated through stretch. Interestingly however, expression of TREK-1 was decreased in twin pregnancy myometrium where levels of stretch are expected to be greatest (Yin et al., 2018). In preterm human myometrium, several splice variants of TREK-1a which lack either the pore or transmembrane domain have also been detected (Wu et al., 2012). These splice variants are capable of physically interacting with the full length wild-type TREK-1 at the

plasma membrane and may form homo or heterodimers. The C and N termini of TREK-1 are important for channel gating and trafficking respectively, hence co-expression of a TREK-1 splice variant with TREK-1 has been hypothesized to affect wildtype TREK-1 channel trafficking or activity (Wu et al., 2012).

Overall, at least in humans, most evidence supports the role of mechanosensitive ion channels in the myometrium to that of maintaining myometrial quiescence as gestation advances and the mechanical load placed on the myometrium increases. Upregulation of TRPV4 and TREK1 expression with pregnancy would support this. As labour approaches, if expression is reduced, as is the case for TREK1, this brake on contraction is removed and the myometrium can transition from quiescence to labouring. In cases of preterm labour, it is postulated that dysregulation of mechanosensitive channel expression occurs, as evidenced by several studies showing different expression in preterm tissues (Buxton et al., 2010; Wu et al., 2012; Yin et al., 2018) or perturbation in factors controlling their trafficking or insertion in membranes. The lipid composition of the cell membrane as well as the interactions between the actin cytoskeleton and extracellular membrane proteins may also affect the activity of mechanosensitive channels (Kim et al., 2007; Romero et al., 2019). In multiple pregnancy where mRNA expression of TREK-1 was reduced compared to singleton pregnancies (Yin et al., 2018), this may indicate a dysregulation of these channels or their activity in these pregnancies or a downregulation of expression and increased excitability in preparedness for labour.

5.3. Effects of stretch on other important contraction proteins

In addition to direct changes on cell excitability by activation of mechanosensitive ion channels, several studies have reported that stretching of primary human uterine myocytes or tissue strips *in vitro* changes expression of several contraction associated proteins (Fig. 1). This is likely through activation of transcription factors e.g. AP-1 system (Oldenhof et al., 2002; Mitchell et al., 2004; Sooranna et al., 2004) and *c-fos*, as well as activation of MAPK cascades. Stretching of primary human uterine myocytes upregulates OTR mRNA expression (Terzidou et al., 2005) and increases maximal responses to oxytocin in tissue explants subjected to prolonged stretch (Tattersall et al., 2012; Moraitis et al., 2015; Aye et al., 2018) which can be inhibited by selective blockade of the OTR (Aye et al., 2018). Stretch may also induce constitutive activation of the oxytocin receptor (OTR). Cyclooxygenase 2 (COX-2) expression is known to increase with the onset of labour. In primary human myocytes exposed to stretch *in vitro*, COX-2 expression and activity is significantly upregulated resulting in increased prostaglandin synthesis (Sooranna et al., 2004). Gap junction proteins such as Connexin (Cx) 26 and 43 which form major communication channels between cells were also shown to be upregulated by stretch in pregnant rat myometrium (Ou et al., 1997). Stretch activation of Cx43 expression may also facilitate signalling between the cervix and myometrium to initiate labour (Vink, 2020). Prolonged stretch in tissues also alters focal adhesion signalling and the phosphorylation of several proteins including ERK1/2 and caldesmon (CaD) (Li et al., 2004, 2009; Aye et al., 2018) which has been shown to increase the activation of contractile filaments required for contraction. Hence stretch may serve to alter protein function in addition to changing its expression. Stretch also reduces expression of progesterone receptor B isoform (Lei et al., 2011) which is the predominant progesterone receptor expressed in pregnancy and the isoform thought to maintain the inhibitory brake on contraction.

Other novel proteins identified with increased uterine stretch include gastrin-releasing peptide (Tattersall et al., 2012), a known smooth muscle stimulating agonist, including in the uterus (Amiot et al., 1993). In pregnant rats, prolonged stretch has also been implicated in changes in expression of MMP-2 and MMP-9 (Yin et al., 2012) which are known to have roles in tissue remodelling and degradation of extracellular matrix proteins, as well as role in mechanotransduction (Ingber, 2006).

5.4. Stretch and inflammation

Inflammation is suggested to play a physiological role in transforming the quiescent myometrium to the contractile state and stretch may facilitate this. Secretion and synthesis of several pro-inflammatory mediators and cytokines is increased in response to myometrial stretch (Fig. 1). In unilaterally pregnant rat horns (Shynlova et al., 2008) levels of CCL-2 were significantly upregulated in the pregnant horn under stretch compared to the empty horn. Using balloon catheters in rhesus macaques, levels of IL-1β, TNF-α, IL-6, IL-8 and CCL-2 were also significantly elevated with stretch (Adams Waldorf et al., 2015). In *in vitro* studies of human and rat myometrial cells, stretch consistently induces pro-inflammatory signalling which is thought to be mediated via MAPK (Loudon et al., 2004; Sooranna et al., 2004) and/or NFκB signalling (Hua et al., 2012). Furthermore, the increased secretion of chemokines and cytokines by uterine myocytes *in vitro* in response to stretch has been shown to activate uterine microvascular endothelial cells and leukocyte recruitment (Lee et al., 2015). Thus *in vivo*, stretch may aid inflammatory cell recruitment from local uterine blood vessels into the myometrium and promote labour onset.

The evidence from *in vitro* and *in vivo* studies of the effect of prolonged stretch on the uterus points largely towards a role in stretch promoting contraction and acting in a pro-labour manner (Fig. 1). In the case of preterm labour, those stretch-sensitive channels which appear to act to maintain uterine quiescence and counterbalance the effect of stretch may be failing prematurely. In the case of multiple pregnancy, excessive uterine stretch may become pathological and factors promoting contraction including increased expression of contraction-associated proteins and inflammatory signalling, may supersede those fighting to maintain relaxation. Differences between species in the effect of stretch on signalling and contraction also highlights the need to use more relevant models of uterine contraction which better resemble the human uterus and its physiology. Pregnant human uterine tissues are clearly the gold standard.

6. Contractile properties and gene expression in twin pregnancy myometrium

Only a few studies have compared the contractile properties and gene expression between singleton and twin pregnancy myometrium. We showed that *ex vivo* uterine contractions from twins are shorter in duration but more frequent than singletons (Turton et al., 2013). The differences were also significantly correlated with increasing neonatal birthweight, used as a surrogate marker for uterine stretch. The findings reflect those from home uterine monitoring devices in twins and triplet pregnancies (Newman et al., 2006). We also showed that response to oxytocin was significantly positively correlated with neonatal birthweight which supports a pro-contractile role for stretch in myometrium and preparation of labour onset. However, Yin et al. (2018) found a reduced response to oxytocin in twin pregnancy myometrium compared to singletons. The decrease in TREK-1 expression (and hence increased excitability) found in twins in their study, however, may be contributing to the increased frequency of spontaneous contractions which we observed.

With the advancement of whole genome sequencing, transcriptome-wide studies have been performed to explore differences in global gene expression between singleton and twin myometrium. Using RNAseq we compared samples form women with singleton and twin pregnancy, delivering preterm and term (all pre-labour). Surprisingly, we found no difference in the transcriptome between singleton and twin myometrium when matched for gestational age (Arrowsmith et al., 2020). Similarly, no change was found in a recent study of preterm singleton and twins, either in labour or non-labouring which examined for differences in 44 selected transcripts known to change with labour onset (Phung et al., 2022). We did however find some differences between preterm singleton and twin term samples which had the greatest

difference in neonatal birth weight (more than double), and hence assumed differences in myometrial stretch. These genes mapped to pathways involved in immune response, regulation of immune response and inflammation, as well as cytokine and chemokine signalling. Changes in genes associated with regulation of myometrial contraction and genes involved in the de-novo synthesis of steroids such as pregnenolone and progesterone which are important for the maintenance of pregnancy were also identified but were on average reduced in the twin group (Arrowsmith et al., 2020).

7. Prevention and treatment of preterm birth in multiple pregnancies

Given the aetiology and underlying pathophysiology in twin pregnancy may be different to singleton pregnancies, its stands to reason that the treatment approaches may need to be tailored as well. A detailed overview of the different pharmacological approaches for prevention or treatment of preterm labour is beyond the scope of this review but see (Wray et al., 2023). In short, prophylactic treatment is largely either pharmacological by vaginal progesterone pessaries (UK) or intramuscular 17- hydroxyprogesterone caproate (17-OHPC, US), or a physical intervention such as cervical cerclage (stitch) to close the cervix, or ARABIN® pessary. This is a small, flexible silicone device inserted into the cervix to support it. It changes the utero-cervical angle which reduces cervical tension and prevents shortening and dilatation. Treatment of preterm labour contractions involves tocolytics to stop or slow contractions in an attempt to sufficiently delay delivery (~48 h) to give antenatal corticosteroids and/or $MgSO_4$ to aid fetal lung maturation and provide neuroprotection in utero respectively, and for the transfer of the woman to a tertiary hospital with appropriate neonatal care facilities.

Daily vaginal or weekly intramuscular progesterone (17-OHPC) therapy has been somewhat successful in reducing rates of preterm birth (<34 weeks) in selected singleton women at high risk of preterm birth (short cervix on ultrasound or previous history of spontaneous preterm birth) (EPPIC group, 2021) but not in twins or triplets (Norman et al., 2016; Dodd et al., 2017). However, following more recent lack of evidence that it reduces the risk of recurrent spontaneous preterm birth (Blackwell et al., 2020) (PROLONG trial) the use of 17-OHPC as a prophylactic in any pregnancy at high-risk of preterm birth has recently lost FDA approval.

In organ bath studies of twins and singleton myometrium, we found progesterone to be less effective in reducing *in vitro* contractions in twin pregnant myometrium than singletons (Arrowsmith et al., 2016). Additionally, *in vitro* studies have also shown that treatment with progesterone does not overcome the stretch-induced effect on myometrial signalling and contraction (Lei et al., 2011). In the case of overcoming the drive on contraction with the additional stretch in multiple pregnancy, a greater concentration of progesterone has been suggested (Rehal et al., 2021).

In women with a twin pregnancy and a short cervix, the arabin pessary has also not been shown to reduce preterm birth or adverse neonatal outcomes (Norman et al., 2021). Findings from trials of cervical cerclage for multiple pregnancy are mixed, with some benefit found for women with a history of preterm birth or short cervix but routine cerclage in twin pregnancies even for a short cervix is not recommended. A faster rate of cervical shortening is associated with greater likelihood of birth before 35 weeks (Melamed et al., 2016). Monitoring cervical length in twin pregnancy may help to identify women at increased risk of preterm delivery.

Many of the current tocolytics available to stop contractions and delay labour do not work reliably with most described as *'probably or possibly effective'* in delaying preterm birth compared with placebo or no treatment and are based on low-certainty evidence (Wilson et al., 2022). Many treatments have also been repurposed as tocolytics for preterm birth given their known potent relaxant effects on other smooth muscles including blood vessels. However, this comes at a cost of maternal and fetal side effects and so tocolytics should only be administered when there is clear benefit to the fetus. There is no current consensus on the most effective pharmacological tocolytic strategy and evidence for use of tocolytics in twin pregnancies is limited and is not currently recommended. Finding therapies with better efficacy and safety profile is clearly a priority. Having a better understanding of the drivers and mechanisms of preterm birth pathophysiology in different patient groups is essential for this process.

8. Conclusion and future directions

Whist multiple pregnancies represent a small fraction of the number of births, they disproportionately contribute to rates of preterm birth. There is an urgent need to better understand the physiological mechanisms that contribute to preterm birth generally, to better prevent and manage preterm labour in all gestation types. However, multiple gestation presents unique challenges, and mechanisms of preterm labour onset are unlikely to be universal. In cases of pregnancies where uterine distention may become pathological such as in multiple pregnancy, improved model systems which better reflect the *in vivo* environment are required to advance our understanding of stretch effects and help to develop novel treatment strategies for preterm birth. For example, it may also be the rate of change in distention that is important. Few *in vitro* models and assays have taken relative tension into account in their studies. Examining cell cultures under tension or application of different tensions to contracting strips of myometrium *ex vivo* to better model the *in vivo* environment are also needed. Moreover, where the effect of stretch on contraction and signalling has been examined, it has largely been acute stretch rather than the more chronic stretch that is more persistent and likely to be occurring *in vivo*. Animal models are also inherently difficult as their findings do not always translate well to human studies; hence human studies should be prioritised.

That several stretch-sensitive channels have been identified in human myometrium and that for some, their expression changes with gestation and/or with stretch, coupled to the fact that their activities can be pharmacologically modulated, gives hope to identifying alternative therapeutic targets to better regulate uterine contractions, facilitate myometrial quiescence and prolong gestation in women who are more at risk to preterm birth. In addition to changes to excitation and gene expression within the myometrial cells from overdistention, other physical changes such as changes to uterine wall thickness (thinning) and in other gestational tissues including fetal membranes which have not been discussed here, may be contributing to higher rates of preterm birth in multiple pregnancies and should also be investigated.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Sarah Arrowsmith: Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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