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Faculty of Health Sciences
Women's Health and Perinatology Research Group

The effect of hypoxaemia on fetal cardiac function
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Amarnath Bhide
A dissertation for the degree of Philosophiae Doctor, August 2017

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Cogito Ergo Sum

These are the famous words of Rene Descartes, and roughly translate in English to 'I think, therefore I am.' The limit of scepticism is to doubt the existence of oneself. I am grateful to Rene Descartes who pointed out that the ability to think proves that one does exist. Consider this another way, it may be argued that one no longer 'exists' if one stops thinking. So, it is important to keep thinking.

I am grateful to my friend and my principal supervisor Ganesh Acharya who planted a seed in my mind that I should register for a doctorate at the University of Tromso. I still remember the day it happened: he had taken me for a long drive outside Tromso through the beautiful countryside of Northern Norway. It is then that he questioned my future plans. He was candid enough to make me realise that it is now or never. I am glad that he did that, for it made me think about my own future career direction. He tolerated my several visits to his home in Tromso, my demands on his precious time, my occupation of his office when I was there, several telephone calls, innumerable face-time calls and Skype conversations. He reassured me that this project is not only possible, but is worthwhile, despite the trips that I would have to make. Without his confidence in me, I would not have even begun, let alone completed the doctorate. What begun with a casual conversation in the Florence ISUOG congress grew into deep and trusting friendship, and I am very grateful for that. I am grateful to Larissa for welcoming both me and my wife in their home, and for tolerating our claim on Ganesh's time.

I am grateful to have Juha as my co-supervisor for this dissertation. It was with trepidations that I proposed to have my dissertation based on Juha's sheep experiments. To my pleasant surprise I had his unconditional support. What started as a visit to the animal laboratory on Oulu for a couple of days out of curiosity, turned into a dissertation for a doctorate. He and his entire team made me feel very welcome, first as a visitor and later as a member of the team. He looked after me during all my visits starting from airport pick-ups and drops. I cannot forget the day-long drives he has made for me and Ganesh just so that our travelling is made easier. His attention to details, critical wise comments and constructive suggestions have significantly improved the manuscript versions I worked on towards submission. His wry sense of humour and undying optimism is second to none. It is indeed my privilege to work with Juha. Working with Juha,

Ganesh and the team does not remain work, but turns into an exciting expedition. There is time for enjoying as well, with visits to the best eating places in Oulu.

List of original publications

1. Bhide A, Vuolteenaho O, Haapsamo M, Erkinaro T, Rasanen J, Acharya G. Effect of Hypoxaemia with or without Increased Placental Vascular Resistance on Fetal Left and Right Ventricular Myocardial Performance Index in Chronically Instrumented Sheep. Ultrasound Med Biol. 2016 Nov;42(11):2589-2598.

2. Bhide A, Rasanen J, Huhta H, Junno J, Erkinaro T, Ohtonen P, Haapsamo M, Acharya G. The effect of hypoxaemia on fetal ventricular deformation in a chronically instrumented sheep model. Ultrasound Med Biol. 2017 May;43(5):967-973.

3. Bhide A, Alanne L, Rasanen J, Huhta H, Junno J, Erkinaro T, Ohtonen P, Haapsamo M, Acharya G. Effect of Sildenafil on fetal pulmonary circulation and cardiovascular function during hypoxaemia in a chronic sheep model. (Submitted)

List of abbreviations

A-V – Atrio-ventricular

ATP – Adenosine tri-phosphate

NADH – Nicotinamide adenine dinucleotide hydride

TAPSE - Tricuspid annular plane systolic excursion

MAPSE – Mitral annular plane systolic excursion

TDI – Tissue Doppler imaging

FS – Fractional shortening

EF – Ejection fraction

ESD – End-systolic diameter

EDD – End-diastolic diameter

LV – Left ventricle

RV – Right ventricle

MPI – Myocardial performance index

ICT – Isovolumic contraction time

IRT – Isovolumic relaxation time

ET – Ejection time

PD – Pulse-wave Doppler

TD – Tissue Doppler

HR – Heart rate

BP – Blood pressure

TTTS – Twin to twin transfusion syndrome

IVCV – Isovolumic contraction velocity

IVRV – isovolumic relaxation velocity

ICC – Intra-class correlation coefficient

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LVCO – Left ventricular cardiac output

RVCO – Right ventricular cardiac output

CCO – Combined cardiac output

STE – Speckle tracking echocardiography

RT-PCR – Reverse transcriptase – Polymerase chain reaction

NO – Nitric oxide

PDE-5 – Phosphodeisterase-5

SC – Sildenafil citrate

IUGR – Intrauterine growth restriction

SPSS – Statistical package for social sciences

SAS - Statistical Analysis System

LMM – Linear mixed model

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1 Abstract

Objectives

The objectives were

1. To study the effect of placental embolisation as well as maternal hypoxaemia on fetal left and right ventricular function as reflected by pulsed-wave Doppler-derived myocardial performance index (MPI).

2. To study myocardial systolic and diastolic function of the fetal left and right ventricles using ultrasound speckle tracking technique to measure global strain and strain rate.

3. To examine if fetal administration of Sildenafil can relieve reactive pulmonary vaso-constriction in a hypoxaemic fetus.

4. To assess the effects of Sildenafil on cardiac function.

Methods

We used pregnant sheep on gestational day 115-129/145 (approximately 4/5th of the length of gestation) for the study. Separate sets of experiments were performed for each component of the study described above. Pregnant sheep were operated under general anaesthesia in order to place measurement equipment in the ewe and the fetus. Catheters were placed in fetal carotid artery and jugular vein. An ultrasonic transit-time flow probe was placed around the ductus arteriosus to measure ductal blood flow. Electrodes were placed to obtain fetal ECG. A catheter was kept in the amniotic cavity to measure the amniotic fluid pressure. Experiments were performed after a recovery period of 48-72 hours. Baseline measurements of fetal blood gases and acid-base status were performed.

In the first set of the experiments, we studied the effect of both placental embolisation and fetal hypoxaemia (caused by maternal hypo-oxygenation) on fetal global cardiac function using ultrasound. We evaluated global myocardial function with pulse-wave Doppler-based myocardial performance index. Stored fetal myocardial tissue was subjected to hypoxic gene panel using polymerase chain reaction (PCR) to assess expression of hypoxaemic genes.

In the second set of experiments we studied the changes in function of the fetal heart in response to hypoxaemia. An angle independent technique for assessing myocardial function (ultrasound speckle tracking) was used for this purpose. The evaluation of strain and strain rate was

performed off-line on stored loops of the ultrasound examinations carried out during the experiments.

In the third set, fetal cardiac systolic function was evaluated using ventricular outputs, global longitudinal strain/strain rate with speckle tracking echocardiography and isovolumic contraction velocities of the ventricular wall at the level of the mitral and tricuspid valves with tissue Doppler. Diastolic function was evaluated using isovolumic relaxation velocities using tissue Doppler and pulsatility index of the ductus venosus with pulsed wave Doppler ultrasound. Pulmonary blood flow was calculated from the ductus arteriosus flow and left ventricular output. Pulsatility index of the right pulmonary artery was measured with pulsed wave Doppler ultrasound. The measurements were obtained at baseline, with hypoxaemia and at recovery. Animals administered saline or Sildenafil after hypoxemia was induced, and the two groups were used for comparison.

Linear mixed model analysis was used to analyse data on repeated measurements taking into account correlation of measurements carried out in an individual fetus at different time-points. Random intercept model was selected.

Results

Placental vascular resistance and umbilical artery PI increased significantly with placental embolization. During hypoxaemia, mean LV MPI increased significantly only in fetuses with an intact placenta, returning to baseline during the recovery phase. Right ventricular MPI was unaffected. Expression of the hypoxaemic genes was no different with or without placental embolisation. Significantly lower expression of genes involved in cardiac contractile function and its regulation were seen in the group with placental embolisation.

Using speckle tracking echocardiography, baseline mean (SD) left and right ventricular global longitudinal strains were -18.7% (3.8) and -14.3% (5.3) respectively (p = 0.003). Hypoxaemia at 30 and 120 minutes led to a significant reduction (less deformation) in the global longitudinal strain of the LV, while the global circumferential and radial strains were not affected by fetal hypoxaemia. During the recovery period, LV global longitudinal strain returned back to baseline level. Right ventricular global longitudinal, circumferential or radial strains did not change significantly.

In the third set of experiments, fetal hypoxaemia did not affect RVCO that remained unchanged in both groups. However, LVCO and combined cardiac output fell significantly in both the

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groups during hypoxaemia and remained significantly lower in recovery phase than at baseline. In both groups, lung volume blood flow decreased and flow across the ductus arteriosus increased significantly during hypoxaemia phases with no difference between the Sildenafil and control groups. During hypoxaemia phases left but not right ventricular global longitudinal deformation was reduced (p = 0.003) in both groups. Both right and left ventricular and isovolumic relaxation velocity (IVRV) decreased significantly during hypoxaemia phases with no apparent effect of Sildenafil. Fetal hypoxaemia led to pulmonary arterial vasoconstriction, decreased lung volume blood flow, increased shunting through the ductus arteriosus, reduced left ventricular cardiac output and was associated with evidence of cardiac dysfunction on echocardiography.

Conclusions

In near-term sheep fetus, fetal left ventricle is more sensitive to acute hypoxaemia than the right ventricle. LV global longitudinal and circumferential deformations are greater as compared to RV. Acute hypoxaemia leads to LV rather than RV dysfunction as demonstrated by decreased deformation. Direct administration of Sildenafil to hypoxic fetuses did not reverse redistribution of cardiac output or ameliorate hypoxaemia induced cardiac dysfunction.

2 Introduction

The history of evolution is fascinating. Geochemical models suggest a marked increase, and then a decline in atmospheric oxygen levels. The extent of the increase is debated, but may have reached 35% compared to the current 21%. Blue-green algae (Cyanobacteria), in a phenomenon known as the great oxygenation event or oxygen catastrophe are thought to be responsible for the oxygen peak, approximately 3.2 billion years ago(1). The fluctuations of the atmospheric O_2 have influenced evolution of life on earth. Complex multicellular organisms evolved, and the greatest challenge to aerobic organisms became oxygen deprivation. The first physiological system to become functional during mammalian development is the circulatory system. Circulation must be established once the embryo becomes too large for O_2 to be delivered simply by diffusion.

Several compensatory or defence mechanisms evolved to protect from hypoxemia. Nowhere else are these mechanisms more important than the fetal life. The fetus not only survives, but also grows in conditions with significantly lower oxygen concentrations as compared to adults. How organisms distribute and utilise 0_2 under normoxaemic as well as hypoxaemic conditions has been the subject of research for many scientists(2). Oxygen deprivation can be experienced by the fetus in the prenatal period due to compromised delivery such as high altitude or maternal anaemia or abnormal placentation. In the intrapartum period, the uterine contractions lead to a hypoxic challenge. Reduction in the blood flow in the maternal intervillous space and/or compression of the umbilical cord can lead to fetal hypoxia. Although most fetuses are able to adapt to and withstand some degree of hypoxic challenge, not all can. Early identification of compromised fetuses is the focus of research into antepartum as well as intrapartum assessment of fetal wellbeing. Fetal circulation is unique, in that the two ventricles work in parallel before birth rather than in series after birth (also see later). There are three sites where shunting occurs in the fetus: a) Ductus venosus where the oxygenated venous blood bypasses the liver, b) Foramen ovale, where umbilical venous blood bypasses the lungs and c) Ductus arteriosus where arterial blood bypasses the lungs. All these three shunts close at, or soon after birth. Accurate knowledge of fetal circulation is necessary to understand fetal responses to hypoxaemia, and also to interpret investigations on fetal circulatory system.

3 Cellular effects of hypoxaemia

Adenosine triphosphate (ATP) is the energy currency of the body, and is the essential link between energy utilisation and energy production. Availability of oxygen is necessary for the maintenance of oxidative phosphorylation leading to energy transfer. Cells have to switch to anaerobic metabolism with lack of oxygen. Anaerobic metabolism is relatively inefficient. One mole of Glucose produces 38 moles of ATP in theory, but 30-32 moles in real life with oxidative metabolism(3). This represents 66% of the total energy stored in a Glucose molecule. In contrast, anaerobic metabolism involves breakdown of one molecule of Glucose into Pyruvate and NADH, with formation of 2 ATPs (just over 3% of total energy in a glucose molecule). The resultant Pyruvate and NADH combine to form lactic acid. Thus, lack of oxygen leads to a build-up of lactic acid (metabolic acidosis).

Oxygen must reach the fetus across the placenta. In the fetal sheep, placental utilisation is 40% of the total uterine oxygen uptake, the remaining being delivered to the fetus. Bonds et al(4) showed that in human pregnancies, placental oxygen consumption is 0.58 mM/min, whereas fetal oxygen consumption was 0.98 mM/min. Thus, placental oxygen consumption is 40% of the total oxygen consumed even in the human pregnancy. The placenta is metabolically much more active (per unit mass of tissue) as compared to the fetus. Factors that determine oxygen transfer to the fetus are affinity and oxygen capacity (Hb concentration) in maternal and fetal blood, blood-flow to the placenta, permeability of trophoblast cells intervening the two circulations, the total surface area available for transfer and oxygen consumption by the placenta(3). In human pregnancies, assuming uterine blood flow of 750 ml/min and maternal Hb of 120 g/L, the O_2 delivery can be calculated to be 5.7mM/min at term. Oxygen uptake of the normally grown human fetus at term is approximately 6.6 ml/kg/min (0.3 mM/kg/min), and is not significantly affected by normal labour and delivery(5). Fetal oxygen consumption can largely be maintained in spite of fluctuations in oxygen delivery. Thus, fetal and maternal placental blood flows and blood oxygen capacities can be altered by as much as 50 % without any major change in fetal oxygen uptake. However, there is a critical threshold below which fetal oxygen uptake becomes dependent on oxygen delivery. In the sheep this corresponds to an oxygen delivery of about 0.6 mM/min/Kg of fetal weight(3).

4 Hypoxaemia and alteration of genes

Response to hypoxaemia is characterised by altered expression of several genes. Hypoxia-inducible factor 1 (HIF-1) functions as a master regulator of oxygen homeostasis by controlling both the delivery and utilization of O_2(6). HIF-1 activity is induced by hypoxia through changes in HIF-1α mRNA and protein levels in the heart amongst other tissues. HIF-1 directly regulates the expression of more than 1,000 human genes(6). HIF-1 regulates vascular responses to hypoxia and ischemia. HIF-1 activates the transcription of multiple genes encoding angiogenic growth factors and cytokines. This effect can be studied, thanks to the availability of modern laboratory techniques such as PCR and Western blotting (please also see Table 1 in Appendix). The function of some of these genes is tabulated in the following Table:

Table 1. Genes modulated in cellular response to Hypoxaemia in cardiomyocytes.

Gene	Name	Function
HIF1-α	Hypoxia inducible factor	Master regulator of oxygen homeostasis(6)
ANGPT-1	Angiopoietin-1	Vascular development and angiogenesis(7)
ANP	Atrial natriuretic peptide	Vasodilator, reduces the water and sodium loads on the circulatory system(8)
BNP	Brain natriuretic peptide	Similar to ANP(8)
TNNC1	Troponin C	Responsible for binding calcium to activate muscle contraction(9)
ADRB1	β1-adrenoreceptor	Increases contractility(10)
PLN	Phospholamban	Activates Ca^{2+} pump(10)
ATP2A2	Sarco/endoplasmic reticulum Ca^{2+}-ATPase	Involved in regulation of the contraction/relaxation cycle(10)
STAT3	Signal transducer and activator of transcription-3	Plays a key role in many cellular processes such as cell growth and apoptosis(11)
CTP1A1	Carnitine palmitoyltransferase	Regulates fatty acid oxidation process(12)

5 Fetal cardiovascular system and Hypoxaemia

Several organ systems show adaptive changes to the lack of oxygen. The fetal cardiovascular system is perhaps the most dynamic system when adaptation to hypoxaemia is concerned. There are several differences between the adult and the fetal circulation. These differences mean that application of concepts derived from adult cardiology cannot be directly applied to fetal circulation. Many non-invasive parameters used in cardiology remain unvalidated in the fetus. Prenatally detected structural defects can be verified after birth. However, functional alterations cannot be ascertained after birth, because neonatal circulation is considerably different from the fetal circulation. Therefore, study of the function of the fetal heart and the circulation remains difficult. A thorough understanding of these differences is important before adaptations to hypoxia can be studied.

The main function of the heart is to deliver sufficient oxygenated blood to meet the metabolic requirements of the tissues(13). The overall performance is dependent on a complex interplay between the heart, blood volume and the vasculature.

5.1 The fetal circulation

Fetal circulation differs considerably as compared to adult. Placenta is an organ functional exclusively before birth, and receives 40% of the combined cardiac output in the sheep fetus(14). In human fetus, the proportion is lower(15). The right and the left ventricles pump in parallel in the fetus as opposed to in series in neonates. Oxygenated blood returning from the placenta is mixed with de-oxygenated blood. Three sites of mixing exist in the fetal circulation: the ductus venosus (umbilical vein to right atrium), foramen ovale (right and left atrium) and the ductus arteriosus (right ventricular outflow to aorta).

The human fetal blood volume is 10-12% of the body weight compared to 7 to 8% in adults(16). At mid-gestation, as much as 50% of the total fetal blood volume may be contained within the placenta, but the fraction is reduced to 20 to 25% at term in fetal sheep(15). In humans, the fraction is 33% at birth(17).

Placental vasculature is thought to account for 55% of the umbilical resistance(18). One must keep in mind the difference between resistance and impedance. Ultrasound Doppler technology is used to measure pulsatility index, which is a measure of vascular impedance. It has been shown that the infusion of vasopressor angiotensin-II to fetal sheep led to extreme

vasoconstriction, and 13-fold increase in umbilical arterial resistance(19). However, in another experiment, infusion of Angiotension II to fetal sheep did not change the pulsatility index of the umbilical artery(20). Therefore, one can conclude that increase in the resistance does not necessarily increase the pulsatility index.

Placental vasculature has no neural regulation. It has been shown that the placental blood flow is not under auto-regulatory control(21). Nor-adrenaline and Angiotensin II both lead to vasoconstriction in the placental vasculature, but the site of their major action is different(19). None lead to an appreciable increase in resistance in the cotyledons. Placental blood flow is maintained with infusion of Noradrenaline, but is significantly reduced with Angiotensin(19). Prostanoids and endothelin lead to vasoconstriction of the placental vasculature, and NO to vasodilatation. However, the exact role of hormonal regulation of placental vasculature is not fully understood(22). Placental vascular resistance progressively decreases with advancing gestation due to extensive development of vascularisation in the placenta.

5.2   Factors affecting fetal cardiac function

Fetal heart rate – Provided that stroke volume is unaltered, an increase in the heart rate increases the cardiac output. Indeed, an increase in the heart rate is an important physiological mechanism to increase the cardiac output in adults in conditions needing an increase such as exercise. Relatively high fetal heart rate is responsible, in part, for higher weight-indexed cardiac output in comparison to the adult. However, rapid pacing of the fetal heart decreases stroke volume as filling time decreases(23, 24). Increase in the heart rate is tolerated poorly by the fetal heart, and the extent to which cardiac output can be increased by increasing fetal heart rate is limited. It is interesting that the cardiac output is unchanged across a wide range of baseline heart rate. In the human fetus, it is only with significant reduction (below 55 BPM) or increase (above 210-220 BPM) of fetal heart rate that fetal cardiac failure leading to fetal hydrops is observed(25, 26).

Myocardial contractility - Contractility of the heart is the ability of the myocardium to generate a certain amount of pressure at a fixed volume. Cardiac excitation-contraction coupling is the process from electrical excitation of the cardiomyocyte to mechanical contraction of the cell. The second messenger Ca^{2+} is essential in cardiac electrical activity and is also the activator of the myofilaments which cause contraction. Ca^{2+} enters the cell through depolarization-activated Ca^{2+} channels during cardiac action potential. Ca^{2+} entry triggers Ca^{2+} release from the

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sarcoplasmic reticulum (SR). The combination of influx of Ca^{2+} from outside the cell and release of intracellular Ca^{2+} allows it to bind to the myofilament protein troponin C. This process switches on the contractile machinery of the cell. For relaxation to occur intracellular free Ca^{2+} must decline, allowing Ca^{2+} to dissociate from troponin. This requires calcium transport out of the cytosol (back into the SR), and is a process requiring energy. It is the free intracellular calcium that is responsible for contraction. Calcium is present in the bound form (to SR), and the ratio of bound to free Ca^{2+} (100:1) suggests powerful buffering(10).

There are two main ways to change the strength of cardiac contraction: by altering the amplitude or duration of the Ca^{2+} transient, and by altering the sensitivity of the myofilaments to Ca^{2+}(10). Myocardial sensitivity can be increased by stretching (Frank-Starling mechanism), Caffeine and certain ionotropic drugs. Sensitivity is reduced by acidosis, hypoxia and beta-adrenergic activation. Although beta adrenergic stimulation reduces myocardial sensitivity (by phosphorylation of Troponin I), overall effect is increased contraction force brought about by higher sarcoplasmic Ca^{2+}, and enhanced relaxation (lusitropy) by accelerated decline of free cytosolic Ca^{2+}(10).

Pre-load - Preload is the force per unit cross-sectional area of the ventricular wall just before contraction. In other words, it is the pre-contraction stretching of the chamber (end-diastolic volume). It is dependent on the blood volume, venous return and atrial contraction. Left and right fetal atrial pressures are equal(27). Increase in the pre-load has a limited ability to increase the cardiac output. Fetal left and right ventricles appear to be operating at the top of pre-load-output curve(27, 28) and do not tolerate volume overload well. Fetal myocardium is known to be less compliant than the adult(29), so that high filling pressure has a limited impact on increasing end-diastolic volume. Moreover, ventricular filling is more dependent on atrial contraction than venous return in the fetus as compared to adults.

Afterload - By definition, afterload is the load which an isolated muscle fibre has to overcome in order to shorten(30). The afterload is best defined as ventricular wall stress during ejection according to La Place's law: Wall stress = PR/2h, where P is intra-cavitary pressure, R is radius of the curvature, and h is the thickness of ventricular wall. Oxygen consumption is a function of the tension that is developed and the velocity of shortening of the unloaded muscle(31). Afterload is mainly reflected by peripheral vascular resistance and arterial impedance. In the intact heart, it is correlated to the arterial pressure, which the ventricles have to overcome, in order to eject blood. The impedance to right ventricular emptying is contributed by resistance in the pulmonary circulation as well as descending aorta supplying the lower body and the

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placenta via the ductus arteriosus. Impedance to the left ventricular emptying is contributed by resistance in cerebral circulation and the upper body. In the lamb fetus, increase in right ventricular afterload was associated with maintained right ventricular stroke volume and cardiac output(32). The ventricular volume was unchanged. Similarly, fetuses with increased placental vascular resistance are able to maintain their cardiac output(33, 34), although left ventricle contributes a higher proportion(35). The afterload (arterial pressure) for fetal left and the right ventricles should be equal, since they pump in parallel. The function of the two ventricles is closely dependent on each other. They share common muscular fibres encircling the chambers. Shunting at the level of foramen ovale allows equalisation of filing pressure (preload). Moreover, presence of the ductus arteriosus leads to equalisation of the afterload.

Neuro-hormonal influences - The fetal heart responds to hypoxia with an acute reduction in the heart rate and hypertension(36). This response is abolished by denervation of the carotid sinus. Variability of the baseline heart rate is reduced following chemical sympathectomy. However, sympathectomy did not alter fetal heart variability between episodes of umbilical cord occlusion, suggesting that this might be under hormonal rather than neural influence(37).

Growth and development - The fetal myocardium is much stiffer (less compliant) as compared to neonates(29). In fact, the compliance progressively increases with advancing gestation. This is due to a higher proportion (60%) of non-contractile elements as compared to adults (30%)(38).

5.3   The cardiac cycle

The normal cardiac cycle has five phases from end-systole to the next end-systole.

1.   Isovolumetric relaxation phase – This is the interval between the end of ventricular contraction, when the myocardial fibres begin to relax identified by closure of the semilunar valves to the beginning of ventricular filling identified by opening of the A-V valves. The volume of the ventricular contents is constant.

2.   Early diastolic filling – This begins with the opening of the A-V valves. The atrial pressure is relatively high due to continued venous return. The A-V valves open when the ventricular pressure becomes lower than the atrial pressure and the ventricular filling begins.

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Active ventricular relaxation continues in this phase, and is responsible for filling of the chamber.

3.   Late diastolic filling – This is due to atrial contraction, and gives the last 'kick' to complete ventricular filling before the onset of ventricular contraction.

4.   Isovolumetric contraction phase – This is the interval between closure of the A-V valves when rising intraventricular pressure exceeds the atrial pressure and the opening of the semilunar valves when the rising intraventricular pressure surpasses that in the aorta and the pulmonary artery.

5.   Ejection phase – This is the phase when the intraventricular blood is ejected in the great arteries.

These main components of the cardiac cycle define the main features of cardiac blood flow movement and myocardial motion and deformation. Heart failure is defined as a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood(39).

6   Assessment of fetal cardiac function using ultrasound

Hypoxaemia is a condition characterised by a reduction in the amount of oxygen carried by the blood. The fetus responds to hypoxic stress in a variety of ways. Heart is an essential organ, and vulnerable to the effect of hypoxaemia. There are several ways to study the function of the fetal heart in the presence of hypoxaemia. Ultrasound is non-invasive, and is extensively used for prenatal evaluation of the human fetus.

Current techniques of evaluation of fetal heart function using ultrasound can be divided into the following(30, 40, 41):

M-mode based techniques

6.1   Fractional shortening (FS) and ejection fraction (EF)

Ventricular end-systolic diameter (ESD) and end-diastolic diameter (EDD) are both obtained from two-dimensional M-mode images in a four-chamber view with the cursor perpendicular to the interventricular septum, just below the tip of the mitral valve leaflets(42). Maximal and minimal distances between the endocardium of the right ventricular anterior wall and the interventricular septum are measured with M-mode imaging. These distances are used to calculate the maximal end-diastolic dimension and minimal end-systolic dimension of the right ventricle, respectively. Similarly, the maximal and minimum distances between the endocardium of the left ventricular posterior wall and interventricular septum are used to calculate the EDD and ESD of the left ventricle (Figure 6-1).

The ventricular FS and EF are derived from the end-diastolic (EDD) and end-systolic (ESD) diameters as follows:

$FS = (EDD-ESD)/EDD$

$EF = (EDD^3 - ESD^3)/EDD^3$
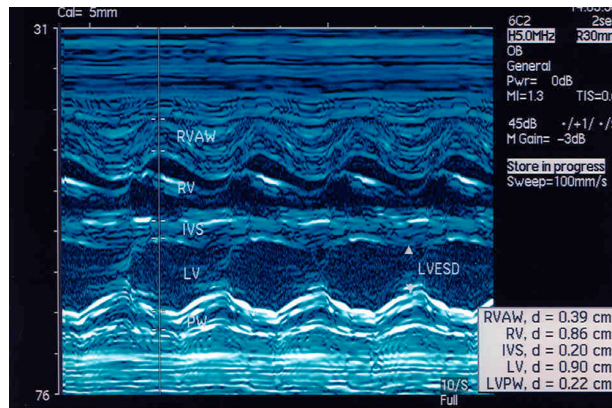
Figure 6-1 M-mode recording of a fetal heart at 24 weeks' gestation demonstrating the measurements of ventricular dimensions

RVAW = right ventricular anterior wall, RV = end-diastolic dimension of the right ventricular cavity, IVS = interventricular septum, LV = end-diastolic dimension of the left ventricular cavity, PW = posterior wall of the left ventricle, LVESD = left ventricular end systolic dimension

Acharya G et al, Current Cardiology Reviews, 2006, 2, 5-20 (Reproduced with permission from the publishers).

Hsieh et al (42) reported that LV FS as well as EF remained unchanged from 10 till 40 weeks. RV FS and EF showed a gradual decline during this period (0.86 to 0.78 for EF, and 0.49 to 0.42 for FS). Normal fractional shortening should be > 0.28(30)

6.2   Tricuspid or mitral annular peak systolic excursion (TAPSE/MAPSE)

M-mode can be used to the movement of the atrio-ventricular annulus. This moves during the cardiac cycle. The peak systolic excursion of the mitral or tricuspid valve annulus during systole has been termed as MAPSE and TAPSE respectively (Figure 6-2). The annular displacement

can also be measured using other techniques such as colour or pulsed-wave TDI and two-dimensional speckle tracking echocardiography(30).
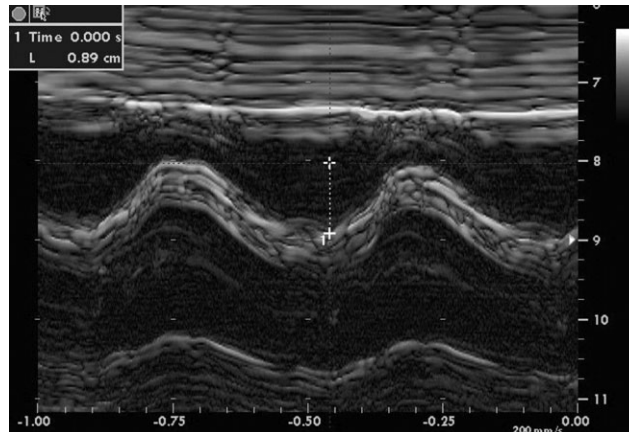
Figure 6-2 Tricuspid annular peak systolic excursion

Acharya, G. Ultrasound Obstet Gynecol 2013; 42: 125–129 (Reproduced with permission from the publishers).

Techniques based on conventional pulsed-wave Doppler

6.3 E/A ratio

Doppler assessment of the flow across atrio-ventricular valves is characterised by two peaks. The first peak is generated by the first rapid filling (E wave), and the second by the atrial contraction or 'kick' (A wave). The ratio of the amplitudes of the E and A waves generates E/A ratio (Figure 6-3). This ratio is independent of the angle of insonation, and can be used to assess fetal heart function(40). Pulse wave Doppler (PD) is used to measure the velocities by placing the sample volume just distal to the A-V valve opening in the left and the right ventricles.
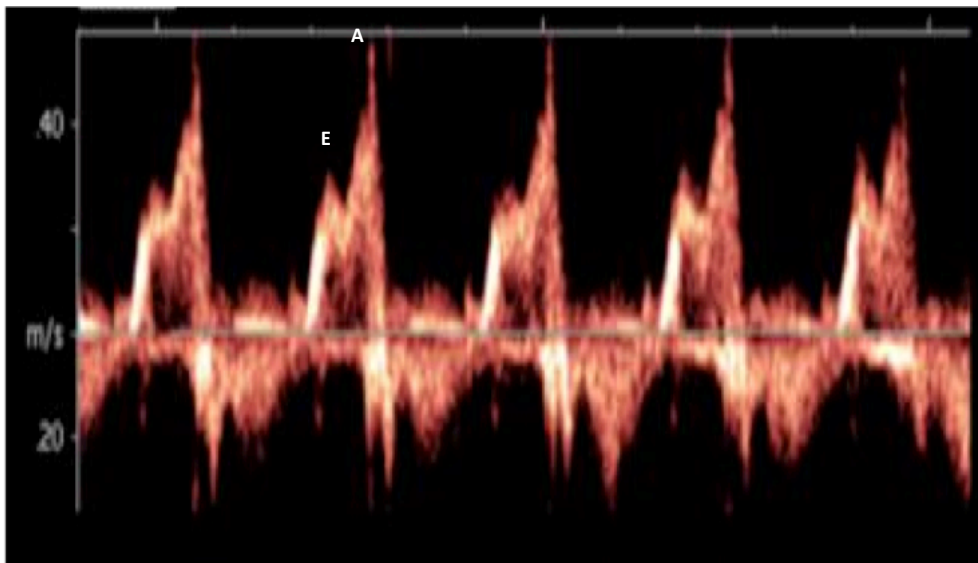
Figure 6-3 Pulse wave Doppler image showing the spectral Doppler waveform used for the calculation of the E/A ratio

6.4 Left and right ventricular output

Ventricular output can be measured with the use of grey scale and pulsed Doppler ultrasound. The diameters of the aortic and pulmonary valves are measured from frozen real-time images during systole by using the leading edge–to–leading edge method. cross sectional area of the vessel (A) is calculated with the assumption of a circular cross section. Blood velocity waveforms are obtained from the aortic and pulmonary valves using pulsed-wave Doppler. FHR (in beats per minute) is measured from the Doppler tracing, and the time-velocity integral TVI (in centimetres) is calculated by integrating the area underneath the Doppler spectrum. Volumetric blood flow (Q; in ml/minute) is calculated with the formula: Q=FHR×A×TVI. Left ventricular output (LVCO) equals the blood flow through the aortic valve and right ventricular output (RVCO) equals the blood flow through the pulmonary valve. Combined cardiac output (CCO) equals the sum of LVCO and RVCO.

6.5 Myocardial performance index for assessing global cardiac function

Myocardial performance index (MPI, also known as Tei index) was originally described to evaluate dilated cardiomyopathy(43). It is defined as the ratio between the sum of the duration of isovolumic contraction and isovolumic relaxation phases to the duration of the ejection phase

(Figure 6-4). Traditionally, this index has been calculated from the pulsed-wave Doppler (PD) recordings of ventricular inflow and outflow blood velocity waveforms. (please see the section on cardiac cycle). Within the index, the ICT and IRT reflect systolic and diastolic cardiac function respectively. The technique was refined by Hernandez-Andrade et al(44). They used the opening 'clicks' of the A-V valves to mark the timings, and showed that this technique was associated with a lower variation and better inter- and intra-observer agreement than the previously used methods.
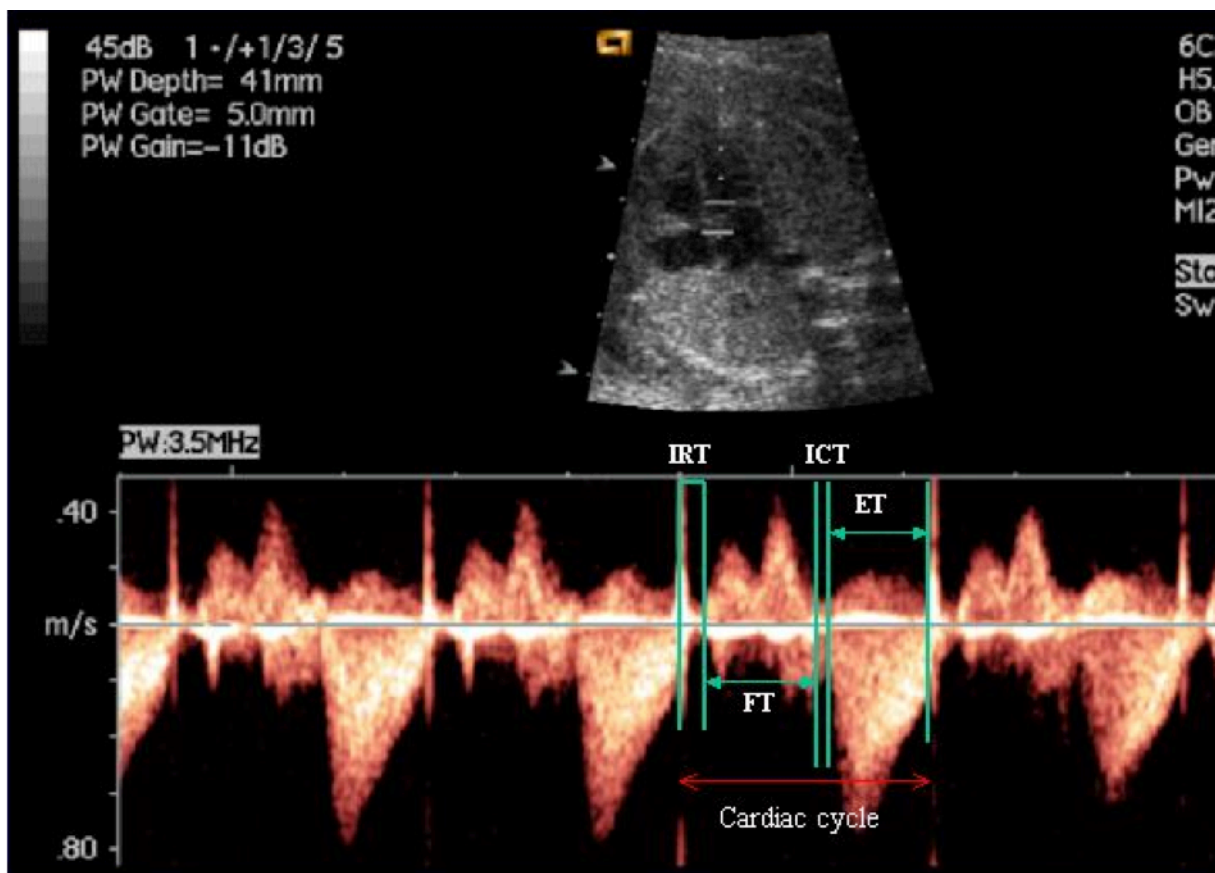
Figure 6-4 Tei index (MPI) evaluation using pulsed Doppler ultrasound.

Left ventricular Tei index measurement using pulsed-wave Doppler-derived blood flow velocity waveforms obtained simultaneously from the mitral and aortic valves. IMP = (ICT + IRT)/ET, where ICT is isovolumic contraction time, IRT is isovolumic relaxation time, and ET is ejection time. FT = ventricular filling time.

To calculate the LV Tei index (MPI), pulsed-wave Doppler (PD) blood velocity waveforms are obtained simultaneously from the inflow and outflow of the left ventricle, and measurements of ICT, IRT and ET can be made from the same cardiac cycle. However, this technique is not applicable for the right ventricle. Simultaneous blood flow velocity waveforms of the right ventricular inflow and outflow cannot easily be obtained because of the anatomical relationship of the right ventricle and its outflow tracts. Therefore, measurements of the components 'a' and

'b' of the cardiac cycle from the PD blood velocity waveforms of the RV inflow and outflow need to be obtained one after another from separate cardiac cycles (Figure 6-5). Consequently, measurement of the individual duration of RV ICT and IRT is not possible using PD.
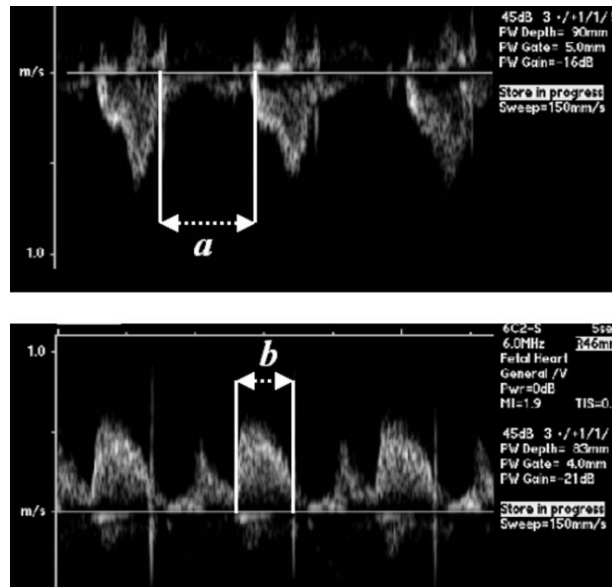
Figure 6-5 Right ventricular Tei index measurement using pulsed-wave Doppler-derived blood flow velocity waveforms obtained from the pulmonary and tricuspid valves in series.

a = time interval from closure to opening of the tricuspid valve; b = time interval from opening to closure of the pulmonary valve (ejection time); Tei index = (a − b)/b.

Acharya G et al, Ultrasound Obstet Gynecol 2008; 31: 406–411 (Reproduced with permission from the publishers).

MPI has been shown to reflect left and right ventricular function in adults, children and neonates. It combines systolic and diastolic function. Initial reports showed that it is independent of age, geometric assumptions, heart rate (HR), and blood pressure (BP)(45). This is not completely true though, and the MPI is influenced by loading conditions. Eidem et al(46) reported that left and right ventricular PD MPIs were no different in fetal life, and the indices were not significantly different from those observed in children between the age of 3 and 18

years. There was no significant change with gestational age. Similar findings have been reported by other groups(47).

Van Mieghem et al(48) validated left ventricular MPI against E/A ratio (diastolic function) and ejection fraction (EF, for systolic function) in normal fetuses as well as fetuses with twin to twin transfusion syndrome (TTTS). They reported an inverse correlation between MPI and EF (Spearman's rho = -0.43, p<0.001), but not with the observed/expected E/A index (P = 0.56).

Tei index has been studied as a possible marker of fetal heart dysfunction(49). However, the fetal circulation is quite different from adult circulation. Adult systemic and pulmonary circulations are connected in series, as opposed to being connected in parallel in the fetus. The dominant ventricle in adult life is the left ventricle. In fetal circulation, it is the right ventricle, which supplies blood to most of the body and the placenta. The fetal left ventricle preferentially perfuses the brain. In the setting of increased placental resistance, the afterload on the right ventricle is high. The resistance in the cerebral circulation, however, remains relatively low. The MPI has been reported to be increased in placental insufficiency, however it is uncertain whether it is a reflection of altered fetal cardiac loading condition or hypoxaemia. There have been a few previous attempts to study the relationship between hypoxaemia and MPI. One such study(50) reported elevated left as well as right ventricular MPI in hypoxia caused by acute cord occlusion. The effect of pure hypoxia (without an increase in the afterload) and the effect of non-acute elevation of placental resistance on fetal MPI have not been studied in experimental setting.

Techniques based on tissue Doppler

6.6   Tissue-Doppler imaging based MPI

Pulsed-tissue Doppler recordings of longitudinal myocardial wall motion are obtained at the level of mitral and tricuspid valve annuli in an apical four-chamber view for the calculation of the tissue Doppler (TD) MPI. For both ventricles, the 'a' component is measured as the time interval from the end of the myocardial lengthening velocity waveform during the late ventricular filling (atrial contraction) phase of diastole (A') to the beginning of the myocardial

lengthening velocity waveform during early ventricular filling (E'). The waves of isovolumic myocardial contraction velocity(IVCV) and relaxation velocity(IVRV) are included in this measurement. The 'b' component is the time interval from the beginning (excluding IVCV) to the end of the myocardial shortening velocity waveform (S') during the ventricular systole of the same cardiac cycle (Figure 6-6). The IRT is measured from the end of the S' wave to the start of the E' wave. The advantage of TD MPI is that simultaneous recordings of isovolumic contraction time, relaxation time and ejection time is possible in the same cardiac cycle for both ventricles. Moreover, individual measurements of ICT and IRT for the right ventricle is possible using TD MPI, which is not possible with PW MPI.
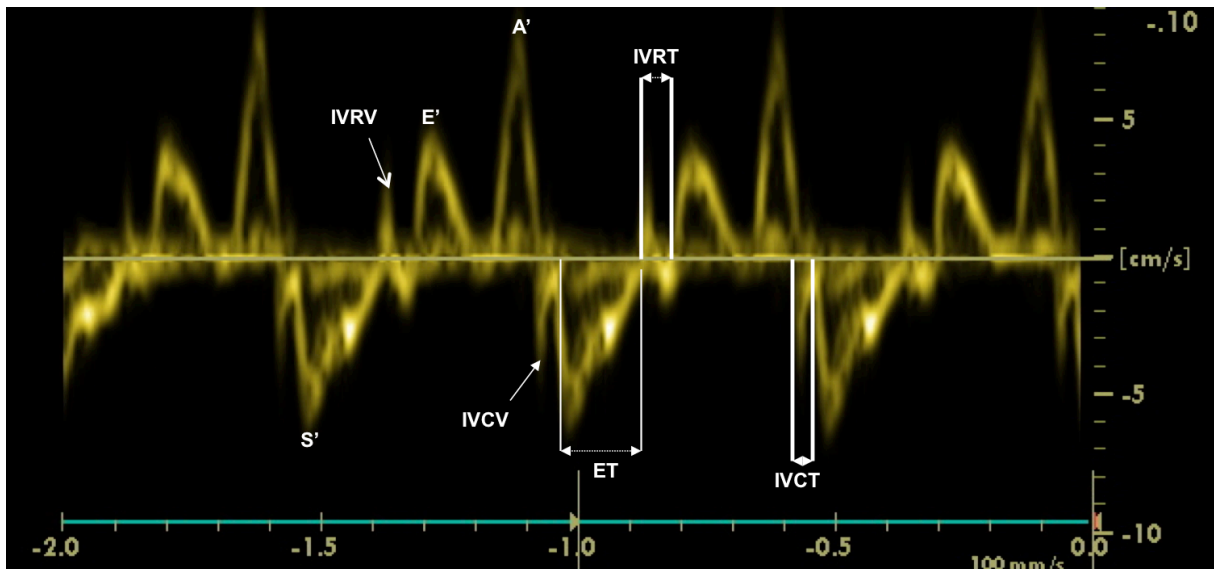
Figure 6-6 Right ventricular Tei index measurement using tissue Doppler-derived longitudinal myocardial wall motion velocities obtained at the tricuspid annulus.

A'= myocardial lengthening velocity during the late ventricular filling (atrial contraction) phase of diastole; a = time interval from the end of the A' wave to the beginning of the E' wave; b = time interval from the beginning to the end of the S' wave; E' = myocardial lengthening velocity during early ventricular filling; ICT = isovolumic contraction time; IRT = isovolumic relaxation time; IVCV = isovolumic myocardial contraction velocity; IVRV = isovolumic myocardial relaxation velocity; S' = myocardial shortening velocity during ventricular systole; Tei index = (a − b)/b.

Acharya G et al, J Pregnancy. 2011;2011:634240. (Open access resource. Permission to reproduce not required).

A comparison of PD MPI and TD MPI in the fetal heart showed that there is significant bias(51). The TD MPI is much larger as compared to PD MPI in the same individual. The most likely reason for the difference is because the myocardial tissue begins movement before there is blood flow. Tissue Doppler detects movement of the tissue, whereas pulsed wave Doppler detects blood flow. The authors reported significant correlation between PD MPI and TD MPI of right, but not the left ventricle. Correlation between MPI measured by PD and TD methods was weak and the agreement between individual measurements was poor. Therefore, the authors reported that PD and TD techniques cannot be used interchangeably to measure fetal

MPI. Reproducibility was comparable for both techniques with the intra-class correlation coefficients (ICCs) close to 0.9 for both techniques and both ventricles. These findings are different from those reported by Comas et al(49). Although they did not directly study the agreement between PD MPI and TD MPI, their mean MPIs with either technique in the IUGR as well as control fetuses were very similar.

6.7   Mitral and Tricuspid isovolumic acceleration and deceleration velocities

Despite reduced myocardial contractility, global cardiac function as measured by stroke volume and cardiac output are maintained with progressive hypoxaemia and metabolic acidosis(52). Isovolumic velocities of the mitral and tricuspid valve (See Figure 6-6) are sensitive indicators of myocardial dysfunction in response to hypoxaemia and metabolic acidosis(53, 54). These are independent of loading conditions and can be used non-invasively to measure myocardial force-frequency relationships(53).

6.8   Ultrasound speckle tracking

Conventional and tissue Doppler-based techniques are dependent on the angle of insonation, since the Doppler shift is sensitive to the angle. Angles lesser than 30^0 are recommended for reliable acquisition of data. Beyond angles of 60^0, the acquisition becomes unreliable. Ultrasound speckle tracking was developed in order to circumvent the problem of angle dependence. The use of speckle-tracking is an approach to study myocardial motion as a surrogate for cardiac function. It uses 2D B-mode echocardiography, and is based on identification of 'speckles'(55). Speckles are natural acoustic markers, spread randomly throughout the myocardium, which are generated by stable interference and back-scatter of the ultrasound signal. Distinctive speckles are identified in consecutive frames of a cine-loop. With a known frame rate, the movement and velocity vector of each speckle can be calculated. Eventually, the strain and strain rate of the chamber can be evaluated segmentally as well as globally. Speckle tracking is usually coupled with an automated border recognition program, so that speckle tracking occurs within the context of the ventricle under investigation. Speckle tracking essentially measures myocardial deformation (change of shape). Changes in strain rate may be able to identify subtle changes in cardiac function not large enough to affect the traditional measures such as the ejection fraction. Speckle tracking has not yet been studied sufficiently for use in evaluation of diastolic function(40). The advantage of speckle tracking

over conventional 2-D ultrasound techniques is that it is more sensitive(56). Another advantage over pulsed wave and tissue Doppler is that it is relatively angle independent.

Studies in adult humans have shown that exposure to normobaric hypoxia leads to an increase of regional myocardial deformation in both ventricles. The contractile reserve during hypoxic exercise is reduced in the left, but not the right ventricle(57).
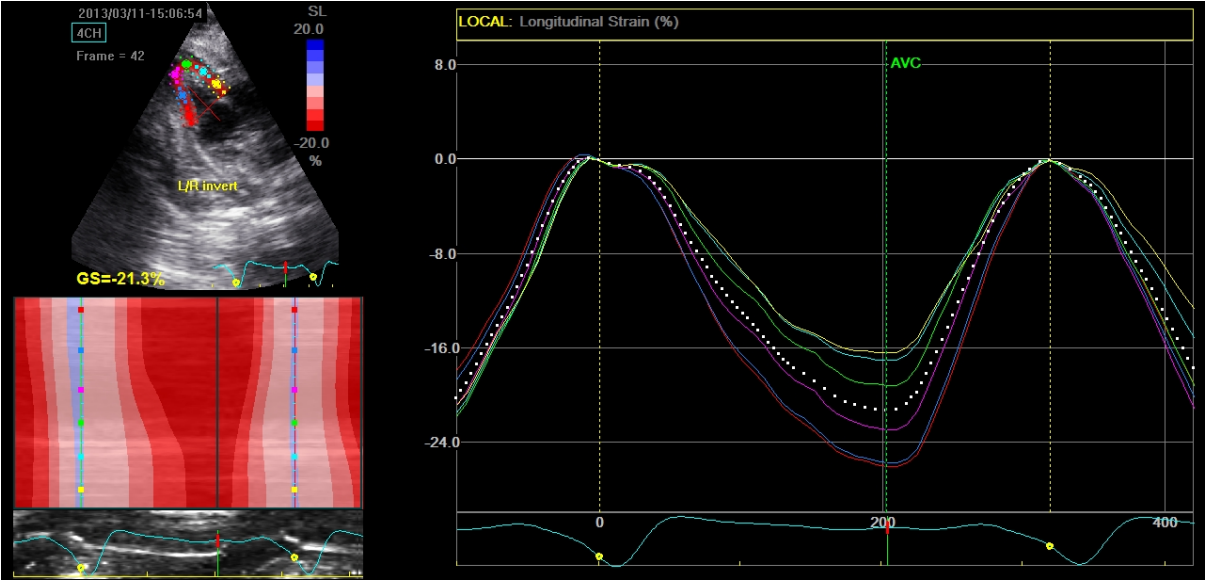
Figure 6-7 Assessment of left ventricular global longitudinal strain using Speckle tracking echocardiography

Bhide A at al, Ultrasound Med Biol. 2017 May;43(5):967-973. (Reproduced with permission from the publishers).
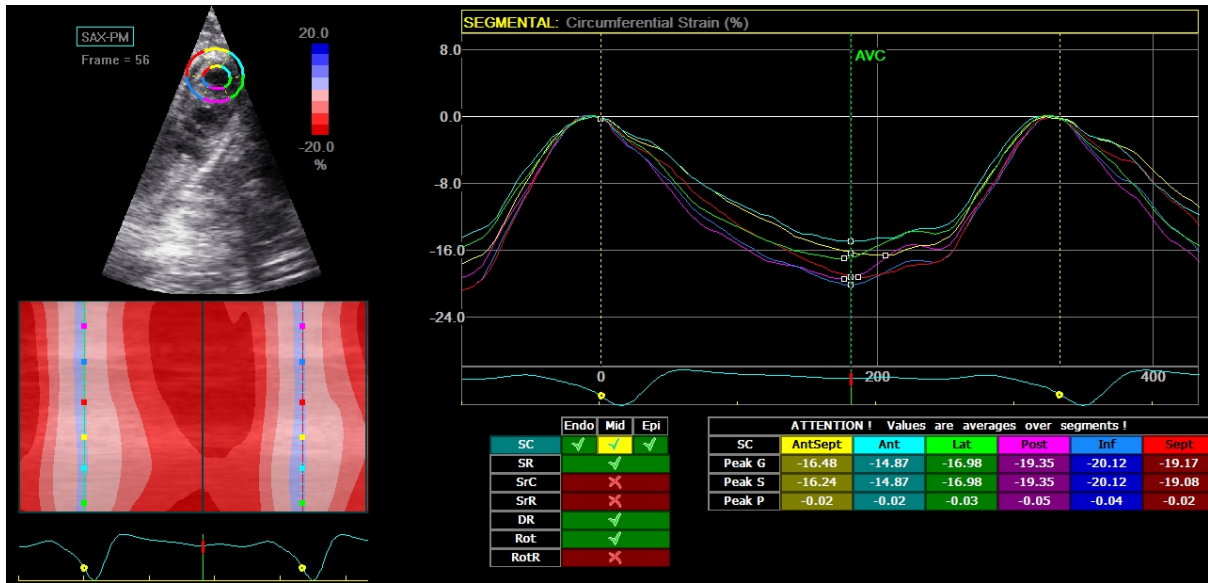
Figure 6-8 Assessment of left ventricular global circumferential strain using Speckle tracking echocardiography

(Global strain is calculated by averaging segmental values)

6.9 Tissue Doppler imaging for the assessment of cardiac cycle time intervals

Koga et al(58) reported that isovolumic contraction time (ICT) increased in pregnancies complicated by placental insufficiency, and the prolongation of ICT predicted adverse effects. This investigation used conventional pulsed-wave Doppler ultrasound. Tissue Doppler Imaging (TDI) provides information on mechanical events of the myocardium as opposed to spectral Doppler providing information on flow. The duration of each of these events can be measured using TDI. Wagstrom et al(59) showed that there is relative prolongation of the pre-ejection (mean 50%) and post-ejection (mean 38%) intervals in lambs subjected to progressive umbilical cord occlusion leading to severe hypoxia and lactic acidosis. The left ventricle and the septum showed the most pronounced differences. The right ventricle exhibited less change in the pre-ejection phase. One of the limitation of this experiment was that hypoxia was produced by occlusion of the umbilical cord. This would inevitably lead to varying venous return to the

heart, and also a change the afterload. Hypoxia caused by occlusion of the umbilical cord does not resemble the pathophysiology of fetal hypoxaemia resulting from placental insufficiency.

Techniques used to study cardiac function are described in the table below:

Table 2. Most commonly used parameters to assess fetal cardiac function (Modified from Crispi & Gratacos, 2012(41), Acharya et al, 2006(30))

Cardiac function	Index	Ultrasound modality
Systolic function	Ventricular output	2-D ultrasound and pulse wave Doppler
	Fractional shortening	M-mode ultrasound
	Ejection fraction	2-D ultrasound
	Systolic annular peak velocity	Tissue Doppler
	Strain/strain rate	Speckle tracking echocardiography
	Isovolumic acceleration velocity	Tissue Doppler
Diastolic function	E/A ratio	Pulsed wave Doppler, Tissue Doppler
	Ductus venosus PI	Pulsed wave Doppler
	Isovolumic relaxation time	Pulsed wave Doppler, Tissue Doppler
	Diastolic annular peak velocity	Tissue Doppler
Global function	TAPSE/MAPSE	M-mode ultrasound, colour or pulsed-wave TDI, speckle tracking echocardiography
	MPI	Pulsed wave Doppler, Tissue Doppler

7 Study of fetal Hypoxaemia

7.1 Choice of the animal for the experimental model to study fetal Hypoxaemia

There is a long tradition for the use of sheep as experimental models for perinatal research. The ewe is similar in size to the human mother. In contrast to many small/medium animals such as rat, mouse or guinea pig, sheep pregnancies can be limited to a single fetus. The size of the sheep fetus at birth is comparable to that of the human fetus. Moreover, sheep have a relatively long gestation and similar ontogeny for all major organ systems. Maternal and fetal blood vessels can be catheterized to directly measure blood gases, nutrient uptakes and metabolism in utero. Structure of the fetal heart and fetal circulation is essentially the same as that of human pregnancies. Another reason why sheep model is preferred is because preterm labour is uncommon even after surgical manipulation. It has a relatively fixed gestation of 145 days. The sheep model has been used to study the features of human placental insufficiency, and to evaluate therapeutic approaches to improve placental function(60).

Important differences between the sheep and humans should be kept in mind. The sheep placenta is not haemochorial like that of the humans. In fact, there are multiple discrete cotyledons. Transfer of respiratory gases and nutrients in sheep is not directly from maternal blood as in humans, but through a lymph-like fluid. The fetal ductus arteriosus is longer than that in humans. Functionally, the ductus venosus delivers oxygenated blood from the umbilical vein to the formaen ovale in the sheep as well as humans. However, the anatomy is different. In the sheep fetus, the ductus venosus joins a relatively long thoracic segment of the inferior vena cava(14). A valvular membrane directs blood to foramen ovale in the sheep fetus. In the human fetus, there is no membrane, and thoracic part of the IVC is almost non-existent. The human fetus is less dependent on the laminar flow arrangement seen in the sheep fetus. The ductus venosus projects umbilical venous blood directly towards the foramen ovale(61).

Rats and mice are easier and relatively inexpensive to maintain. The length of gestation for the mouse or rat pregnancies is short (20 and 21 days respectively). This limits the gestational age window in which to perform experiments. The pregnancies produce multiple litter. The size of each fetus is tiny, making both examination and instrumentation very difficult. The average heart rate of a mouse fetus is 500 BPM as opposed to 140 BPM in the sheep or human fetuses. It is possible that the mechanism of heart pumping is not directly comparable when the heart rates are so vastly differing. In pigs, the size of the mother is comparable to humans, but there

are multiple fetuses. Each fetus is small, and instrumentation is difficult. Although rhesus macaques(62) and baboons have been used in perinatal research, they are expensive to maintain.

7.2   Experimental models of fetal Hypoxaemia

There are several methods of creating hypoxaemia described in published literature, which suggests that no single one is ideal. Methods commonly described in the published literature are listed below. This is not an exhaustive list, but enumerates techniques commonly used by previous investigators in their publications.

1.   Reducing the partial pressure of oxygen in air inhaled by the mother – This can be achieved by attaching the mother to a re-breathing circuit(20, 62). This is an efficient method of creating acute fetal hypoxaemia, and is suitable for acute (short-term) experiments. This is the method that was used in the current research. The advantages of this method are that it does not require instrumentation of maternal or fetal vessels, and loss of animals in the preparation phase is minimal. Examination of the fetus by ultrasound scan performed through the abdomen of the ewe is extremely difficult if not impossible while the animal is awake. General anaesthesia would make this possible. The disadvantages are that animals have to be anaesthetised. Anaesthetised animals may not accurately reflect human un-anaesthetised pathophysiology. The other disadvantage is that fetal hypoxaemia in human pregnancies is rarely as a result of the mother inhaling air containing reduced oxygen content.

2.   In order to avoid subjecting the animals to anaesthetic, hypobaric chambers have been used for experimentation in animals(63). The advantage is that animals are not subjected to anaesthetic, therefore study conditions are not artificial. The disadvantages are that hypobaric chambers are expensive to build and maintain, and are available at only a few selected establishments. Moreover, continuous monitoring of maternal/fetal physiology was not possible until recently(63). The challenge of performing ultrasound examination in awake animals still remains.

3.   Experiments have been performed in human(64) and animal(65) pregnancies at high altitude. The partial pressure of oxygen at high altitude is low. This is a natural experiment in adapting to hypoxaemia. Such conditions are limited by geography and are not

available everywhere. The conditions are not similar to the vast proportion of human and animal pregnancies, and findings are not directly applicable to pathology.

4.	Restricting uteroplacental blood flow by placement of occluders around uterine vessels – Snare occluders can be placed around the maternal hypogastric arteries(66, 67). Closing of the occluder leading to a 30% reduction in uterine blood flow brings about a significant reduction in fetal arterial pO_2. Ligation of one of the uterine arteries has also been described(68).

5.	Embolising the placental circulation – In this technique, the fetus is instrumented. Through a hysterotomy, the lower half of the fetal body is exteriorised, and catheters are placed in the descending aorta through the fetal femoral artery. Bolus doses of 1 ml of 45–150 μm microspheres are injected via the femoral artery catheter into the descending aorta every 15 minutes till a fall in fetal arterial pO_2 is achieved(33, 69). This model is a good reflection of pathophysiology of chronic placental insufficiency. The disadvantage is that the use is associated with some fetal mortality, and loss of experimental material makes it more expensive. Very often, a single embolisation is insufficient, and embolisation is needed every other or every third day to achieve fetal hypoxaemia.

6.	Embolising the uterine circulation – A midline laparotomy is performed under general anaesthesia in the ewe. The main uterine arteries are catheterized via a distal arterial branch. After 3 days' recovery, hypoxaemia is induced by up to twice daily embolization of the uterine arteries with polystyrene microspheres. The frequency and volume of injections are titrated against fetal PaO_2 and lactate levels. Embolization is withheld if fetal PaO_2 was <14 mm Hg or fetal arterial lactate is >4 mmol/L(70).

7.	Placement of vascular occluders around the umbilical vessels – There are several reports describing creation of fetal hypoxaemia in acute(50, 71) as well as chronic(72) setting using umbilical cord occlusion. Occlusion of the umbilical cord has effects other than producing fetal hypoxaemia. The first vessel to get occluded in the umbilical cord is the low pressure umbilical vein. This leads to a reduction in the venous return to the fetal heart (reduced preload), hypotension and reflex increase in the heart rate. This model is suitable for

investigating the effects of umbilical cord compression in labour. However, it is not an accurate reflection of hypoxaemia resulting from placental dysfunction.

8.   Hypotension – Epidural anaesthesia is used to cause maternal hypotension. This leads to a reduction in the uteroplacental blood flow and results in fetal hypoxaemia. This technique has been used in the sheep model to create fetal hypoxaemia(73).

8 Sildenafil and fetal cardiovascular physiology

Sidenafil citrate (Sildenafil) has been developed for the treatment of erectile dysfunction(74). In normal pregnancy, the vasodilator nitric oxide contributes to the increased vasodilation and reduced vascular resistance seen in the uteroplacental circulation. The nitric oxide second messenger cGMP is enzymatically degraded by phosphodiesterases. Sildenafil, an inhibitor of phosphodiesterase-5 (PDE5), is able to enhance the vasodilatory action of nitric oxide(75). In-vitro studies have shown that exposure to Sildenafil reduces contractility in the spiral arterioles. Wareing et al(76) showed in an in-vitro experiment that myometrial small artery function was abnormal in pregnancies affected with intrauterine growth restriction (IUGR), and incubation with Sildenafil citrate reversed the increase in contraction and significantly improved endothelial-dependent vasodilatation in arteries from pregnancies complicated by IUGR. Several studies are planned where Sildenafil will be used to improve placental function in human severe early onset IUGR with dismal prognosis(77). In a recent study in sheep, fetal growth restriction was induced by embolization of the uterine artery. The lamb and placental weights of Sildenafil treated fetuses of embolised ewes were comparable to the non-embolised ewes. Lamb and placental weights of embolised but untreated fetuses were significantly lower as compared to non-embolised controls. Thus, treatment with Sildenafil was associated with higher lamb and placental weights compared to those treated with vehicle(70). On the other hand, another study(68) reported significant fetal hypotension and tachycardia in response to Sildenafil. Sildenafil is usually administered to the mother in pregnancy. Maternal Sildenafil treatment may have effects on fetal physiology. Although the effects of Sildenafil on maternal physiology are studied well, there is relative paucity of information on the effects of Sildenafil on the fetus.

8.1 Placental transfer of Sildenafil

The molecular structure of Sildenafil suggests that it should be able to cross the placenta. The work of Pellicer et al(78) demonstrates that this is actually true. These authors administered 4 mg/kg/day of Sildenafil dissolved in the drinking water to pregnant rats. They found Sildenafil citrate (SC) and its metabolite desmethyl SC in the livers of fetal rats. The levels in the fetal liver correlated significantly with maternal serum levels of SC and desmethyl SC. This work shows that Sildenafil crosses the placental barrier at least in the rat. Rat placenta, like human placenta is haemochorial, in that fetally-derived trophoblast tissue is directly bathed in maternal

blood(79). The rodent (rat and mouse) placenta has three trophoblast layers between the maternal and fetal circulations. Therefore, placental transfer of Sildenafil is likely to be easier in the human placenta, where there is a single trophoblast layer between the maternal and fetal circulations(79). We were unable to find any published reports of measurements of Sildenafil levels in the fetal blood following maternal administration. Thus, the exact fraction of maternally administered Sildenafil reaching the fetus is currently uncertain.

8.2   Is there substrate for Sildenafil in the placenta and the fetus?

Maharaj(80) and co-workers examined this issue. They took samples of the second-order chorionic plate arteries from the placentas obtained after term vaginal or Caesarean delivery. RT-PCR in homogenised chorionic arteries demonstrated the presence of Phosphodiesterase-5 mRNA. Western blotting and immunohistochemistry localised phosphodiesterase protein in chorionic plate arteries. They also investigated the effect of Sildenafil on chorionic arteries by attaching the vascular rings to a force transducer and exposing them to either Saline or Sildenafil. They showed a dose dependent relaxation of the chorionic arteries with Sildenafil in chorionic arteries pre-constricted with U46619, a Thromboxane analogue. The investigators were blinded to the presence of the active drug. This study shows that Phosphodiesterase is present in the chorionic arteries, and that it is sensitive to Sildenafil in a dose-dependent manner. These investigators also showed that Sildenafil significantly enhances the vasodilation produced by the NO donor sodium nitroprusside(80). This suggests that mechanism of action of Sildenafil involves increased responsiveness to nitric oxide.

8.3   Sildenafil and teratogenesis

Abott & co-workers(81) reviewed the safety profile of Sildenafil. They gave daily doses of Sildenafil, within and far beyond the human therapeutic range to dogs for up to one year and to rodents for up to two years. They reported very low risk of toxicity for Sildenafil. Rats and rabbits were given high doses of Sildenafil (200 mg/kg/day) during organogenesis. No teratogenicity was observed(81). In comparison, the typical human dose is 1.43 mg/kg/day. This demonstrates excellent safety profile as far as teratogenesis of Sildenafil is concerned. We

were unable to find any publications on the safety profile of a related compound Tadalafil, a longer acting PDE5 inhibitor(82).

8.4   Effects of Sildenafil on fetal heart rate, blood pressure and feto-placental circulation

Work from adult medicine shows that Sildenafil has widespread effects on smooth muscles. In clinical practice it is used to relax the pulmonary vasculature in neonates(83) and adults(84) with pulmonary hypertension. Sildenafil is known to relax myometrial fibres(85). Placental transfer of Sildenafil may have effects on fetal physiology, as the smooth muscle relaxant properties are unlikely to be restricted only to maternal circulation. Virtually all tissues and cell types express PDE5(86). Miller & co-workers induced fetal growth restriction in six sheep fetuses by ligation of one of the two umbilical arteries, and compared them to controls at 0.7 gestation. Both groups were given Sildenafil (intravenous bolus of 100 mg). They measured uterine blood flow before and after Sildenafil administration. Maternal administration of Sildenafil led to immediate reduction (-1h compared to 1h) in fetal mean arterial pressure in both groups(68). They also reported that there was no difference in the pre-treatment fetal heart rates, but Sildenafil administration was associated with an acute increase in fetal heart rate in both groups. Similar results (reduction in the blood pressure and increase in the heart rate) were seen with maternal parameters. It should be kept in mind that Miller and co-workers reported on the short-term effects of Sildenafil on fetal physiology. Proposed maternal administration of Sildenafil for maternal pulmonary hypertension or improvement in fetal growth by increase in the utero-placental blood flow is long-term. Therefore, the results of Miller et al may not be directly applicable where long-term administration of Sildenafil is being considered. Oyston & co-workers(70) also worked on the sheep model of IUGR, but did not report similar findings. They had 27 animals in three groups: controls, IUGR + Sildenafil and IUGR + vehicle. Their method of creating growth restriction was not the same as that reported by Miller et al. They induced IUGR was by embolisation of maternal uterine arteries. Sildenafil was administered to the mother by daily subcutaneous infusion of 150 mg over 12 hours using an infusion pump for 21 days. They did not report on either maternal or fetal haemodynamic parameters. Therefore, the effect of Sildenafil on haemodynamic parameters of the mother or the fetus on slower

administration of Sildenafil in this study is unknown. However, they reported an improvement in the umbilical artery blood flow impedance and higher fetal weights with Sildenafil treatment.

8.5   Effect of Sildenafil on fetal pulmonary circulation

Sildenafil is clinically used to treat pulmonary hypertension in children and adults. Fetal pulmonary vasculature is known to be sensitive to the administration of Sildenafil. Sildenafil infusion to the fetus led to 60% reduction in pulmonary vascular resistance in normoxaemic fetuses in the chronically instrumented sheep model(87). The action of Sildenafil in hypoxaemic conditions is unknown. It may be possible to increase the left ventricular output by the administration of Sildenafil if Sildenafil could increase the pulmonary blood flow, and consequently venous return to the left atrium.

8.6   Alteration of feto-placental blood flow with fetal growth restriction and the influence of Sildenafil

In the adult circulation, the left and the right ventricle are arranged in series. Therefore, by necessity, both pump the same volume of blood in a unit time. In the fetus, the ventricles are arranged in parallel. A sum of the output of both ventricles is referred to as the combined cardiac output (CCO). In the sheep model, combined cardiac output is 450 ml/min/Kg in the latter half of the pregnancy(14). The right ventricle ejects about 60-65%, and the left only 35-40% of CCO. The lungs receive 7-8% of CCO, and the remaining 55% of CCO ejected by the right ventricle passes through the ductus arteriosus(14). These measurements were obtained by the microsphere method or by electromagnetic flow transducers applied around the pulmonary trunk and ascending aorta. The placenta receives 40% of CCO in sheep (200 ml/min per kg fetal body weight).

Invasive measurements are not possible in human pregnancies. However, ultrasound has been used to estimate cardiac outputs and flows to the placenta and lungs. In the human pregnancy the combined cardiac output is about 400 ml/min/Kg, and is almost unchanged from 18-41 weeks (88). Normally, one third of the fetal combined cardiac output is distributed to the placenta in most of the second half of human pregnancy, and one fifth near term. In placental compromise this fraction is reduced while CCO/kg is maintained at normal levels, signifying

an increased recirculation of umbilical blood in the fetal body without being delivered to the placenta for oxygenation(88). Fetal pulmonary circulation is known to be increasingly responsive to blood oxygen levels with advancing gestation in the lamb(88). In normal human fetuses, the Doppler-measured impedance to flow in the peripheral pulmonary circulation decreases with advancing gestation(89). This decrease is seen till 30 weeks, and then remains stable(90). Human fetal pulmonary circulation has an important role in the distribution of the fetal cardiac output(90). The reactivity of the human fetal pulmonary circulation to maternal hyper-oxygenation increases with advancing gestation(89). This suggests that fetal pulmonary circulation is under acquired vasoconstriction at least after 31 to 36 weeks of gestation. Impedance to flow in the lungs is elevated in the presence of IUGR and this increase is related to the severity of fetal hypoxia(91). Presumably, the increase in the impedance to flow in the lungs is a compensatory mechanism for the presence of hypoxaemia seen with IUGR. Sildenafil is known to be pulmonary vasodilator. Therefore, fetal exposure to Sildenafil may change the distribution of the left and the right ventricular output, and interfere with this compensatory mechanism. There is insufficient information about fetal cardiac output of Sildenafil exposed fetuses. An alteration in the relative left and right ventricular outputs may be detrimental to the growth-restricted fetus. Future studies with Sildenafil use in pregnancy should collect information on the fetal left and right ventricular outputs.

The target of Sildenafil (PDE5) is present in fetal and placental tissues. Short-term exposure to, or bolus administration of Sildenafil leads to a reduction in fetal mean arterial pressure, and a reduction in feto-placental blood flow(68). Fetal effects of long term administration on fetal cardiovascular parameters are unknown. There are no reports of detrimental effects of long-term Sildenafil use on fetal growth. Future studies on Sildenafil administration to women during pregnancy should collect data on the effects of Sildenafil on fetal physiology.

9   Hypotheses

1.   Increased placental vascular resistance with chronic hypoxaemia leads to an increase in fetal left ventricular MPI, and an acute reduction in fetal pO_2 has a greater effect on left ventricular MPI in fetuses with increased placental resistance compared to control fetuses.

2.   Hypoxaemia is associated with a reduction in global myocardial deformation (measured by speckle tracking) and that the fetal left ventricle is more susceptible to the effect of hypoxaemia than the right ventricle.

3.   Sildenafil, a PDE inhibitor used to treat placental dysfunction, reverses pulmonary vasoconstriction resulting from hypoxaemia, increases the pulmonary venous return to the left atrium and increases left ventricular output.

4.   Sildenafil improves the myocardial dysfunction caused by hypoxaemia.

10 Aims and objectives

The overarching objective of this thesis is to examine the effect of hypoxaemia on fetal cardiac function. The specific aims are:

1.   To study the effect of both placental embolisation and maternal hypoxaemia on fetal left and right ventricular function as reflected by pulsed-wave Doppler derived myocardial performance index (MPI).

2.   To study myocardial systolic and diastolic function of the fetal left and right ventricles using ultrasound speckle tracking technique to measure global strain and strain rate.

3.   To examine if Sildenafil (Phospho-diesterase-5 inhibitor) can at least partially relieve reactive pulmonary vasoconstriction in a hypoxaemic fetus. This could improve pulmonary venous return to the left atrium and augment cerebral blood flow.

4.   To assess the effects of Sildenafil on ventricular function.

11 Methodology

This study was conducted in the Animal Laboratory of the University Hospital of Oulu, Finland. University Hospital Oulu has an established animal laboratory and active research program. The project leader has a long-standing collaboration with the University of Oulu and Oulu University Hospital, Oulu, Finland, and has been successfully performing animal experiments there for more than 14 years.

11.1 Experimental animal model

Time-dated pregnant sheep at 4/5th of the length of gestation (approximately day 115-129) were used for the study. The duration of sheep gestation is 145 days.

11.2 Study design

Separate sets of experiments were performed for each component of the study described above. The experiments where the placenta was embolized had a separate control group with intact placenta for comparison. In other experiments, baseline measurements were performed before producing hypoxaemia by maternal hypo-oxygenation and each animal was its own control. Similarly, measurements were carried out in the recovery phase after the reversal of hypoxaemia, and evaluation of the fetal cardiac function was repeated. In fetuses exposed to Sildenafil, saline was used in the control group.

11.3 Sample size

Experiments were designed in compliance with the 3-R principles (Reduction, Replacement and refinement) for using animals for experimental research. We used the resource equation method to calculate the sample size(92). According to this method, a value "E" is measured, which is the degree of freedom of analysis of variance (ANOVA). The value of E should lie between 10 and 20(92). Any sample size, which keeps E between 10 and 20 should be considered as adequate. E can be calculated as follows:

E = Total number of animals − Total number of groups

For the first set of experiments there were four groups. Therefore, we included 12 animals in the intervention and 12 in the control group. For other two sets of experiments we had two

groups. Therefore, we used approximately 10-12 animals in each group. There were several phases of the experiment, and not all fetuses survived the entire experiment. The use of linear mixed model ensured that all the data were used, and no case was excluded from the analysis due to missing data.

11.4 Research Methods

Details of the preparation of animal models have been documented in the published papers (Please refer to the appendix). All experiments took place at the animal laboratory of the University Hospital of Oulu (Figure 11-1)

Figure 11-1 The animal laboratory of the university Hospital of Oulu

In brief, the pregnant sheep of Finnish origin were operated under general endotracheal anaesthesia in order to place measurement equipment (arterial and venous catheters and flow

probes where appropriate) in the mother and the fetus. The animals were allowed to recover from the anaesthetic.

Figure 11-2 Pregnant sheep recovering from the operation

Experiments were performed after 4-5 days' recovery. In the sub-set of animals where placental embolization was performed in order to increase placental vascular resistance and simulate placental insufficiency, this was carried out 24 hours before the experiments. Baseline assessment of fetal cardiac function was performed. Replacing oxygen by medical air in the maternal re-breathing circuit produced fetal hypoxaemia, and the assessment was repeated. Re-instating oxygen reversed hypoxia, and a third set of assessment of fetal cardiac function was performed.

In the first set of the experiments, we studied the effect of both placental embolisation and fetal hypoxaemia on fetal global cardiac function using pulsed-wave Doppler ultrasound. Left ventricular inflow and outflow Doppler velocity waveforms were recorded simultaneously and the myocardial performance index [(Left ventricular isovolumic contraction time + isovolumic relaxation time)/Ejection time] was measured. Fetal myocardial tissue was stored at -80^0C. Tissue was subjected to hypoxic gene panel using polymerase chain reaction (PCR) to assess expression of hypoxic genes. We measured the mRNA levels in the fetal heart by qRT-PCR of 11 genes, representing various aspects of cardiac physiology: direct responses to hypoxia (HIF1a, angiopoietin 1), contractile function (troponin C, SERCA, phospholamban), metabolic function (carnitine palmitoyltransferase), endocrine function (ANP, BNP), neural regulation

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(beta-1 adrenoreceptor, tachykinin) and cytokine regulation (signal transducer and activator of transcription 3, STAT3).

In the second set of the experiments, we studied the changes in regional myocardial function of the fetal heart in response to hypoxaemia. Ultrasound speckle tracking, which is an angle independent technique for assessing myocardial function, was used for this purpose. A commercially available software (Echopac®) was used for the calculation of global strain and strain rates. The evaluation of strain and strain rate was performed off-line on stored loops of the ultrasound examinations carried out during the experiments. The measurements of global strain and strain rates were performed in duplicate in order to assess reproducibility.

In the third set of the experiments, we compared the effect of fetal hypoxaemia on left and right ventricular cardiac function and its modification by administration of Sildenafil. In this experiment, pregnant sheep were operated under general anaesthetic for instrumentation of the fetus. A midline laparotomy was performed

An ultrasonic transit-time flow probe (Figure 11-3) was placed around the ductus arteriosus (Figure 11-4) to measure ductal blood flow.
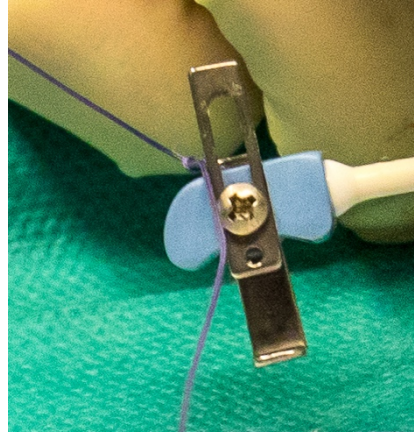
Figure 11-3 Ultrasonic transit-time flow probe used in the experiment

Figure 11-4 Specimen of the fetal heart in sheep shown after the end of the experiment

A red rubber band is put around the ductus arteriosus.

The ductus arteriosus was dissected (Figure 11-5) to allow placement of the flow probe around the ductus arteriosus (Figure 11-6).
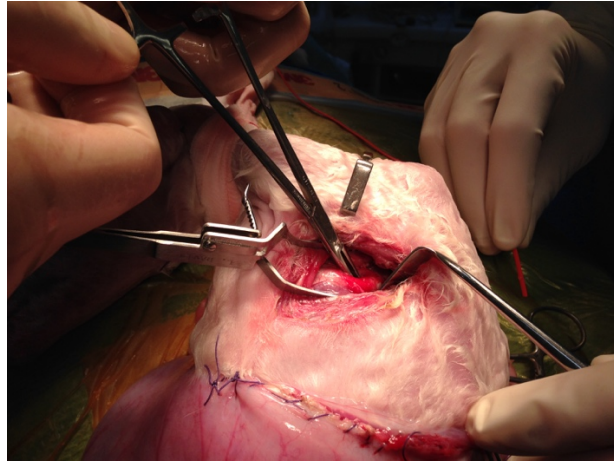
Figure 11-5 Ductus arteriosus is dissected in the fetal thorax following a thoracotomy.
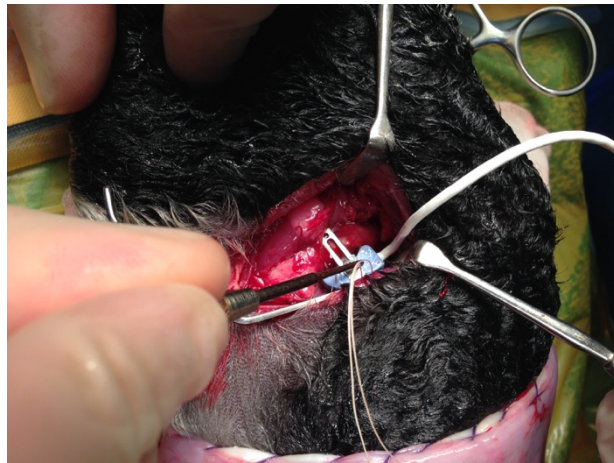
Figure 11-6 Ultrasonic transit time flow probe is secured around the ductus arteriosus

The systolic function was evaluated using ventricular outputs, global longitudinal strain/strain rate with speckle tracking echocardiography and isovolumetric contraction velocities of the ventricular wall at the level of the mitral and tricuspid valves with tissue Doppler. Diastolic function was evaluated using isovolumetric relaxation velocities using tissue Doppler and pulsatility index of the ductus venosus with pulsed wave Doppler ultrasound. Pulmonary blood flow was calculated by subtracting the flow through the ductus arteriosus from right ventricular output. Pulsatility index of the right pulmonary artery was measured with pulsed wave Doppler ultrasound. The measurements were obtained at baseline, with hypoxaemia and at recovery in

fetuses receiving intravenous Sildenafil citrate and compared to those receiving intravenous saline infusion.

11.5 Ethics approval

All these experiments are a part of an on-going project investigating fetal cardiovascular function in the chronically instrumented sheep model. The research protocol is approved by the National Animal Experiment Board in Finland (Approval no. ESAVI/1007/04.10.07/2014, Date of approval:13.03.2014, valid until 14.03.2017). The animal care and experimental procedures were conducted according to the national legislation and the EU Directive 2010/63/EU(93).

11.6 Data Analysis

Collected data were entered into a database, and checked for quality and completeness. Data analysis was performed using commercial statistical software (SPSS and SAS). Data distribution was tested for significant deviation from normality using Kolmogorov-Smirnoff test. To study differences between groups, independent sample student-t test was used for parametric and Mann-Whitney U test for nonparametric data. Paired t-test was used for longitudinal data with two states. Chi-square test or Fisher's exact test were used to compare categorical data. Linear mixed model analysis was used to analyse data on repeated measurements taking into account correlation of measurements carried out in an individual fetus at different time-points. Random intercept model was selected. $p < 0.05$ was considered significant.

12 Summary of results

12.1 Paper 1

In this experiment we evaluated global myocardial function with pulse-wave Doppler-based myocardial performance index. Placental vascular resistance and umbilical artery PI increased significantly with placental embolization. This was accompanied by significant fetal hypoxaemia. Fetal pH and base excess were no different. Left, right ventricular and combined cardiac outputs were comparable. Left and right ventricular MPI were no different at baseline.

Attaching the mother to a re-breathing circuit led to significant reduction of fetal arterial pO_2 in both embolized and control groups. Fetal heart rate and ventricular outputs did not show significant changes. During hypoxaemia, mean LV MPI increased significantly only in fetuses with an intact placenta, returning to baseline during the recovery phase. Right ventricular MPI was unaffected. We also studied the expression of hypoxic genes in fetuses with and without placental embolisation. In general, expression of the hypoxaemic genes (HIF1α and ANGPT1) was no different with or without placental embolisation. Significantly lower expression of genes involved in cardiac contractile function (TNNC1 and ATP2A2), as well as its regulation (ADRB1) were seen in the group with placental embolisation. An explanation of the findings is that hypoxaemia leads to upregulation of cardiac genes in both groups, but less so with prior placental embolization. The ewes (and fetuses) were exposed to hypoxaemia for 15 minutes. It is possible that this period was too short to have an effect on the expression of hypoxic genes. Lower expression of genes involved with contractile function suggests that exposure to hypoxaemia has an effect on myocardial cellular calcium handling.

We concluded that fetal left ventricle is more sensitive to acute hypoxaemia than the right ventricle. Exposure to chronic hypoxaemia could pre-condition the fetal heart and protect its function with worsening hypoxaemia.

12.2 Paper 2

In this manuscript we examine the effects of Hypoxaemia on fetal myocardial contractility using speckle tracking echocardiography. We hypothesized that in near-term sheep fetuses

hypoxaemia changes myocardial function that is reflected in altered ventricular deformation on speckle-tracking echocardiography.

Baseline mean (SD) LV and RV global longitudinal strains were -18.7% (3.8) and -14.3% (5.3). Baseline RV global longitudinal and circumferential deformations were less compared to LV (p=0.016 and p<0.005). Although baseline right ventricular output is more than the left, baseline right ventricular global longitudinal and circumferential deformations are less compared to the left ventricle. LV, but not RV global longitudinal deformation reduces with Hypoxaemia compared to baseline. Circumferential and radial strains do not show significant changes.

We concluded that, in near-term sheep fetus, LV global longitudinal and circumferential strains are more negative compared to RV. Acute hypoxaemia leads to LV rather than RV dysfunction as demonstrated by decreased deformation.

12.3 Paper 3

We examined the effect of acute hypoxaemia on fetal central cardiovascular parameters by a variety of indices, and its modification by administration of Sildenafil. We tested the hypotheses that Sildenafil reverses pulmonary vasoconstriction resulting from hypoxaemia, increases left ventricular output by increasing pulmonary venous return and ameliorates myocardial dysfunction caused by hypoxaemia. We show that exposure to acute hypoxaemia influences fetal central cardiovascular function in the chronically instrumented fetal sheep model. We examined 24 pregnant sheep. Fetuses were made hypoxaemic and then given intravenous Sildenafil or saline infusion. Blood flow through ductus arteriosus was measured with an ultrasonic transit-time probe. Fetal cardiac function was examined using echocardiography.

Fetal hypoxaemia led to pulmonary arterial vasoconstriction, decreased lung volume blood flow, increased shunting through the ductus arteriosus, and reduced left ventricular cardiac output. Mean arterial pressure and fetal pO_2 were lower in the Sildenafil group at the end of the experiment. Sildenafil, when given directly to a hypoxaemic fetus, could not reverse this

redistribution of cardiac output. Furthermore, fetal cardiac systolic and diastolic dysfunction observed during hypoxaemia could not be ameliorated by Sildenafil treatment.

13 Discussion

Fetal hypoxaemia is encountered commonly in fetal life. Fetal supply of oxygen from the mother is by diffusion, and not by active transport. Therefore, all fetuses are significantly hypoxaemic as compared to their mother. Further restriction of oxygen can be a challenge to the fetus, and there are several adaptive mechanisms. As mentioned previously, fetal and maternal placental blood flows and blood oxygen capacities can be altered by as much as 50% without any major change in fetal oxygen uptake(3).

Fetal hypoxaemia can potentially result from reduced oxygen content of maternal blood (maternal anaemia, pregnancy at high altitude, maternal cyanotic heart disease), reduced rate of oxygen supply due to compromised utero-placental blood flow (maternal vascular disease such as pre-eclampsia), placental dysfunction (loss of placental villi), reduced fetal blood reaching the placenta for exchange (umbilical cord compression, typically intrapartum), reduced oxygenation of fetal blood (fetal anaemia) or reduced utilisation of the oxygen by the fetus (congenital fetal metabolic disorders). Moreover, available oxygen may be insufficient due to high fetal demands (macrosomia/diabetic fetus). Detection of significant fetal hypoxaemia is important for the diagnosis of fetal compromise.

In the current series of experiments, we assessed the fetal cardiovascular response to hypoxaemia in an experimental model of sheep pregnancy with the view to understand the pathophysiology. We used two methods to create acute and chronic hypoxaemia. Chronic hypoxaemia was created by embolisation of the placental circulation by placing catheters in the fetal descending aorta. Acute hypoxaemia was created by reducing the oxygen content of the air breathed by the ewe. Placental embolisation and creation of hypoxaemia mimics the pathophysiology of fetal growth restriction resulting from placental dysfunction. General anaesthesia is necessary to subject the animals to ultrasound examinations. Therefore, manipulation of oxygen inhaled by the mother is a convenient method to create fetal Hypoxaemia.

Fetal MPI is known to reflect global myocardial function(43). It is also known to be a sensitive parameter of cardiac dysfunction(94, 95). MPI is elevated in fetuses affected with placental insufficiency(49). However, it is uncertain whether the change in MPI is a reflection of hypoxaemia, changes in cardiac loading conditions or a direct effect of myocardial cell damage. Placental vascular resistance is increased in fetuses with placental insufficiency, and can affect MPI due to increased afterload. In a previous report demonstrating increased left and right

ventricular MPI with hypoxaemia, umbilical cord occlusion was used to create hypoxaemia(50). We showed that left and right ventricular MPI was comparable in the two groups of fetus with and without increased placental vascular resistance, although fetuses with embolised placentas had significantly lower arterial pO_2. Placentas were embolised at least 24 hours before the experiment. MPI was measured using pulsed wave Doppler. Therefore, fetal myocardial function appears to adapt to increased placental vascular resistance and hypoxaemia. We further showed that acute hypoxaemia led to a significant increase in the left (but not right) ventricular MPI, but only in fetuses with intact placentas. LV MPI changes were not observed in the group of fetuses where the placental vascular resistance was already elevated by placental embolization. This shows that LV MPI changes may not be seen with significant further hypoxaemia in fetuses already compromised. We speculate that prior compromise by placental embolisation could pre-condition the fetal heart so that further hypoxaemia is tolerated without myocardial dysfunction. There is evidence in the literature for this pre-conditioning theory.

Significant hypoxaemia leads to cellular injury and cellular death. Tissues respond to sub-lethal levels of hypoxaemia by upregulation of genes involved with cell survival. This hypoxaemia may be local or systemic. The ability of various tissue to withstand hypoxaemia is different. Therefore, significant hypoxaemia should be defined in relation to oxygen tension normally experienced by a cell type or tissue. hypoxia inducible factors play a central role in the ability of tissues/cells to withstand further episodes of hypoxaemia. Induction of HIF during repeated short episodes of ischaemia appears to provide resistance to a subsequent protracted ischaemic event(96).

LV MPI appears to be a good test for acute rather than long-standing hypoxaemia. There is a modest overlap between the two groups of normoxaemic and hypoxaemic fetuses. Therefore, an absolute value is not particularly useful, and serial assessment have the advantage of each fetus acting as its own control.

It has been shown in human fetuses that global longitudinal strain is similar in both ventricles at 20-24 weeks of gestation(97). Later in pregnancy LV global longitudinal strain does not change, however, corresponding RV strain or deformation decreases(98). This is explained by an increase in the RV preload. A reduction in the LV global longitudinal deformation with hypoxaemia may be a result of reduced LV preload, direct LV myocardial dysfunction or a combination of the two. It has been shown that pulmonary circulation is sensitive to fetal oxygenation during the last trimester of pregnancy(89), and that hypoxaemia leads to

pulmonary vasoconstriction(62). Indeed, in the current experiment RPA PI values increased significantly with hypoxaemia suggesting that impedance to pulmonary blood flow increased during hypoxaemia, although the reduction in the estimated pulmonary volume blood flow was not statistically significant. It has been shown in a near-term sheep model of acute ductus arteriosus occlusion that the fetus is unable to increase volume blood flow across the foramen ovale(99), thus the reduction in the LVCO is most likely a consequence of a drop in pulmonary volume blood flow leading to a decrease in LV preload. If the change in preload would be the major factor in the strain formation, we should have observed an opposite finding in the LV global longitudinal strain. Therefore, our results indicate that the direct effect of hypoxaemia on intrinsic contractile properties of the LV is likely responsible for decreased global longitudinal strain.

13.1 Validity of the results

The sheep model has been extensively used for research in fetal hemodynamics. Guorang(50) et al reported on the effect of hypoxaemia with umbilical cord compression on the left and right ventricular MPI. The lowest fetal arterial pO_2 reported by those authors in the cord occlusion group was 21.7 mm Hg (2.85 kPa). In contrast, the pO_2 levels in the intact placenta group in our experiment were 2.3 kPa (17.5 mm Hg) and in the placental embolisation group was 1.2 kPa (9.1 mm Hg). The decline in pO_2 in the study of Guorong et al was 20% compared to 30% in the intact placenta fetuses and 38% in placental embolisation group in our study. The RV and LV MPI at baseline in the study by Guorong et al were 0.29 and 0.31 respectively, compared to 0.24 and 0.34 respectively, in our study. Although the LV MPI values in the two studies are very similar to each other, the RV MPI values are quite different. Right ventricular MPI needs to be calculated from separate cardiac cycles, because it is not possible to image the tricuspid and pulmonary valve simultaneously. Therefore, when pulsed wave Doppler is used, the accuracy of measurement is inferior for right ventricular MPI as compared to the left

ventricle. This is the most likely reason why the RV MPI values in the two studies are so dissimilar.

Our results are in agreement with human fetal studies of placental insufficiency indicating comparable weight-indexed cardiac outputs for fetuses with normal placental hemodynamic findings(88).

We used Sildenafil as an intravenous infusion to the fetus. Studies on the use of Sildenafil in placental insufficiency administer the drug to the mother over a period of days to weeks(70, 100). Oyston et al, in a sheep model used continuous subcutaneous infusion to the ewe(70). Oral administration to the mother was used in humans(100). There is published evidence showing fetal transfer of maternally administered Sildenafil(78). However, extrapolation of these results to human pregnancy should be done cautiously. The use of anaesthesia may have affected the results of the experiment. The experimental conditions are different from normal physiology, because the sheep and the fetus are under a general anaesthetic. Isoflurane can modify fetal cardiovascular regulation. However, newborn lambs under isoflurane anaesthesia are able to increase cardiovascular performance during stress(101). Previous work has shown that uterine and placental volume blood flows are comparable before and after general anaesthetic(34), suggesting unaltered cardiovascular response. Therefore, the results are likely to be close to physiologic conditions.

Indexed cardiac output, baseline arterial pO_2 and arterial pO_2 following hypoxaemia are remarkably similar to those reported by Cohn et al(102), where the animals were not anaesthetised.

13.2 Strengths and weaknesses

The surgical procedures may constitute a significant stress on the sheep fetuses, and it may be argued that the conditions are quite different from human fetuses exposed to hypoxaemia. Normal arterial blood gas values at the baseline stage suggest conditions close to physiologic circulatory state(34). The study was carried out in a narrow gestational age window of 115-129 days, and may limit the validity and significance outside this time period. Extrapolation of these results to human pregnancy should be done cautiously. The sheep model has been extensively

used for research in fetal hemodynamics, and the myocardial strain curves are similar in normal ovine and human pregnancy.

We wished to assess the effect of fetal hypoxia caused by reducing the oxygen delivered to the mother on duration of events in the cardiac cycle. This has obvious implications on developing more sensitive tests for subtle cardiac dysfunction resulting from fetal hypoxia. Use of animal model allowed us to measure (and control) fetal oxygenation using invasive monitoring. It could be argued that the reduction in LV global longitudinal strain could just reflect a drop in cerebral vascular resistance during hypoxaemia. Hypoxaemia leads to pulmonary vasoconstriction, reduction in pulmonary volume blood flow and therefore less pulmonary venous return to the left atrium. The capacity to increase shunting across foramen ovale to compensate reduced left atrial filling is limited(103, 104). So, hypoxaemia could reduce left ventricular output by reduced pre-load. We did not monitor cerebral blood flow during the experiment. This is because the vascular catheters were placed in the left carotid artery and the left jugular vein of the fetus to measure fetal blood pressure and obtain blood gases and the presence of the catheter in the carotid artery would mean that measurements changes in MCA PI would be unreliable and difficult to interpret. However, we measured the fetal blood pressure from the carotid artery, which is a direct reflection of left ventricular afterload.

Analysis of the ultrasound images was performed by examiners blinded to the allocation. Therefore, it is unlikely to result in a bias. All volume flow measurements were indexed to fetal weight, eliminating the effect of the size of the fetus. The surgical procedures may constitute a significant stress on the sheep fetuses, and it may be argued that the conditions are quite different from human fetuses exposed to hypoxaemia. Normal arterial blood gas values at the baseline stage suggest conditions close to physiologic circulatory state. Availability of invasive data and oxygenation lend important validation to the results. This is usually impossible in human setting.

14 Conclusions

1. Placental vascular resistance and umbilical artery PI increase significantly with placental embolization. This is accompanied by significant fetal hypoxaemia. Fetal pH and base excess as well left, right and combined ventricular cardiac outputs are maintained.

2. Fetal left ventricle is more sensitive to acute hypoxaemia than the right ventricle. Exposure to chronic hypoxaemia could pre-condition the fetal heart and protect its function with worsening hypoxaemia.

3. Hypoxaemia may lead to upregulation of cardiac hypoxic genes in both groups with and without placental embolisation, but less so with prior placental embolization.

4. In the near-term sheep fetus, left ventricular (LV) global longitudinal and circumferential deformations are greater compared to the right ventricle (RV).

5. Acute hypoxaemia leads to LV rather than RV dysfunction as demonstrated by reduced deformation assessed using speckle tracking echocardiography.

6. Acute hypoxaemia is associated with pulmonary vasoconstriction, and increased shunting through the ductus arteriosus. It is also associated with reduced myocardial deformation as well as reduced isovolumetric contraction and relaxation velocities.

7. Sildenafil does not alter the fetal pulmonary vasoconstriction in response to hypoxaemia. Sildenafil does not alter the parameters of myocardial function.

8. Fetuses exposed to Sildenafil had a lower arterial pO_2 in the recovery phase of the experiment. This potentially detrimental effect needs further investigation.

15 Future perspectives

Addition of biomarkers to ultrasound evaluation may improve the assessment of response of the fetal heart function to hypoxaemia. Myocardial global longitudinal strain measurements for clinical use in suspected hypoxaemia in human pregnancies need validation studies. Future studies on the use of Sildenafil will benefit by the results of this study that pulmonary blood flow is unaffected by the intervention. The potentially detrimental finding that fetuses exposed to Sildenafil had a lower arterial pO_2 in the recovery phase needs further investigation. Future studies should include the assessment of fetal cerebral blood flow to make sure that the adaptive fetal blood flow redistribution is not disturbed by the use of Sildenafil in complicated pregnancies.

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17 Supplementary tables

17.1 Primers and bifunctional probes (5'-FAM, 3'-TAMRA) used in the qRT-PCR reactions.

The sequences are listed in the 5' to 3' direction. (Supplementary table in Paper I).

| Gene	Forward primer	Reverse primer	Probe
ADRB1	AGACGCTCACCAACCTCTTCA	GGCACTACCAGCAGACCCA	CTGGCCAGCGCAGACCTGGT
ANGPT1	GCAAATGTGCCCTCATGCT	TCCATTTAGATTGGATGGGCC	ACAGGAGGCTGGTGGTTTGATGCC
ATP2a2	TGCTCTTTTCTGGCACAAACA	TAACGCCAGTTGCCACCAC	TGCTGCTGGCAAAGCCATGGG
CPT1A	CCAGTTCACAGTCACCCCG	GATCTGCCTGAGGGCCTCA	ATGGCATCGACCTCCGAATGAGCC
HIF1A	CATGCCCCAGATTCAAGATCA	TGGGACTGTTAGGCTCAGGTG	CCAGCTAGTCCTTCTGATGGAAGCACCA
NPPA	TTCCTCCTCTTTGTGGCGTT	CACAGAGCCATACACGGGATT	CAGCTCCCAGGGCAAACAGGAGC
NPPB	GACCCCCAGAAGGCGC	TAGCAGCGACAGGTGCAAGA	TCCCGAACGCTCCTGCTTCTCCT
PLN	AATACTATATAAGAGACCACTTCCTAAATAGAAGAA	GCAGATGAATACGTTTGCTAACAATT	TTTGTGAAAAGGTCGAGATTTGGAACAGAGC
STAT3	ATCGACCTGGAGACGCACTC	AGGCGTTGGGCATCTGAC	TTGCCAGTGGTGGTGATCTCCAACA
TAC1	GCCCTTTGAGCATCTTCTGC	GCCCATCAATCCAAAGAACTG	ATCGCTCGGAGACCCAAGCCTCA
TNNC1	TGAGGAGCTGCAGGAGATGA	CATCAAAGTCCACTGTGCCG	TGACGAGGTGGATGAGGACGGCA

18 Supplementary figures
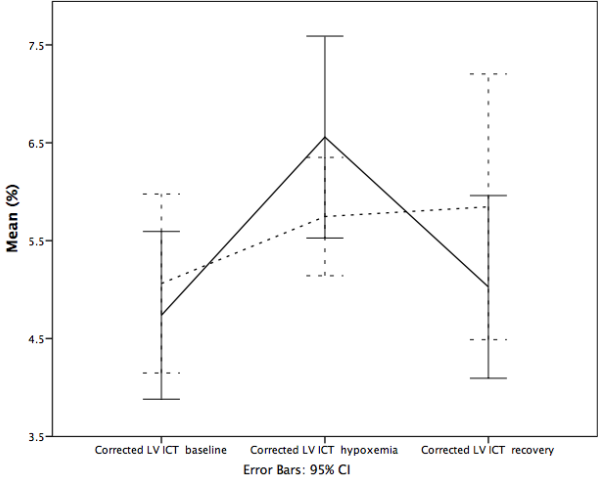
Figure 18-1 Left ventricular isovolumic contraction time with hypoxaemia and placental embolisation

(corrected for the heart rate, and expressed as a percentage of the duration of the entire cardiac cycle) Solid line: Intact placenta, Broken line: Placental embolization

(Intact placenta group: Baseline v/s hypoxaemia, p = 0.007; Hypoxaemia v/s recovery, p = 0.04). Changes not significant in the group with placental embolization (p = 0.62 and 0.37 respectively).
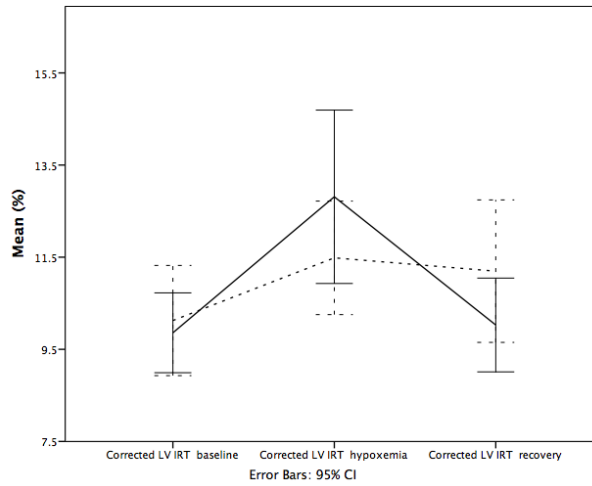
Figure 18-2 Left ventricular isovolumic relaxation time with hypoxaemia and placental embolisation

(corrected for the heart rate, and expressed as a percentage of the duration of the entire cardiac cycle) Solid line: Intact placenta, Broken line: Placental embolization

(Intact placenta group: Baseline v/s hypoxaemia, p = 0.001; Hypoxaemia v/s recovery, p = 0.024). Changes not significant in the group with placental embolization (p = 0.17 and 1.0 respectively).
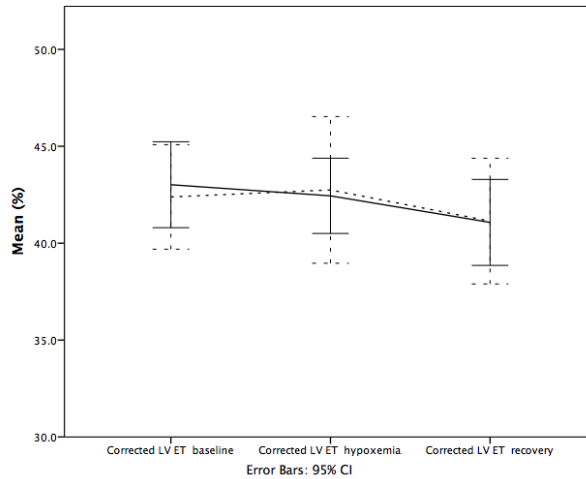
Figure 18-3 Left ventricular ejection time with hypoxaemia and placental embolisation

(corrected for the heart rate, and expressed as a percentage of the duration of the entire cardiac cycle). Solid line: Intact placenta, Broken line: Placental embolization.

No significant difference between groups or with time.
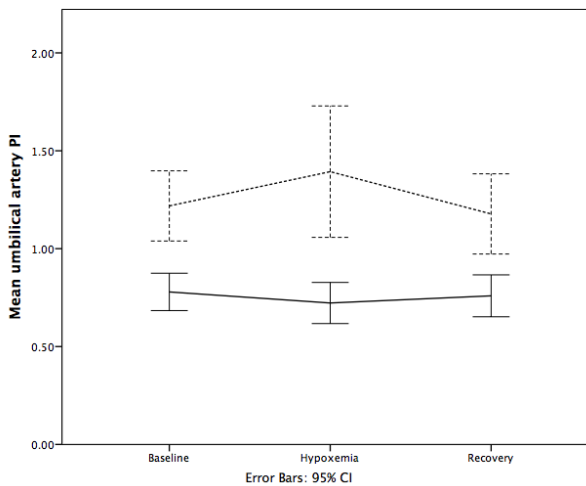
Figure 18-4 Umbilical artery PI in the two groups (with and without placental embolisation)

Solid line: Intact placenta, Broken line: Placental embolization. Significant difference between fetuses with intact placenta versus those with placental embolisation ($p < 0.0005$). No significant difference with time ($p = 0.27$).
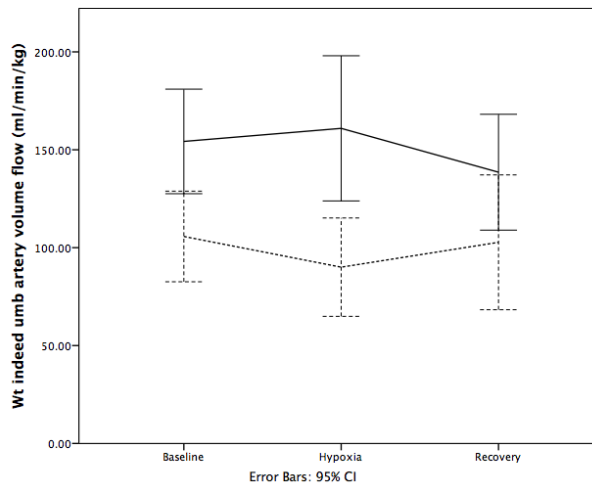
Figure 18-5 Weight indexed umbilical artery volume blood flow with hypoxaemia and placental embolisation

Solid line: Intact placenta, Broken line: Placental embolization. Significant difference between fetuses with intact placenta versus those with placental embolisation (p=0.009). No significant difference with time (p = 0.23). Significant interaction was seen (p = 0.007)

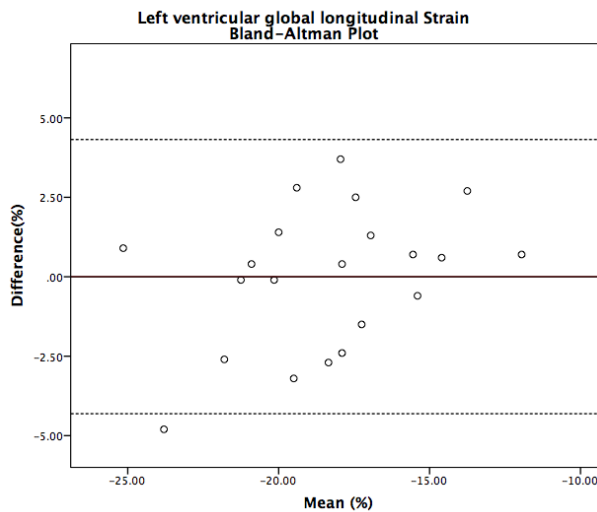Figure 18-6 Reproducibility of left ventricular global longitudinal strain measurement

Horizontal line in red shows mean difference. Dotted lines represent 95% limits of agreement.
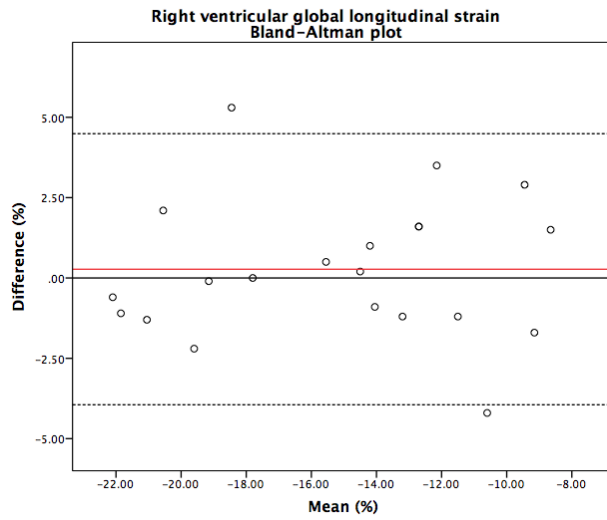
Figure 18-7 Reproducibility of right ventricular global longitudinal strain measurement

Horizontal line in red shows mean difference. Dotted lines represent 95% limits of agreement
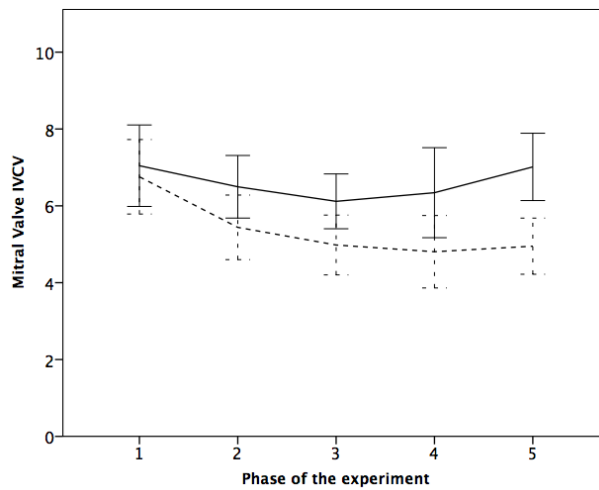

Figure 18-8 Mitral valve isovolumic contraction velocity with Sildenafil

Dotted line – Sildenafil, Solid line – Saline. Error bars indicate SEM. No significant difference was seen between groups (p = 0.318) or with time (p = 0.222)
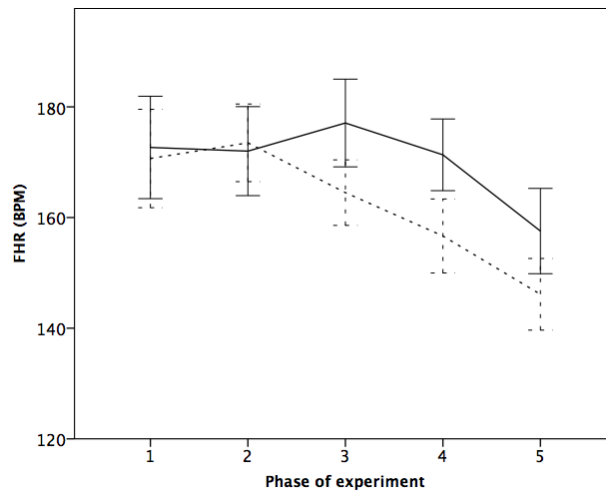
Figure 18-9 Fetal heart rate with hypoxaemia and Sildenafil

Dotted line – Sildenafil, Solid line – Saline. Error bars indicate SEM. No significant difference was seen between groups (p = 0.506), but significant effect of time was seen (p = 0.011)

19 Papers I – III

Paper 1