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The Pathophysiology of Threatened Miscarriage and its Effect on Pregnancy Outcome

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Abstract

The presented thesis is an investigation of the incidence and pathophysiology of first trimester threatened miscarriage and its outcomes. First trimester threatened miscarriage is the commonest complication of pregnancy, affecting 10-20% of women with clinically recognised pregnancies, and the incidence and mechanisms for long term adverse outcomes are poorly understood.

Early placental development requires a delicate balance between the entry of oxygenated maternal blood and the capacity of the villous trophoblast to metabolise oxygen and eliminate its metabolites (free radicals). There is a rapid increase in placental markers of oxidative stress as the maternal circulation is established, which may serve a physiological role in stimulating placental differentiation, but which equally may result in free radical damage if antioxidant defences are depleted. In normal early pregnancy, the rapid increase in oxygen tension is paralleled by a rise in the expression of placental antioxidant enzymes.

Bleeding in early pregnancy could change the delicate equilibrium of placental production of reactive oxygen species and its natural antioxidant defences, leading to disruption of normal development of the early placenta and placental membranes. This disruption results in a range of adverse pregnancy outcomes, from

miscarriage in the first trimester, to pre-term pre-labour rupture of the membranes, pre-term labour, fetal growth restriction and pre-eclampsia in the third. This study examines in detail the incidence and possible mechanisms of adverse outcome in women with threatened miscarriage; and the role of placental function, in terms of placental hormone production and markers of oxidative stress, in both the causation of threatened miscarriage and the subsequent outcome of the pregnancy. It confirms an association between threatened miscarriage and adverse outcome, and provides potential markers of placental damage or stress to add to a growing body of research elucidating the role of oxidative stress in the developing placenta and later pregnancy complications.

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Table of Contents

Abbreviations

AF	Amniotic fluid
CF	Coelomic fluid
CRL	Crown rump length
DHA	Dehydroascorbic Acid
E2	Oestradiol
ECC	Excocoelomic coelom
EPU	Early pregnancy unit
ESR	Electron spin resonance
FAS	Fetal alcohol syndrome
FGR	Fetal growth restriction
FSH	Follicle stimulating hormone
FRAP	Ferric reducing ability of plasma
GPX	Glutathione peroxidase
GSD	Gestational sac diameter
GSH	Glutathione
GTD	Gestational trophoblastic disease
hCG	Human chorionic gonadotrophin
HPLC	High pressure liquid chromatography
IUFD	Intrauterine fetal demise
IUH	Intrauterine haematoma
IVF	In-vitro fertilisation
IVS	Intervillous space
LBW	Low birth weight
LH	Luteinising Hormone

LMP	Last menstrual period
MoM	Multiples of median
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NTD	Neural tube defect
PAPP-A	Pregnancy associated plasma protein A
PET	Pre-eclamptic toxaemia
PIH	Pregnancy induced hypertension
PPROM	Pre-term pre-labour rupture of membranes
PTL	Pre-term labour
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TAC	Total antioxidant capacity
TAP	Total antioxidant power
TGF	Transforming growth factor
TSH	Thyroid stimulating hormone
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Figure Legends

Figure 3.1: Diagram representing placentation in normal first-trimester pregnancy (A) and miscarriage (B). Note trophoblast plugging of maternal spiral arteries and trophoblast invasion of the decidua and superficial myometrium in the central area of the normally developing placenta (A). By contrast, in the miscarriage (B) there is a shallow trophoblastic invasion and the plugs are loose allowing premature entry of maternal blood (arrows).

Figure 3.2: Diagrammatic representation of early embryonic nutrition. Secretions from the maternal uterine glands are delivered into the intervillous space (IVS), which are then taken up by the syncitiotrophoblast and secondary yolk sac via the exocoelomic fluid.

Figure 3.3: Working hypothesis that premature influx of oxygenated blood will cause syncitiotrophoblast (SCTB) stress, resulting in a range of pregnancy outcomes dependent on the degree of placental recovery.

Figure 3.4: The free radical cascade. The superoxide free radical ($O_2^{\bullet -}$) is detoxified to hydrogen peroxide (H_2O_2) and then to water. Reactive oxygen and nitrogen species can cause indiscriminate damage to cells, lipids and proteins in the absence of adequate antioxidant defences.

Figure 3.5: Endogenous (red) and exogenous (yellow) antioxidant systems, convert highly reactive oxygen and nitrogen species into

Chapter 1

Early Pregnancy Complications and Adverse Pregnancy Outcome

First trimester miscarriage is the most common complication of pregnancy and affects 10-20% of women with clinically recognised pregnancies[2-4]. The true incidence of early pregnancy failure, including chemical pregnancies (miscarriages within the first 5 weeks of pregnancy) and all clinically recognised pregnancies up to 14 weeks, could be as high as 60%[5]. Miscarriage accounts for approximately 50 000 hospital admissions annually in the United Kingdom[6] and the cost to the National Health Service is high.

Threatened miscarriage, defined as vaginal bleeding before 24 weeks gestation, is a common complication affecting 15-20% of ongoing pregnancies[7] and is the most common reason for emergency gynaecological GP referrals. There are approximately 673 000 births in England and Wales annually[8] and therefore an estimated 100-135 000 women each year will present to a health care practitioner with bleeding in the first trimester. Women who present in the first trimester with threatened miscarriage are usually offered an ultrasound scan to confirm whether there is a viable intrauterine pregnancy. At this initial scan, many practitioners will report the presence of an intrauterine haematoma or subchorionic

bleed. First trimester threatened miscarriage has been associated with several adverse outcomes including an increased risk of spontaneous miscarriage[7;9-11], stillbirth, abruption, pre-term labour[9;12;13] and low birth weight[9;12;13], but an association with complications such as pre-term pre-labour rupture of the membranes (PPROM), fetal growth restriction (FGR) and pregnancy induced hypertension (PIH) has yet to be confirmed[12;14;15]. The presence of an intrauterine haematoma has also been associated with these complications[12;16;17]. The difficulty with many of these studies however is that they are retrospective, uncontrolled or rely on patient recall. The mechanisms for the association between first trimester bleeding and adverse outcomes in later pregnancy are uncertain and once the initial bleeding episode has resolved, these pregnancies are not considered high risk or to require increased surveillance.

1.1 Threatened Miscarriage and Pregnancy Outcome

The mechanism linking threatened miscarriage with pre-term pre-labour rupture of the membranes (PPROM), fetal growth restriction (FGR) and pregnancy induced hypertension (PIH) has not been confirmed, but there is growing evidence for a common role for reactive oxygen species (ROS) in the pathogenesis of many of these complications[18;19]. Bleeding in early pregnancy, with or without the development of an intrauterine haematoma, could affect pregnancy

outcome in several ways. A large haematoma involving the placental bed may lead to a failure of the placenta and subsequent expulsion of the pregnancy within days or a few weeks after the initial bleed. Bleeding at a later stage may be associated with a chronic inflammatory reaction within the decidua and placental membranes, with minimal or no disruption to the development of the early placenta, causing weakening and eventual PPROM and pre-term delivery.

1.2 Mechanisms of Early Placental Failure

First trimester miscarriages can be broadly categorised into two main groups, 'sporadic' miscarriages which occur in up to 25% of the population[20] and recurrent miscarriages. Recurrent miscarriage is defined as three or more consecutive pregnancy losses before 20 weeks gestation and occurs in of 1% of couples trying to conceive[21]. The causes for recurrent miscarriage have been extensively investigated and reported. The causes, diagnosis and management of recurrent miscarriage are outside the scope of this thesis and will not therefore be discussed in detail.

Around 50% of early pregnancy failures are linked to a chromosomal abnormality and, related to this, the risk of miscarriage increases with advancing maternal age[22]. The role of abnormal karyotype in repeated miscarriage is less clear. The causes for idiopathic sporadic miscarriage, not related to chromosomal

abnormality, remain uncertain although evidence is emerging that suggests that abnormal placental development is the essential pathophysiological mechanism in early pregnancy failure[23-25]. Placentation in humans is mainly characterised by infiltration of the uterine endometrium and superficial myometrium by extravillous trophoblastic cells. These cells act as a barrier and subsequently an interface between the maternal and fetal circulations. Early placental development requires a delicate balance between the entry of maternal blood carrying oxygen and the capacity of the villous trophoblast to metabolise the oxygen and eliminate its metabolites (free radicals). There is a rapid increase in placental markers of oxidative stress as the maternal circulation is established,[26] which may serve a physiological role in stimulating placental differentiation, but which equally may result in free radical damage if antioxidant defences are depleted. In normal early pregnancy, the rapid increase in oxygen tension that occurs is a paralleled by a rise in the expression of antioxidant enzymes within the placental tissues[27;28]. Any alteration to the delicate balance in early pregnancy could result in damage to early placental development resulting in a spectrum of effects on pregnancy development and outcome depending on the degree and timing of the insult.

It is known that in about two-thirds of early pregnancy failures there is defective placentation which is mainly characterised by a thinner and fragmented trophoblast shell, and reduced cytotrophoblast invasion of the lumen at the tips of the spiral

arteries[24]. As a result of this there appears to be premature influx of maternal blood[29] and consequent high oxygen concentrations. There is also evidence that later pregnancy complications such as pre-eclampsia (PET)[30], pre-term labour[31] and more recently PPROM[32] are associated with impaired placentation and failure of physiological invasion of the spiral arteries.

If impaired placental development, or bleeding in early pregnancy does result in the premature influx of maternal blood, the rise in oxygen tension will inevitably result in an increase in reactive oxygen species (ROS), as a by-product of aerobic respiration. If the increased demand is not met by an increase in antioxidant defences an imbalance will occur and tissue damage will result in the loss of the pregnancy or later pregnancy complications.

Chapter 2

Hypothesis, Aims and Objectives

2.1 Hypothesis

Bleeding in early pregnancy changes the delicate equilibrium of placental production of reactive oxygen species and its natural antioxidant defences, leading to disruption of the normal development of the early placenta and placental membranes. This disruption will result in a range of adverse pregnancy outcomes, from miscarriage in the first trimester, to pre-term pre-labour rupture of the membranes, pre-term labour, fetal growth restriction and pre-eclampsia in the third trimester.

2.2 Aims of the Study

1. To determine the risk of adverse pregnancy outcome after threatened miscarriage in our population of women presenting to the early pregnancy unit (EPU) at University College London Hospital (UCLH).
2. To prospectively follow a cohort of women presenting with threatened miscarriage to confirm an association with complications

of pregnancy that have been described in retrospective series, such as PPROM, PTL and PET.

3. To investigate placental function in women with threatened miscarriage by examining markers of placental trophoblast activity and comparing them with normal controls.
4. To investigate the presence and activity of antioxidant substances in maternal plasma and fetal fluid (coelomic and amniotic) between 7 and 12 weeks of gestation.
5. To compare antioxidant activity in pregnancies presenting with threatened miscarriage to those found in normal controls.

2.3 Ethics Approval

All studies on women presenting with threatened miscarriage, and on women undergoing therapeutic termination of pregnancy at UCLH have been reviewed by The Joint UCL/UCLH Committees on the Ethics of Human Research.

Chapter 3

Background

3.1 Normal Early Human Placental Development

In order to start to understand the mechanisms leading to early pregnancy loss and later pregnancy complications, it is necessary to understand the development of the early placenta and placental membranes. After implantation, the embryo is completely surrounded by proliferative cytotrophoblast cells. Subsequently extravillous trophoblast (EVT) invades into the decidual and myometrial layers where it surrounds and invades the maternal spiral arteries[33]. This results in transformation of the arteries, with a loss of the musculoelastic structure of the vessel walls and an increase in vessel diameter converting them into low resistance, high capacitance vessels[34].

Traditional teaching is that maternal blood circulates in the intervillous space from a very early stage in placental development[35]. The primitive uteroplacental circulation has always been thought to begin within the first two weeks after conception, with functioning primary chorionic villi present by the third week (Figure 3.1B).

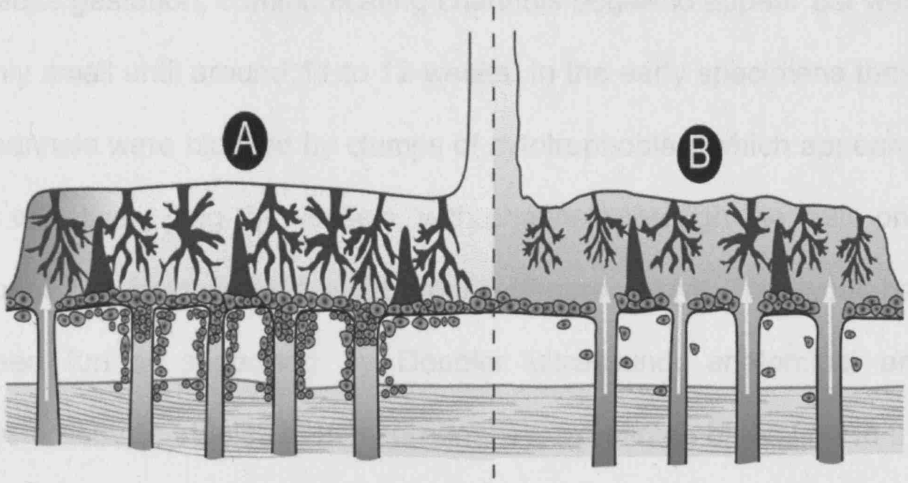


Figure 3.1: Diagram representing placentation in normal first-trimester pregnancy (A) and miscarriage (B). Note trophoblast plugging of maternal spiral arteries and trophoblast invasion of the decidua and superficial myometrium in the central area of the normally developing placenta (A). By contrast, in the miscarriage (B) there is a shallow trophoblastic invasion and the plugs are loose allowing premature entry of maternal blood (arrows).

Original work by Hamilton and Boyd, examining hysterectomy specimens in the first half of pregnancy, challenged this theory, suggesting that trophoblastic cells from the cytotrophoblastic shell form plugs in the endometrial arteries in the early part of pregnancy[36;37]. They concluded that blood flow in the intervillous space was sluggish in early pregnancy. This theory was largely disregarded until recently, when the 'Boyd Collection' was re-examined[38]. Twelve of the specimens were re-examined and the investigators specifically examined the decidua basalis and therefore the endometrial arteries under the implantation site. In the early specimens, no direct communications could be identified between the maternal vessels and the intervillous space. After around eight

weeks gestation, communicating channels began to appear but were only small until around 11 to 12 weeks. In the early specimens these channels were blocked by clumps of cytotrophoblast which appeared to effectively plug the vessels, with channels through the cells only appearing from around eight weeks (Figure 3.1A). This work has been further supported by Doppler ultrasound, anatomical and hysteroscopic studies in early pregnancy, examining blood flow in the intervillous space (IVS)[23;36;39-41], confirming that there is minimal flow before approximately 11 weeks gestation. It has also been shown that greater trophoblast invasion of the spiral arteries occurs in the central region of the placental bed[42] suggesting that the plugs are more extensive and complete in this area. Recent studies have confirmed that dissipation of the plugs occurs first at the periphery of the placenta in the majority of normal pregnancies[43]. This flow of blood in the intervillous space at the periphery of the placenta, and subsequent 'physiological' oxidative stress has been suggested as the trigger responsible for the development of the placental membranes or 'chorion laeve' in human pregnancy[25].

Early Placental Oxygen, Antioxidant Defences

Measurements of placental messenger RNA (mRNA) concentrations and activity of the principal intracellular antioxidant enzymes Copper/Zinc superoxide dismutase and catalase have shown that they are present only in very low levels in the syncitiotrophoblast of

early pregnancy, suggesting a limited requirement in the first trimester[28;44;45]. As pregnancy progresses there is a sharp rise in the activity of these enzymes. Oxygen tensions in the intervillous space rise steeply from below 20mmHg at eight weeks gestation to over 50mmHg at 12 weeks[26], coinciding with the changes in the uterine arteries and the increase in antioxidant enzyme activity. This rise in oxygen tension is also accompanied by a rapid increase in the expression of heat shock protein 70 (HSP70) and other markers of oxidative stress[25]. It is thought that the tightly controlled oxygen tension in the early placenta might regulate cytotrophoblast differentiation. In-vitro studies of cytotrophoblast cell cultures have shown that cytotrophoblast proliferates under conditions of low oxygen tension, but that they adopt an invasive phenotype in higher oxygen tensions[46;47]. This change could be critical for the invasion and conversion of the maternal spiral arteries into low resistance vessels. It has also been suggested that the substantial gradient between the higher oxygen tension in the maternal endometrium and the much lower tension in the intervillous space favours proliferative cytotrophoblast in the intervillous space and the formation of the trophoblastic shell; and the more invasive phenotype in the endometrium; essential for implantation and subsequent feto-maternal exchange[48].

Early Pregnancy Environment and Nutrition

Although there is still some debate over this theory[49;50] evidence points towards the conclusion that the maternal circulation is established gradually towards the end of the first trimester, with dissipation of the trophoblastic plugs, commencing at the periphery of the placenta (Figure 3.1.A). The early human embryo must therefore develop in a relatively low oxygen environment that is tightly controlled, probably with a minimal requirement for antioxidant defences. This state of low oxygen tension remains until the gradual 'unplugging' of the maternal spiral arteries and consequent increase in the oxygen tension as maternal blood is delivered to the primary villi. If this is correct however, how does the early embryo obtain essential micronutrients in the early stages of pregnancy? It becomes obvious that there must be an alternative source of nutrition to the early embryo, which is independent from the developing placenta. Recent work by Burton and colleagues[51;52] suggests that early embryonic nutrition is derived in part from the maternal uterine glands, which have been shown to deliver their secretions freely into the intervillous space in early pregnancy. These secretions are taken up by placental syncitotrophoblasts and also by the secondary yolk sac via the exocoelomic coelom (ECC) (Figure 3.2).

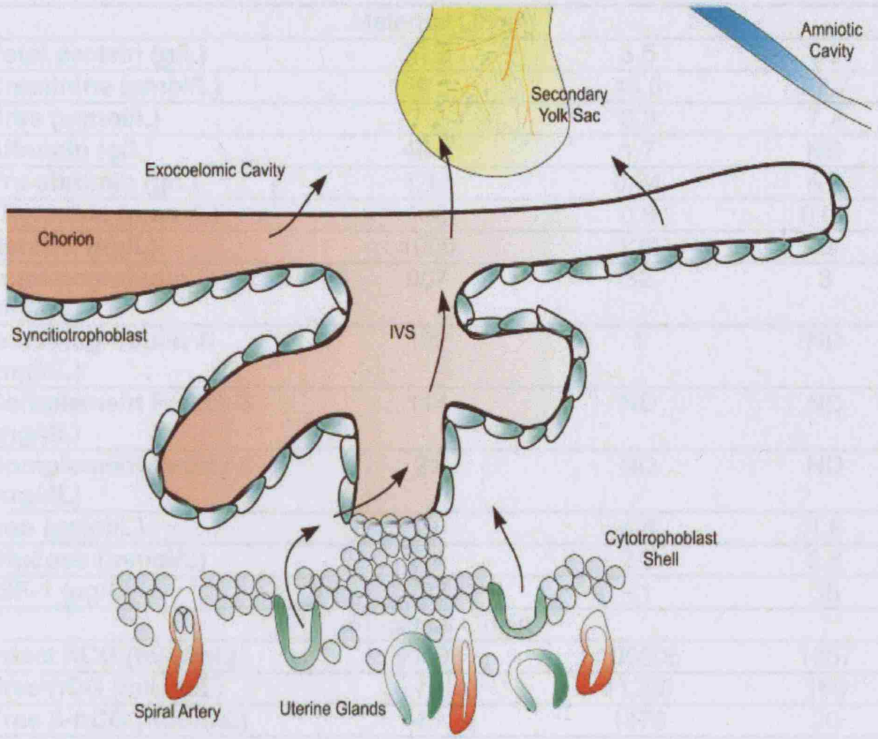


Figure 3.2: Diagrammatic representation of early embryonic nutrition. Secretions from the maternal uterine glands are delivered into the intervillous space (IVS), which are then taken up by the syncitiotrophoblast and secondary yolk sac via the exocoelomic fluid.

The fluid in the ECC (coelomic fluid) has been found to be an ultrafiltrate of maternal serum[53]; rich in amino acids, immunoglobulins[54] and placental products such as human chorionic gonadotrophin (hCG)[55], oestradiol, oestriol and progesterone[56;57] (Table 3.1).

Molecules	Maternal Serum	Coelomic Fluid	Amniotic Fluid
	Maternal Origin		
Total protein (g/L)	71.3	3.5	0.2
Creatinine (µmol/L)	50.1	43.6	27.7
Urea (mmol/L)	7.2	8.3	7.2
Albumin (g/L)	45.5	1.7	ND
Pre-albumin (g/L)	1.14	0.04	ND
Thyroxine (nmol/L)	180	0.9	0.02
Relaxin (ng/L)	1000	122	9
Immunoglobulin G (mg/dL)	907	32	3
Immunoglobulin A (mg/dL)	122	1	ND
Complement Factor 3 (mg/dL)	114	ND	ND
Complement Factor 4 (mg/dL)	21	ND	ND
Iron (µmol/L)	21	4.8	1.8
Glucose (mmol/L)	3.4	2.7	2.8
IGF-1 (µg/L)	233	41	38
	Placental Origin		
Intact hCG (mIU/mL)	80193	105605	1057
Free hCG (mIU/mL)	70	11200	169
Free β-hCG (mIU/mL)	45	1478	20
Human Placental Lactogen (ng/mL)	210	80	30
Progesterone (pg/mL)	17	240	8
Oestradiol (pg/mL)	917	8469	1898
Activin A (ng/mL)	0.68	0.98	0.09
Inhibin B (pg/mL)	5.9	24.3	6.3
β2 microglobulin (mg/L)	0.9	4.7	ND
Lactate (mmol/L)	0.3	0.6	0.9
IGF-II (µg/L)	687	199	40
	Decidual Origin		
Vitamin B12 (ng/mL)	405	3680	987
Prolactin (mU/L)	709	371	40
Placental Protein 14 (µg/L)	642	4416	77
Interleukin-6 (ng/mL)	40	88	17
IGFBP-1 (µg/L)	76	150	16
IGFBP-2 (µg/L)	123	167	49
	Secondary Yolk Sac Origin		
Alpha-fetoprotein (kIU/L)	1.4	21816	27096
Erythropoietin (mU/mL)	15.4	15.5	5.0
	Fetal Origin		
Γglutamyltransferase (U/L)	9	2	25
Ferritin (µg/L)	49	287	2.0
Ca125 (U/mL)	35	35	496

Table 3.1: Concentration of various proteins and other molecules in embryonic fluids and maternal serum according to their main site of production or origin during the first trimester of pregnancy. (Modified with kind permission from Jauniaux E, Gulbis B. Fluid compartments of the embryonic environment. *Hum Reprod Update 2000*; 6: 268-278[58])

The presence of glycoproteins such as glycodelin in the secondary yolk sac also suggests a role for the exocoelomic space in nutrient transfer and embryo protection in early human pregnancy[51].

Evidence is now emerging that impairment of this protection system, which tightly regulates placental oxygen tension in the early embryo, may be involved in the pathophysiology of early pregnancy failure, with uncontrolled oxygen delivery to the delicate embryo and resulting free radical damage. This mechanism may also be responsible for the later pregnancy complications that occur after threatened miscarriage, where the developing placenta is able to mount a sufficient response to this early insult and the pregnancy continues, but where disruption to normal development sets up a state of chronic inflammation or oxidative stress, resulting in disorders such as PET and PPROM (Figure 3.3).

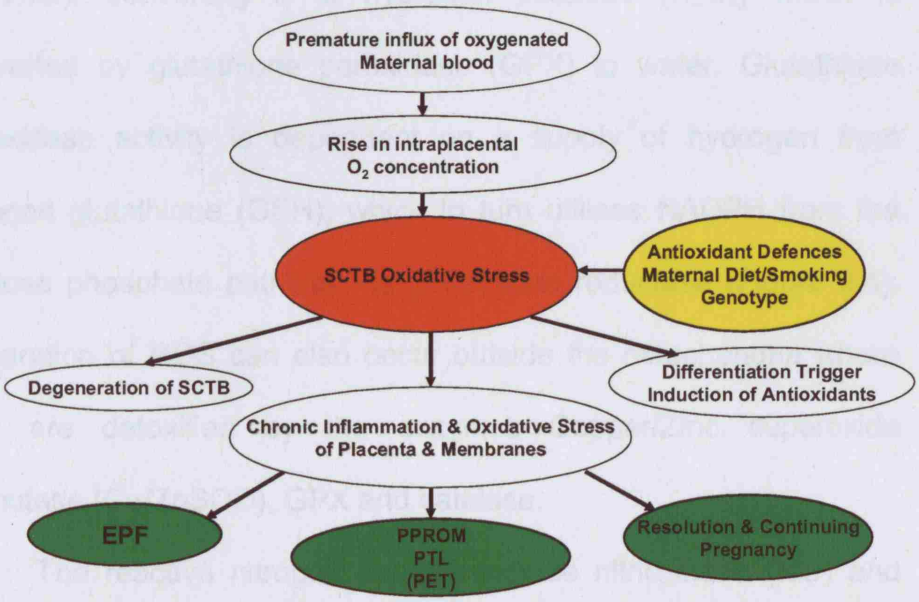


Figure 3.3: Working hypothesis that premature influx of oxygenated blood will cause syncitiotrophoblast (SCTB) stress, resulting in a range of pregnancy outcomes dependent on the degree of placental recovery.

3.2 Free Radicals, Tissue Damage and Defences

Free Radicals in Biological Systems

Free radicals are atoms or molecules with one or more unpaired electrons. The major free radicals in biological systems are the reactive oxygen (ROS) and the reactive nitrogen species (RNS). The predominant intracellular source of ROS is the mitochondria through leakage of electrons from the respiratory chain to molecular oxygen[59;60]. Stepwise single electron additions (reduction) to molecular oxygen generate more reactive intermediates such as the superoxide radical ($O_2^{\bullet -}$). The superoxide radical is detoxified within the mitochondrial matrix by manganese superoxide dismutase (MnSOD), converting it to hydrogen peroxide (H_2O_2) which is converted by glutathione peroxidase (GPX) to water. Glutathione peroxidase activity is dependent on a supply of hydrogen from reduced glutathione (GSH), which in turn utilises NADPH from the pentose phosphate pathway via glutathione reductase (Figure 3.4). Generation of ROS can also occur outside the mitochondria where they are detoxified by the enzymes Copper/Zinc superoxide dismutase (Cu/ZnSOD), GPX and catalase.

The reactive nitrogen species include nitric oxide (NO) and peroxynitrite ($ONOO^-$). Nitric oxide is generated in the mitochondria by nitric oxide synthase[61] (NOS). Nitric oxide can also react with the superoxide ion to produce peroxynitrite (ONOO-). Peroxynitrite is a

powerful oxidant which causes lipid peroxidation and DNA strand breakage. It is detoxified by GPX or reacts directly with GSH. It is thought that at physiological levels the ROS and RNS may be involved in intracellular signalling processes including regulation of transcription factors such as the expression of pro-inflammatory cytokines[62-64].

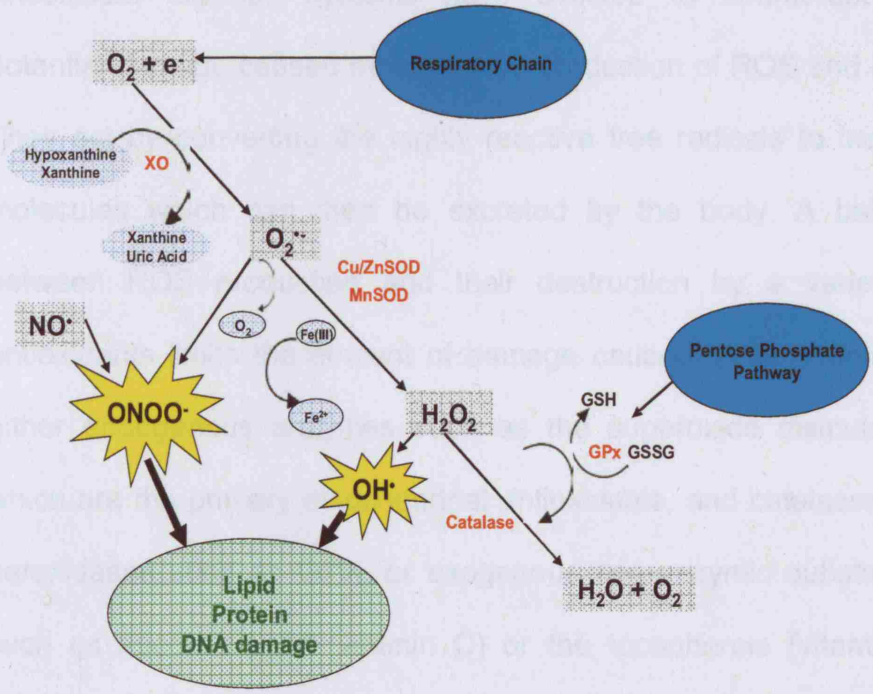


Figure 3.4: The free radical cascade. The superoxide free radical ($O_2^{\bullet-}$) is detoxified to hydrogen peroxide (H_2O_2) and then to water. Reactive oxygen and nitrogen species can cause indiscriminate damage to cells, lipids and proteins in the absence of adequate antioxidant defences.

If generation of these ROS and RNS *exceeds* cellular antioxidant defences oxidative stress will occur, with indiscriminate damage to cells, lipids and proteins. An imbalance in the metabolism of $O_2^{\bullet-}$ and

H_2O_2 can result in the production of the even more reactive hydroxyl ion ($OH^{\bullet}$)[59;60], catalysed by free ferrous ions. Hydrogen atoms are the best electron donors and therefore free radicals can cause widespread tissue damage by causing lipid peroxidation to cell membranes, increasing intracellular calcium and damaging DNA.

Human Antioxidant Defence Systems

Antioxidant defence systems have evolved to counteract any potential damage caused by excessive production of ROS and RNS. They act by converting the highly reactive free radicals to inactive molecules which can then be excreted by the body. A balance between ROS production and their destruction by a variety of antioxidants limits the amount of damage caused. Antioxidants are either endogenous enzymes such as the superoxide dismutases, which are the primary mitochondrial antioxidants, and catalases and peroxidases such as GPX, or exogenous non-enzymic substances such as ascorbic acid (vitamin C) or the tocopherols (vitamin E) (Figure 3.5).

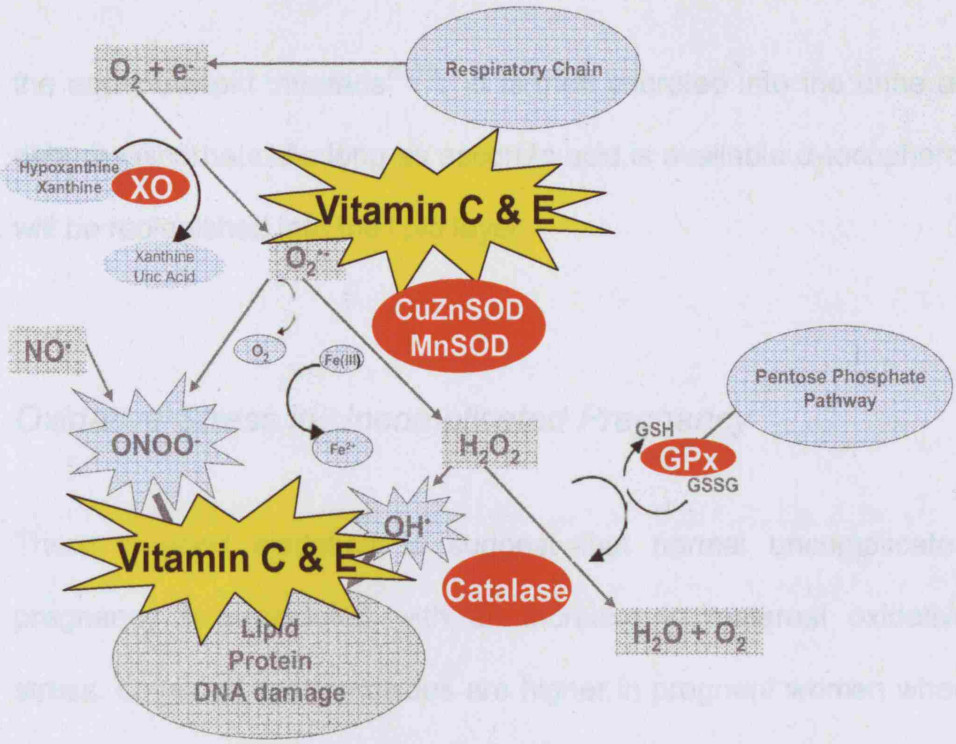


Figure 3.5: Endogenous (red) and exogenous (yellow) antioxidant systems, convert highly reactive oxygen and nitrogen species into inactive molecule that can then be safely excreted from the body.

Vitamin E is the major non-enzymic lipid soluble chain breaking antioxidant[65], protecting lipid membrane and lipoprotein structures against lipid peroxidation. Vitamin E is a generic term, referring to all tocol compounds with the same biological activity as α-tocopherol[66], the difference referring to the number and position of the methyl group on the chroman ring. Gamma-tocopherol is the most prevalent form of vitamin E in the western diet[67] and α-tocopherol is the commonest form to be found in supplements[68].

Vitamin C is an essential water soluble antioxidant in plasma and acts as a reducing agent. Vitamin C works in synergy with vitamin E, reducing the tocopheryl free radical to α-tocopherol-OH at

the aqueous lipid interface[69;70]. It is then excreted into the urine as dehydroascorbate. As long as ascorbic acid is available α-tocopherol will be replenished into the lipid layer.

Oxidative Stress in Uncomplicated Pregnancy

There is good evidence to suggest that normal uncomplicated pregnancy is associated with an increase in maternal oxidative stress. Levels of lipid peroxides are higher in pregnant women when compared with non-pregnant controls[71] and it has been shown that levels of lipid peroxidation increase in pregnancy from the first trimester to term. This increase is directly related to the increase in metabolic rate in pregnancy. Along with this there appears to be a rise in maternal enzymic antioxidant levels as an apparent adaptive measure[28;44;72]. Culture of syncitiotrophoblast from early pregnancy specimens shows that at early gestations, it is extremely sensitive to oxygen mediated damage, an effect that becomes less marked as pregnancy progresses[45]. The placental syncytiotrophoblast brush border membrane is most susceptible to lipid peroxidation as it is continuously exposed to the high oxygen content of the maternal blood, acting as an interface between the maternal and fetal circulations[73]. It has been shown that vitamin E is concentrated in this brush border membrane in early pregnancy[74], suggesting a crucial role in the protection of the early embryo. In their study of trophoblast invasion in normal and abnormal pregnancy[25], Jauniaux

and colleagues also examined the morphology of the trophoblast cells in normal pregnancies for markers of syncitial stress. Using immunohistochemical techniques they found a greater proportion of unstressed syncytiotrophoblast in the central region of the placenta compared to the periphery. Markers of oxidative damage were higher in the periphery, where maternal blood flow is higher and it has been postulated that these oxygen mediated processes may be involved in regression of peripheral villi and the formation of the chorion laeve[25].

3.3 Abnormal Placental Development and Miscarriage

Epidemiology

As previously stated, first trimester miscarriage is the most common complication of pregnancy and affects 10-20% of women with clinically recognised pregnancies[2-4]. Miscarriage can be broadly categorised into two main groups, 'sporadic' affecting 20-25% of the population[20] and recurrent (defined as 3 or more consecutive miscarriages before 20 weeks gestation) affecting approximately 1% of couples trying to conceive[21]. The causes for recurrent miscarriage have been traditionally divided in to six main categories: anatomical, endocrine, genetic, infectious, immune and idiopathic, however it is apparent that in many cases the cause is multifactorial, and the emergence of inherited thrombophillic defects as a frequent cause of

recurrent miscarriage has dramatically changed the investigation and management of these women.

Around 50% of early pregnancy failures are chromosomal in origin and the risk increases with advancing maternal age[22]. The role of abnormal karyotype in repeated miscarriage is less clear and there is good evidence that there is a higher rate of subsequent miscarriage in women who have miscarried a fetus with a normal karyotype[75;76] and that women with recurrent miscarriages have a higher number of chromosomally normal miscarriages compared with controls[77;78]. Maternal age and number of previous miscarriages are independent risk factors for early pregnancy loss[4] . The causes for sporadic miscarriage, not related to chromosomal abnormality, remain uncertain and the potential role of abnormal placental development will be discussed in detail below.

The Pathophysiology of Early Pregnancy Failure

There is good evidence that early pregnancy failure is associated with defective development of the placental bed[29;79;80]. Placental bed tissue shows defective trophoblast invasion, with limited maternal arterial 'transformation' in women with missed miscarriages in both the first and second trimester[29]. These findings have been confirmed in a larger study[24] where spontaneous miscarriage specimens were associated with a thinner discontinuous trophoblastic shell and defective intravascular trophoblastic plugs. In two thirds of these

cases, spiral artery transformation was significantly reduced. Doppler studies[23;41] have shown pulsatile flow in the IVS in pregnancies that have gone on to miscarry and more recent work examining intervillous blood flow in normal and abnormal early pregnancies has shown that in normal pregnancy, intervillous blood flow increases gradually from eight weeks gestation[25]. In abnormal early pregnancy however, flow can be detected from a much earlier gestation. In normal early pregnancy maternal blood flow starts at the periphery of the placenta, possibly where plugging by the trophoblast is less complete. In missed miscarriage, this process appears to be reversed with flow in the IVS being more commonly seen in the central region of the placenta (Figure 3.1.B, page 19). A study of miscarriage specimens from women with antiphospholipid syndrome has also shown that the normal endovascular trophoblastic plugging described above could only be identified in 23% of cases compared to 75% of normal controls[81]. These findings appeared to be in agreement with early work on placental development[33;42] which showed that trophoblastic migration and morphological changes in the uteroplacental arteries are more extensive in the central area of the placental bed, with invasion starting at the centre of the implantation site and extending to the periphery in an annular pattern; trophoblast invasion of the endometrial arteries is also most extensive i.e. deep in the central regions[42].

Early syncitiotrophoblast is extremely sensitive to oxygen mediated damage[45]. The brush border membrane is most

susceptible to lipid peroxidation and is continuously exposed to the high oxygen content of the maternal blood, acting as an interface between the maternal and fetal circulations[73]. In their study of trophoblast invasion in normal and abnormal pregnancy[25], Jauniaux and colleagues found a greater proportion of unstressed syncytiotrophoblast in the central region of the placenta compared to the periphery. Levels of heat shock protein 70 (HSP_{70}) were higher in the periphery, where maternal blood flow is higher. Samples examined from missed miscarriage specimens showed signs of syncitial stress in the central regions of the placenta and the markers of oxidative damage were significantly higher than in controls. They concluded that oxidative damage caused by widespread premature influx of maternal blood secondary to impaired trophoblast invasion was an important mechanism in early pregnancy loss.

Impairment of the trophoblast shell in abnormal early pregnancy with premature onset of the maternal circulation, such as in threatened miscarriage, could well result in oxidative damage to the developing placenta and embryo, resulting in miscarriage. Lesser degrees of impairment of early placental or membrane development with influx of oxygenated maternal blood may result in a chronic state of oxidative stress resulting from inflammation and the presence of substances which form free radicals (such as free ferrous ions), resulting in later complications of pregnancy such as PET, PPROM, fetal growth restriction (FGR) and pre-term labour.

Studies of maternal antioxidant status and oxidative stress in

early pregnancy failure are abundant but can be difficult to interpret. Study design varies and is often retrospective, subjects are frequently investigated in the non-pregnant state or immediately post-miscarriage and many different markers of oxidative stress and antioxidants have been measured. Few studies assess antioxidant status in pregnancy prior to miscarriage or in cases of threatened miscarriage. Assessment of maternal antioxidant levels or levels of oxidative stress can be difficult and may not fully represent the human body's response to stress and pregnancy.

Reduced serum selenium levels, an integral component of the antioxidant enzyme GSH, have been found in women who are miscarrying[82] and selenium levels are lower in women with first trimester sporadic miscarriages compared with women with a history of recurrent miscarriage[83]. Hair selenium levels have also been found to be lower in women with recurrent miscarriage[84]. Other studies have shown reduced plasma levels of ascorbic acid, α-tocopherol and red cell GSH in women with recurrent miscarriage[85], reduced levels of the antioxidant enzyme superoxide dismutase in first trimester miscarriage[86] and an increase in markers of lipid peroxidation in women who have had a spontaneous miscarriage[87;88]. In general, data seem to suggest that in early pregnancy failure there is an increase in markers of oxidative stress and a probable decrease in maternal antioxidant defences. Whether this decrease is constitutional or as a result of excessive consumption as a result of the 'disease' process is unclear.

Pregnancy related disorders associated with oxidative stress

In terms of pregnancy related disorders associated with increased oxidative stress, pre-eclampsia is by far the most extensively investigated[89]. Maternal lipid peroxide levels are significantly increased in PET when compared with normal pregnancy[90;91]. Maternal antioxidant levels have also been found to be decreased[92-96] as has placental antioxidant capacity[93;97], mRNA expression of Cu/Zn superoxide dismutase and glutathione peroxidase[93;98] and placental vitamin E levels[98]. A pilot randomised trial of the antioxidants vitamin C and vitamin E in women at high risk of PET demonstrated a significant reduction in PET compared with placebo[99] with improvement in maternal biochemical indices for the disease[100]. Unfortunately, the follow-on multi-centre study did not show a reduction in the incidence of PET in high risk women[101]. Results of the effect on biochemical indices are awaited.

A role for oxidative stress is emerging in other pregnancy related diseases such as maternal diabetes and drug related teratogenicity. Congenital abnormalities are 2-3 times more common in infants of diabetic mothers compared with normal controls[102]. Miscarriage rates are also increased in Type 1 diabetes[103;104]. Diabetic embryopathy has been associated with increased levels of oxidative stress[105;106] and vitamin C and E supplementation of

diabetic rats has been shown to reduce the incidence of malformations[107-109]. Raised glucose levels have also been found to disturb the expression of regulatory genes in embryonic development and cell-cycle progression leading to premature cell death in animal models[110]. In this study, when this occurred at the pre-implantation stage, spontaneous miscarriage or congenital abnormalities were the result. At the post-implantation stage, alterations in gene expression resulted *specifically* in neural tube, musculoskeletal and cardiac defects.

It is well know that thalidomide administration to pregnant women produces a multitude of birth defects and ethanol has long been recognised as a teratogen, resulting in the Fetal Alcohol Syndrome (FAS), a spectrum of disorders, one of the mechanisms for which has recently been postulated to be oxidative damage[111;112]. In vitro work on animal models has found low levels of antioxidants, an exaggerated response to pro-oxidant stimuli and evidence of mitochondrially mediated cell death in fetuses exposed to alcohol in utero[113;114].

3.4 Threatened Miscarriage and Obstetric Outcome

3.4.1 Epidemiology of Threatened Miscarriage

Threatened miscarriage is defined as vaginal bleeding before 24 weeks gestation and occurs in 15-20% of ongoing pregnancies[7]. It is the commonest reason for emergency gynaecology General Practitioner referrals. There are approximately 673 000 births in England and Wales annually[8] and therefore an estimated 100-135 000 women each year will present to a health care professional with bleeding and pain in the first trimester. Threatened miscarriage in the first trimester has been associated with many adverse pregnancy outcomes. There is an increased risk of miscarriage[16;17;115], particularly when bleeding occurs before 8 weeks[10]. If not associated with immediate fetal loss, threatened miscarriage has been linked to fetal loss later in the pregnancy[7;77] as well as abruption[9;13;16], fetal growth restriction (FGR), pre-term labour (PTL)[9;13;16], pre-term pre-labour rupture of the membranes (PPROM)[9], pre-eclampsia (PET)[9] and low birth weight[12] (LBW). A recent retrospective study reported that the only risk factor found to be independently associated with pre-term delivery in women with threatened miscarriage was unexplained second or third trimester haemorrhage[13].

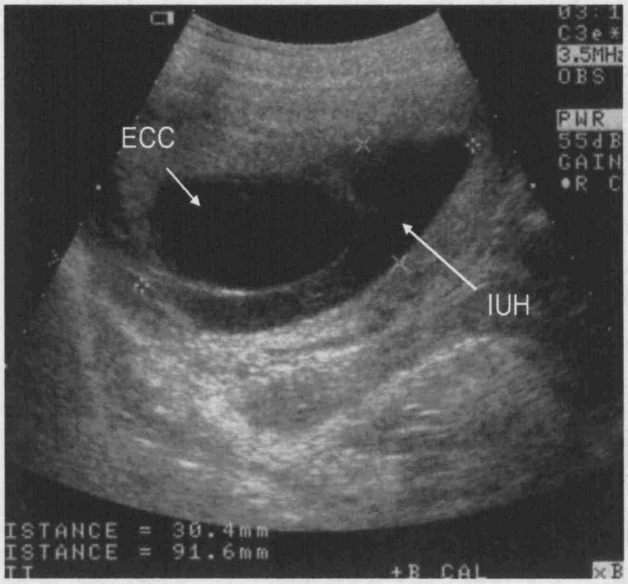
The problem with the majority of these reports is that they are

conducted retrospectively, are uncontrolled, or rely on patient recall, often recruiting later in the pregnancy. The largest study on threatened miscarriage and obstetric outcome was conducted by Weiss et al[9] as part of the FASTER Consortium. This study concluded that first trimester bleeding was an independent risk factor for adverse obstetric outcome and that the risk was also dependent on the amount of vaginal bleeding experienced. In women with heavy bleeding (defined as similar to menses), there was an increased incidence of FGR, PTL, PPROM and abruption. In this study, women were recruited at 10-14 weeks gestation, and divided into subsets based on whether there was a history of bleeding in the four weeks prior to enrolment. The spontaneous pregnancy loss rates before 24 weeks in both the cases and controls were extremely low in their study and may be explained by the later recruitment of the cases, when many of the early miscarriages will have already occurred.

Intrauterine Haematoma

Women presenting with vaginal bleeding in the first trimester are commonly investigated using ultrasonography to assess the viability of the pregnancy and to exclude alternative diagnoses such as ectopic pregnancy. A frequent ultrasound finding in women with bleeding in the first trimester is the presence of subchorionic bleeding or intrauterine haematoma (IUH). Intrauterine haematoma is defined as an echo-free area that lies between the placental membranes and

the uterine wall[15] (Figure 3.6). These IUH are present in approximately 18% of women with threatened miscarriage[116;117] and there has been much debate over the resolution and clinical significance of their presence on ultrasound. Many of the early studies examining pregnancy outcome were small or uncontrolled[14]. The incidence of miscarriage has been variably reported and ranges from 4-33 % depending on the gestation at presentation[14]. A recent retrospective study of intrauterine haematoma in the first trimester which found that the risk of spontaneous miscarriage was significantly increased if the haematoma was diagnosed before nine weeks[11], this is in keeping however with the consistent finding that bleeding alone, with or without a haematoma, before eight weeks results in an increased incidence of miscarriage[10], and the significance of the finding of these haematomas has yet to be confirmed. Intrauterine haematomas have been shown to be associated with an increased incidence of pre-term labour and low birth weight[12], but an association with complications such as premature rupture of membranes (PROM), fetal growth restriction and pregnancy-induced hypertension is still debated[12;14;15;118-120]. A summary of the available data on the incidence and outcomes of IUH can be found in Table 3.2.



IUH = intrauterine haematoma ECC = exocoelomic space

Figure 3.6: Transvaginal ultrasound image of an intrauterine haematoma (IUH) in an early ongoing pregnancy. The well defined crescent shaped area can be seen adjacent to the coelomic cavity of the gestation sac (ECC).

Table 3.2: Studies examining the outcome of pregnancies complicated by intra-uterine haematoma (search criteria: intrauterine haematoma, subchorionic bleed and threatened miscarriage - using PubMed and Medline)

Authors	Year	Study Type	Entry Criteria	N	Outcome	Comments
Mantoni & Pedersen[15]	1981	Observational	TM	10	3 patients with IUH >50mls miscarried or PTL	
Goldstein et al[117]	1983	Prospective observational	TM	10	20% loss rate	
Ylostalo et al[121]	1984	Prospective observational	TM	16	Shorter duration of pregnancy	Incidence 16%
Jouppila[122]	1985	Prospective observational	TM	33	18.7% miscarriage, 9.4% PTL	No correlation between volume of IUH & outcome
Sauerbrei & Pham[123]	1986	Prospective observational	TM	30	23% PTL, 13% SB, 10% miscarriage	
Abu-Yousef et al[124]	1987	Prospective observational	IUH on US	21	71 % adverse outcome	
Borlum et al[17]	1989	Case-control	TM	86	Significantly increased risk of miscarriage (22.1%)	
Bloch et al[125]	1989	Unclear	TM	31	6.5% PTL, 9.7% 1st trimester miscarriage	No correlation between volume of IUH & outcome
Stabile et al[126]	1989	Prospective observational	TM	22	No increase in miscarriage	5.4% incidence of IUH
Mandruzzato et al[127]	1989	Unclear	TM	?	No increased incidence of miscarriage (12.9%)	11% incidence of IUH
Pedersen & Mantoni[1]	1990	Prospective observational	TM	23	No increased risk of miscarriage or PTL	IUH ≥ 50 mls only
Pedersen & Mantoni[128]	1990	Prospective observational	TM	62	11% miscarriage, 11% PTL (similar to those without IUH)	18% incidence of IUH No correlation between volume of IUH & outcome
Glavind et al[129]	1991	Unclear	IUH on US	60	12% miscarriage, 10% PTL	No correlation between volume of IUH & outcome
Dickey et al[130]	1992	Retrospective	Routine US	230	No increase in miscarriage	Assisted conception
Kurjak et al[119]	1996	Case-control	IUH on US	59	Significantly increased risk of miscarriage (17%)	Site but not size of IUH affected outcome
Ball et al[16]	1996	Retrospective case-control	IUH on US	?	Increased risk of miscarriage (OR 2.8), SB (OR 4.5) abruption (OR 11.2) and PTL (OR 2.6)	1.3% incidence of IUH Bleeding alone also increases risk of miscarriage
Bennett et al[10]	1996	Retrospective	IUH on US	516	9.3% miscarriage (13.7% if bleeding< 8 weeks)	Large IUH - 3-fold increase in miscarriage
Tower & Regan[118]	2001	Prospective	IUH on US	41	No increase in miscarriage or LB rate APL antibodies more common in IUH group	Recurrent miscarriage population 12% incidence of IUH
Nagy et al[120]	2003	Prospective case-control	IUH on US	187	Increased incidence PIH (RR 2.1), PET (RR 4.0), abruption (RR5.6), PTL (RR 2.3), FGR (RR 2.4)	3% incidence, retroplacental IUH associated with worse outcome
Johns et al[131]	2003	Retrospective case-control	TM	51	IUH not associated with adverse pregnancy outcome	TM independently associated with adverse pregnancy outcome
Sharma et al[132]	2003	Retrospective	IUH on US	129	18.6% PTL	No correlation between volume of IUH & outcome
Maso et al[11]	2005	Unclear	IUH on US	182	14.3% miscarriage, 7.7% FGR, 6.6% PTL	2.4-fold increase risk of adverse outcome if IUH detected < 9 weeks gestation

IUH = intrauterine haematoma, PTL = pre-term labour, SB = stillbirth, US = ultrasound, LB = live birth, FGR = fetal growth restriction, APL = antiphospholipid, PIH = pregnancy induced hypertension, PET = pre-eclampsia, TM = threatened miscarriage

If the link between first trimester threatened miscarriage and PET, FGR, PPROM and subsequent PTL is confirmed, it will have major implications for health care resources and provision. Pre-term delivery occurs with an incidence of 7-11% and is the leading cause of death of normal infants[133]. Forty percent of pre-term deliveries are preceded by PPROM[134]. The incidence of PTL has remained largely unchanged despite advances in obstetric care and in the antenatal diagnosis and management of fetal abnormality and pregnancy complications such as PET and FGR. Identification of women at risk remains the only strategy for reducing the incidence of premature delivery and if 15-20% of ongoing pregnancies are complicated by threatened miscarriage, a large number of potentially high risk women are going unidentified. Increased antenatal surveillance might identify women within this group who are at increased risk and whilst there are no effective interventions currently, identification would enable closer monitoring and timely administration of antenatal corticosteroids.

Pathophysiology of Adverse Outcome in Threatened Miscarriage

The mechanism by which intrauterine haematomas and bleeding in the first trimester can affect pregnancy outcome is uncertain. Despite the many studies linking threatened miscarriage with adverse pregnancy outcome, little work has been done to investigate why

such a link occurs. From the described evidence above in 2.3.2., a role for damage by reactive oxygen species seems a possibility. The role of oxidative stress in the pathogenesis of PET has been extensively investigated and there is good evidence supporting increased levels of lipid peroxidation and reduced antioxidant activity in PET[97;135-141].

Recently, evidence for a role for increased free radical production in PPROM is becoming apparent[142;143]. Epidemiological evidence for a link with rupture of the membranes is well established. Cigarette smoking which is a potent inducer of ROS increases the risk of pre-term rupture of the membranes 2 to 4 fold compared with non-smokers[144] and maternal smoking increases the risk of spontaneous miscarriage by 33% compared with non-smoking controls[145-149]. Bleeding in the second trimester of pregnancy is also associated with premature rupture of the membranes[150]. Red blood cell haemolysis results in the release of free iron, which can catalyse the generation of the highly damaging $OH^{\bullet}$ radical via the Fenton reaction; potentially damaging the placental membranes and causing premature rupture. The presence of infection is also a well recognised cause of PPROM and activated macrophages and neutrophils generate increased levels of superoxide free radicals such as hypochlorous acid (HOCl) during phagocytosis[151]. This in combination with the proteases produced by bacteria including group B streptococcus[152] may impair membrane integrity and result in PPROM.

In vitro evidence suggests that vitamin C is involved in several biochemical processes involved in collagen synthesis and maintenance[153;154]. Collagen provides strength to the placental membranes and at least five different types can be found within them[143]. The main component of amnion is type III collagen and a reduction has been associated with PPROM[155]. Culturing amnion cells and amnion in hydrogen peroxide has been shown to induce apoptosis, cytokine and prostaglandin production[156], and a recent study has shown that pre-treatment of placental membranes with vitamin C and E protect against oxidative damage in vitro[157]. It has also been shown that women with PPROM have lower levels of vitamin C than controls, suggesting a possible preventative role for antioxidants in those at risk[158;159]. The picture is still however far from clear cut as it has also been reported that vitamin C alone may result in a dose dependent increase in membrane rupture[156]. A recent report has suggested that vitamin C may have a pro-oxidant effect[160] if used alone, again in a dose dependent manner. In this study there was an increased rate of adenine base mutations in non-pregnant subjects. Study design and data interpretation have however been widely criticised and a recent review suggests that vitamin C does not have any detrimental effect[161].

It can be seen from the above evidence that threatened miscarriage could affect pregnancy outcome in several ways. Theoretically, a large haematoma could be a threat to the continuance of the pregnancy by a direct pressure/volume effect.

This may well depend upon the site of the haematoma and its distance from the placental site, or the volume of the haematoma. The presence of subchorionic bleeding could also result in a chronic inflammatory reaction in the decidua and consequent persistent myometrial activity and expulsion of the pregnancy. If the pregnancy continues, subchorionic bleeding will result in an increase in the amount of both oxygenated blood and free iron available, potentially increasing the production of reactive oxygen species with subsequent damage to the placental membranes and eventual rupture.

3.5 Measuring Placental Function and Oxidative Stress

The human feto-placental unit and corpus luteum produce a vast number of substances, including hormones which have been studied extensively looking for markers and predictors of adverse pregnancy outcome and for assessment of placental function.

Screening for neural tube defects and trisomy 21 using a combination of maternal serum markers and ultrasound parameters has become standard practice in many developed countries. More recently an association has been identified between second trimester maternal serum markers and adverse pregnancy outcomes other than congenital anomalies and chromosomal aneuploidies[162;163], FGR[164-166], LBW[167], IUFD[168], PTL[166;167;169] and PET[167;170;171].

It has long been known that deviations in the levels of certain maternal serum markers in early pregnancy can be associated with early placental failure and pregnancy loss. Many maternal serum markers have been investigated in attempts to predict the outcome of pregnancy in the first trimester and in particular the likelihood of subsequent miscarriage, with varying degrees of success. Single or multiple markers in combination have been examined. Many of the early studies were conducted after embryonic demise had already occurred[172;173] and have limited clinical application with the advent of

high resolution ultrasound in the diagnosis of early pregnancy failure. Recently the development of highly sensitive urinary hCG assays and greater awareness of the availability of early pregnancy ultrasound amongst health care professionals and women alike has resulted in ever earlier presentation, leading to an increase in the number of inconclusive scans. As a result of this there has been an increase in the requirement for repeat assessments to determine both pregnancy location and viability at ever increasing cost to the NHS. In this context, a role for maternal serum markers, in combination with ultrasound features for the prediction of pregnancy outcome is re-emerging.

Maternal serum markers, combined with ultrasound parameters, maternal age, smoking habits, obstetric history and the occurrence of vaginal bleeding have all been combined in multivariate analyses, with mixed results[174;175]. First trimester human chorionic gonadotrophin (hCG) and more recently progesterone remain the only consistent markers of early pregnancy failure[173;176;177] but their predictive value is low[178].

The role of first trimester markers in the prediction of later pregnancy complications is even less clear. Most studies examine serum markers in the late first trimester as part of Down's Syndrome screening and associations have been made between abnormal levels of several hormones and adverse pregnancy outcome[179-181].

Human Chorionic Gonadotrophin (hCG)

Human chorionic gonadotrophin is a 39 kDa glycoprotein and is a member of a family of hormones including luteinising hormone (LH), follicle stimulating hormone (FSH) and thyrotrophin-stimulating hormone (TSH). Each consists of an α-subunit which is almost identical in all four hormones and a β-subunit unique to each individual hormone. Human chorionic gonadotrophin is produced by the placental syncytiotrophoblast[182], with small amounts also being produced by the pituitary gland and fetal liver. The two sub-units are combined in the syncytiotrophoblast and rapidly released into the maternal circulation, with levels reaching a peak at eight weeks. Levels plateau at 8-12 weeks and decline until 18 weeks where they remain constant until term[183;184]. hCG is probably the major luteotrophic signal from the implanting embryo in early pregnancy, promoting synthesis and release of progesterone from the corpus luteum. In vitro studies have also shown that hCG can regulate the differentiation of cytotrophoblasts into syncitiotrophoblasts, thereby increasing its own production[185]. Thirty percent of hCG is excreted in bile and urine, the rest being metabolised by the kidney and liver. Intact hCG has a half life of 6-24 hours, the β sub-unit a half life of 40 minutes. Several studies have looked at the value of both single and serial hCG measurements in predicting early pregnancy loss and ectopic pregnancy and hCG is one of the first 'maternal serum markers' to have practical applications. It's role in the management and follow-up of gestational trophoblastic disease is well established

and it's place in screening pregnancies for gestational trophoblastic disease (GTD) is promising[186;187]. A single serum βhCG estimation in pregnancy has a sensitivity of 88% with a positive predictive value of 83% for early pregnancy failure[188]. Several recent studies have confirmed the association between a low initial hCG and early pregnancy failure[173;189;190]. In miscarriages, there is a decrease in the expression of the hCG α and β_A subunit gene in villous tissue compared with controls suggesting that down-regulation occurs in the hCG gene after embryonic demise[191]. The relationship between first trimester hCG values and later pregnancy complications is uncertain[189;192] and associations have been made between low levels and FGR[180;181;190;192], and pregnancy induced hypertension (PIH)[181]. Increased levels of hCG have been found in association with PET[193;194] and it has been suggested that increased levels of hCG in abnormal pregnancies may occur as a result of abnormal placental development[195]. A recent study has suggested that hCG levels can be correlated with oxidative stress levels in term pregnant women with PET and suggest that circulating levels of hCG may be useful for screening for diseases associated with increased oxidative stress, like PET, in pregnancy[196].

Pregnancy Associated Plasma Protein-A (PAPP-A)

PAPP-A is a macromolecular glycoprotein. It has recently been identified as insulin-like growth factor binding protein-4 protease

produced by human fibroblasts and ovarian granulosa cells[197;198]. It appears to play a role in follicle selection and corpus luteum formation, by increasing the availability of insulin-like growth factor-II[198]. Serum levels increase steeply during early pregnancy from day 30 onwards[173]. Studies examining its use in predicting early pregnancy failure with a live fetus on ultrasound have shown that PAPP-A levels do decrease in miscarriage but that they have a low predictive value for fetal demise[173;199-201]. PAPP-A is also depressed in Downs' Syndrome and other chromosomal abnormalities[202-204] and has been examined extensively for its role in first and second trimester screening for Downs' Syndrome. In addition to this role, it has been found to be low in the first trimester in association with pregnancy induced hypertension (PIH)[179;190;205] and FGR[180;190;205;206] and possibly pre-term labour although findings are inconsistent[179;205;207;208].

Inhibins, Activins and Follistatin

The inhibins and activins are dimeric glycoproteins of the transforming growth factor- β (TGF-β) family (Figure 3.7), synthesised by the placenta[209;210], decidua and fetal membranes in pregnancy[211;212], as well as the ovary, pituitary and adrenals.

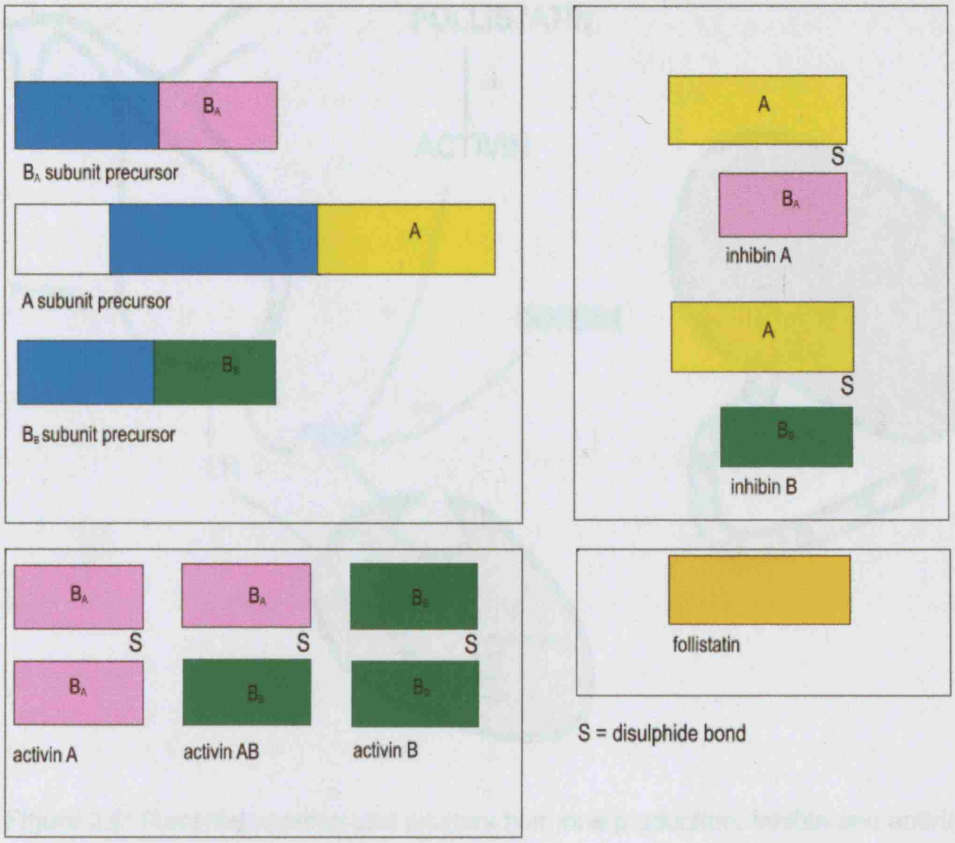


Figure 3.7: The inhibin/activin 'superfamily' of the transforming growth factor β family.

After first trimester termination of pregnancy, inhibin A levels fall rapidly to luteal phase levels within a few hours suggesting that the feto-placental unit is the primary source of inhibin A in the first trimester[213]. Inhibin A is an α-βA dimer and inhibin B is an α-βB dimer. Activin A is a βA-βA dimer, activin B a βB-βB dimer and activin AB a βA-βB dimer. These glycoproteins were initially characterised by their regulatory effect on the pituitary production of follicle stimulating hormone (FSH)[214] as inhibin A and activin A have opposite effects on follicular development (Figure 3.8).

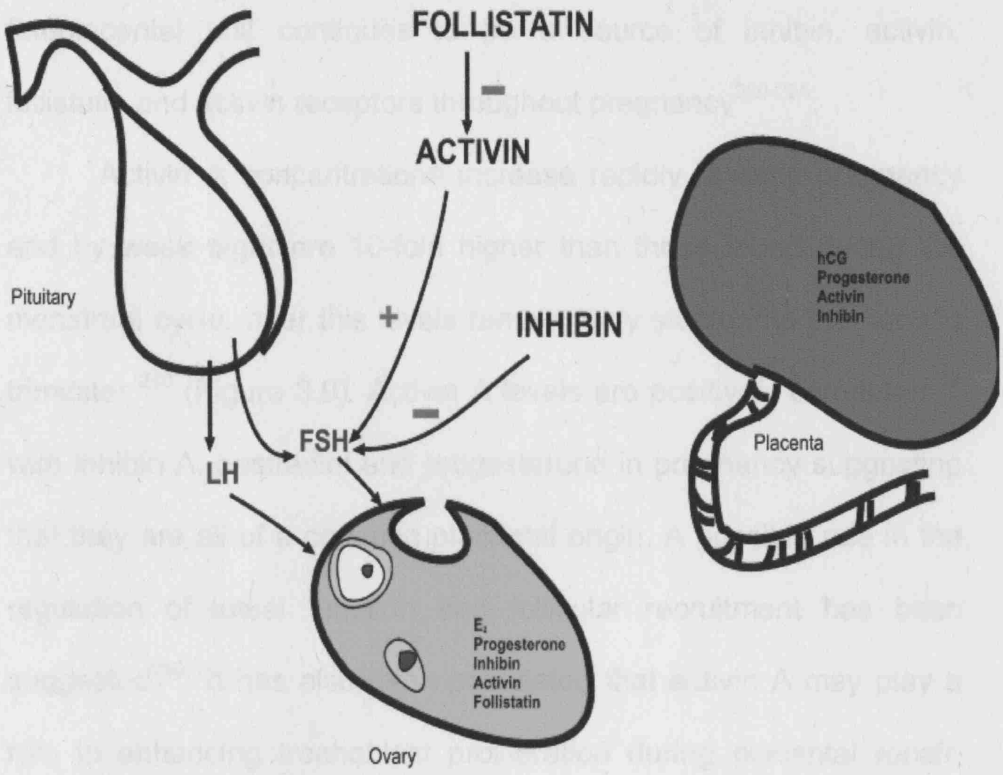


Figure 3.8: Placental, ovarian and pituitary hormone production. Inhibin and activin have opposite effects on follicular development by regulating pituitary production of FSH.

Follistatin is a single chain glycoprotein which is structurally distinct from activin A and the inhibins, but which has a functional similarity with inhibin, by neutralising activin bioactivity[215-217] and therefore suppressing FSH release. It is a high affinity binding protein, modifying the biological activity of activin at the target cell level[215].

Inhibin A (the predominant molecular form of inhibin in the maternal circulation in pregnancy), activin A and follistatin are present in high levels in maternal serum in normal human pregnancy[209;218]. Initially, in early pregnancy, inhibin A is produced by both the fetoplacental unit[213] and the corpus luteum[219], later the

fetoplacental unit continues to be a source of inhibin, activin, follistatin and activin receptors throughout pregnancy[220-224].

Activin A concentrations increase rapidly in early pregnancy and by week eight are 10-fold higher than those found during the menstrual cycle, after this levels remain fairly stable into the second trimester [210] (Figure 3.9). Activin A levels are positively correlated[225] with inhibin A, oestradiol and progesterone in pregnancy suggesting that they are all of a common placental origin. A possible role in the regulation of luteal function and follicular recruitment has been suggested[210]. It has also been postulated that activin A may play a role in enhancing trophoblast proliferation during placental repair. High levels are found in multiple pregnancy and levels decrease rapidly in pregnancy failure. Inhibin A levels increase from 5 weeks to reach a peak at 8 weeks[226]. Levels then gradually decline and remain low throughout the second trimester[209]. Both inhibin A and activin A levels increase markedly in the third trimester to peak at 36 and 38 weeks respectively[209;218]. Levels of inhibin B in the maternal circulation remain low throughout pregnancy[218]. As with activin A, the role of inhibin A in pregnancy is uncertain. It has been shown to have a regulatory effect on the secretion of other placental hormones, reducing the secretion of hCG and progesterone from cultured placental trophoblasts[227-229].

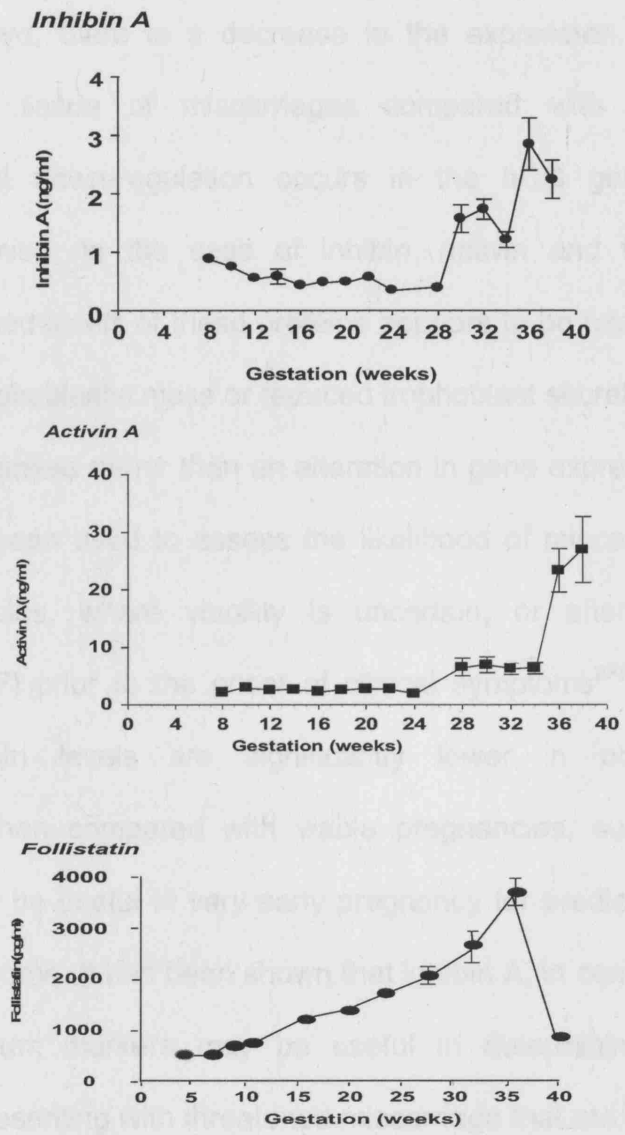


Figure 3.9: Inhibin A[209], activin A[225] and follistatin[218] levels in pregnancy.

Miscarriage

Several groups have studied inhibin A and activin A in early pregnancy loss and maternal serum concentrations of inhibin A have

been found to be lower in miscarriages when compared with gestation matched control pregnancies[230-235]. With hCG as mentioned above, there is a decrease in the expression of hCG genes in the tissue of miscarriages compared with controls suggesting that down-regulation occurs in the hCG gene after embryonic demise. In the case of inhibin, activin and follistatin however, reduced levels of these proteins appears to be related to a decrease in trophoblastic mass or reduced trophoblast secretion prior to embryonic demise rather than an alteration in gene expression[236]. Inhibin A has been used to assess the likelihood of miscarriage in early pregnancies, where viability is uncertain, or after in-vitro fertilisation (IVF) prior to the onset of clinical symptoms[237]. In IVF patients, inhibin levels are significantly lower in non-viable pregnancies when compared with viable pregnancies, suggesting that levels may be useful in very early pregnancy for prediction of a successful outcome. It has been shown that inhibin A, in combination with other serum markers may be useful in determining those pregnancies presenting with threatened miscarriage that are destined to fail[238], however the predictive value for subsequent miscarriage is low under these circumstances[219]. Overall, inhibin A levels appear to add little to progesterone and hCG measurements for the prediction of miscarriage in asymptomatic women, and the role of these hormones in predicting the likelihood of first trimester miscarriage in confirmed ongoing pregnancies after threatened miscarriage is even less clear.

Down's Syndrome

Inhibin A levels are increased in the serum of pregnancies affected by Down's Syndrome and contribute significantly to the sensitivity of second trimester screening tests[239-244]. For a five percent false positive rate, the addition of inhibin A to serum screening improves the detection rate for Down's Syndrome from 77% to 83%[245]. Placental inhibin α-subunit mRNA expression is elevated in Down's syndrome affected pregnancies. Activin A levels and mRNA expression are not altered in pregnancies affected by Down's Syndrome[246] when compared with normal controls.

Adverse Pregnancy Outcome

Increased maternal levels of inhibins and activins have been found in women suffering from pregnancy complications such as pre-eclampsia[247-253] (PET) and fetal growth restriction (FGR)[254;255] and alterations in gene and protein expression of inhibin and activin have been demonstrated in placentas complicated by pre-eclampsia[256;257]. In pre-eclampsia, levels of inhibin A and activin A have been found to be elevated prior to the onset of the disease[249]. Cases of gestational hypertension in the same study group were found to have similar inhibin A levels to controls suggesting that the rise in inhibin A was specific to PET. An association between inhibins, activins and pre-term labour has yet to be established[258-260], with maternal serum

levels of activin A being higher in both term and pre-term labours than in term pregnant women who are not in labour[261]. In vitro work has shown that activin A increases prostaglandin E2 secretion from amniotic cells[212] and immunoreactive oxytocin from placental trophoblast[262] suggesting a possible role for activin A in the initiation of labour. Follistatin levels have not been found to be altered in PET[263].

Oestradiol and progesterone

The role of oestradiol (E2) in early pregnancy is yet to be elucidated. It is produced by the ovarian follicle, corpus luteum and the developing placenta in early pregnancy[264], in particular by the syncitiotrophoblast[265;266]. It is thought to play an important role in the successful outcome of IVF, with higher levels being associated with increased fertilisation rates but with a possible reduction in implantation rates secondary to alteration or impairment of uterine receptivity[267;268]. There is also evidence to suggest that it plays a role in the functional transformation of the syncitiotrophoblast, resulting in an up-regulation of progesterone biosynthesis and therefore the maintenance of pregnancy and the maturation of fetal adreno-corticoid activity[269].

Normal placental development requires extensive angiogenesis, and reduced vascular development has been associated with early pregnancy loss[270], fetal growth restriction[271]

and pre-eclampsia[30]. Oestradiol has been shown to increase vascular endothelial growth factor receptor-2 expression (VEGFR-2), promoting angiogenesis[272-274], suggesting a role for E2 in endometrial development in the menstrual cycle and the normal development of the feto-maternal interface in pregnancy[275;276]. In later pregnancy, progesterone and E2 exert opposing effects on myometrial contractility[277], with progesterone withdrawal playing an important role in the initiation of spontaneous labour.

The evidence discussed in Chapter 3.4 has already suggested an association between first trimester threatened miscarriage and later complications of pregnancy. If this is the case and if suppressed or elevated levels of these hormones reflect changes in placental development and function, it is possible that measuring them in women presenting in the first trimester with threatened miscarriage will not only help to predict the likelihood of early miscarriage[238], but may help identify those women at risk of later pregnancy complications such as PET, PTL and PPROM. Early identification of such women will assist in the planning of antenatal surveillance for example the requirement for ultrasound scanning for fetal growth or uterine artery Dopplers to assess subsequent risk or to decide upon frequency of visits and even enable possible interventions.

Measuring Oxidative Stress in Human Blood and Tissues

One of the main reasons for the difficulties in assessing the role of

oxidative stress in disease processes is the problem of finding a reliable marker of such stress[278] (Table 3.3). It is apparent that an individual's overall level of oxidative stress is dependent on a combination of factors all working in synergy. A reduction in antioxidant capacity can be determined by measuring individual antioxidants such as vitamins C and E, GSH or its components such as selenium. Vitamin C and E work synergistically[70] however and single measurements may not represent the true balance of these antioxidant enzymes. Alternatively, markers of lipid and protein peroxidation can be measured[18;71] or measures of DNA oxidation[279]. More recently, techniques for measuring 'total antioxidant capacity' (TAC)[280] such as the ferric reducing ability of plasma (FRAP) assay[281]; copper reduction capacity assays, electron spin resonance (ESR), which measures the 'magnetism' of an unpaired electron; and spin trapping, a measure of 'trapped' free radicals, have been developed. Many of these methods are time consuming and require specialised equipment limiting their use as routine screening tests. Simple colourimetric assays such as some of the TAC and FRAP assays are quicker and cheaper but need further evaluation and may not represent the total antioxidant status in the stressed state. It would appear that there is no single reliable method for determining oxidative stress and measurement of multiple markers may be the most accurate method[18;282].

Table 3.3: Published methods of measuring oxidative stress in human tissues and fluids

Method	*Measure*	*Comments*
Antioxidants	Vitamin C Vitamin E	Low levels may purely indicate low dietary intake. HPLC required.
Trace Elements	Zn/Cu (SOD's) Se (GSH)	As above. Non-specific and potential for error.
Total Antioxidant Capacity (TAC) **Limited application in biological systems so far**[282].	Hydrogen Atom Transfer (HAT reactions)	Competitive reaction scheme, antioxidant & substrate compete for peroxyl radicals. e.g. ORAC/TRAP
	Electron Transfer (ET reactions)	Measure capacity of antioxidant in reduction of an oxidant which changes colour when reduced. e.g. TEAC/FRAP/TAP (Cu II)
Electron Spin Resonance (ESR)	Free Radicals	Detects magnetism of unpaired electron. Limited use in biological tissues[278].
Spin Trapping	Free Radicals	Converts highly reactive free radicals into inert radicals using 'spin trapping agent' then detected by ESR. Limited application so far.
Lipid Peroxidation	Lipid peroxides	Controversial[283] but more reproducible than TBA.
	Isoprostanes	
	TBA-reactive substances (TBARS)	Non-specific measure of lipid peroxidation. Measures 2° metabolites by colourimetry.
	Lipid breakdown products • MDA[a] • Ethane/Pentane[b] • 4-hydroxynonenal	[a]Most commonly used. HPLC [b]Ethane/pentane in expired air – 'breath test', requires careful preparation of subject[284].
Protein Peroxidation	Protein Carbonyls Free thiol groups (e.g. GSH)	Numerous protein oxidation products formed.
DNA Oxidation	8-hydroxydeoxyguanosine in extracted DNA[279]	HPLC. Complex methodology. Excreted in urine – non-invasive.
Indirect Measures	Salicylate metabolites	Only patients on high dose salycilate.
	Phenylalanine metabolites Allantoin[285]	Little data available.

TBA – Thiobarbituric Acid-reactive substances, MDA – Malonealdehyde, HPLC – High Pressure Liquid Chromatography, ORAC – Oxygen Radical Absorbance Capacity, TRAP – Total Radical Antioxidant Parameter, TEAC – Trolox Equivalence Antioxidant Capacity, FRAP – Ferric ion Reducing Antioxidant Power, TAP – Total Antioxidant Potential

Chapter 4

Threatened Miscarriage and Obstetric Outcome

4.1 Introduction

In chapter three, the potential effects of first trimester threatened miscarriage were discussed in detail. Threatened miscarriage is common and has been associated with many adverse pregnancy outcomes. There appears to be an increased risk of first trimester miscarriage[16;17;115], particularly when bleeding occurs before 8 weeks[10]. If not associated with immediate fetal loss, threatened miscarriage has been linked to fetal loss later in pregnancy[7;77] as well as abruption[9;13;16], FGR, PTL[9;13;16], PPROM[9], PET[9] and LBW[12]. Although these associations have been made in the past, many of these studies are small and retrospective in nature. Long term prospective studies examining both short and long-term outcomes after threatened miscarriage, with and without evidence of an IUH are lacking.

The relationship between ultrasound parameters in early pregnancy and the risk of subsequent miscarriage has been the subject of interest for many years.

Table 4.1 Studies comparing gestational sac diameter (GSD) with pregnancy outcome. (Search criteria: GSD, gestational sac, miscarriage, outcome. Databases: PubMed and Medline)

Authors	Year	Study Type	Entry Criteria	Parameters	N	Outcome
Robinson[286]	1975	Prospective	6-13 weeks	GSV	319	Small GSV useful in diagnosis of anembryonic pregnancies
Nyberg et al[287]	1987	Prospective	No demonstrable embryo	GSD growth	83	Growth ≤0.6mm/day is abnormal
Bromley et al[288]	1991	Prospective case control	Normal FHR	MSD-CRL <5mm	68	Small MSD - 94% mc rate. Normal MSD - 8% mc rate.
Tadmor et al[289]	1994	Prospective	Low risk population	GSD:CRL,FHR	603	GSD: CRL ratio predicts mc (sensitivity 78.3%, specificity 97.8%, FPR 2.2%)
Dickey et al[290]	1994	Prospective case-control	Sub fertile population	GSD	700	GSD >50th centile predicts normal outcome in 90-95%
Oh et al[291]	2002	Prospective cohort	From 4-5 weeks gestation	GSD	67	MSD significantly smaller >35 days in miscarriage group (52%)
Makrydimas et al[175]	2003	Prospective	6-10 weeks low risk population	GSD, FHR, CRL	866	Small GSD - significant independent contribution
Falco et al[292]	2003	Prospective Cohort	Threatened Miscarriage GSD < 16mm, no embryo	GSD	50	>90% mc if GSD < 1.34 SD
Choong et al[174]	2003	Prospective Cross Sectional	Sub fertile population	CRL, MSD MSD-CRL	322	MSD-CRL low predictive value Multivariate model gave best results
Elson et al[293]	2003	Prospective Observational	No demonstrable embryo	Demographic data MSD, serum markers	200	GSD – overlap between viable and non-viable pregnancies Age, progesterone and GSD: Diagnostic of viable pregnancy - sensitivity 99.2%, specificity 70.7%.

GSV=Gestational Sac Volume GSD = Gestational Sac Diameter MSD = Mean Sac Diameter CRL = Crown Rump Length LMP = Last Menstrual Period FHR = Fetal Heart Rate MC = miscarriage

There is little data on the value of ultrasound parameters such as crown-rump length (CRL) and gestational sac diameter (GSD) for the prediction of pregnancy outcome after threatened miscarriage, or for longer term outcomes such as growth restriction or pre-term labour. Table 4.1 demonstrates how published study populations vary; including low risk asymptomatic women, women with threatened miscarriage and women undergoing assisted conception techniques.

Demographic differences are often not accounted for and clearly these data should not be directly compared, and findings should not be applied to all populations. Studies involving women undergoing assisted conception are likely to be the most reliable in terms of accurate dating of the pregnancies, particularly as miscarriage rates for such pregnancies are now believed to be comparable to the fertile population[294;295]. Overall multivariate analyses appear to provide the most sensitive predictors of pregnancy outcome and GSD features strongly in combination with one or two other parameters in all of these models[174;293]. There are a few studies examining the use of ultrasound parameters in predicting outcome in cases of threatened miscarriage[115;292;296;297]; however two of these studies concentrate on pregnancies where no embryo can be identified on ultrasound[115;292]. A third examined several variables and found a miscarriage rate of 6% when threatened miscarriage was the only variable to a maximum of 84% when an additional three ultrasound variables (fetal bradycardia, low GSD-CRL ratio and discrepancy between menstrual and sonographic estimation of

gestation) were present[297].

Gestational sac diameter (GSD)

Serial ultrasound assessments in early pregnancy have shown that gestational sac growth is slower in women who go on to miscarry[287] (1.13mm/day versus 0.7mm/day), although the variation in GSD in 'normal' early pregnancy is wide, limiting it's use for the prediction of miscarriage[286]. Once a gestational sac has been seen on ultrasound, subsequent miscarriage in the embryonic period is around 11%[298]. A smaller than expected GSD can be a predictor of poor pregnancy outcome, both alone and in combination with other parameters such as the CRL, even in the presence of embryonic cardiac activity[175;288;290;292;293]. In very early pregnancy, there appears to be no difference in GSD when compared with pregnancy outcome[291], the difference only becoming apparent from five weeks onwards. Small GSD can also be associated with chromosomal abnormality, such as triploidy and trisomies. Overall, the predictive value of the GSD in isolation is variable and dependent upon other clinical and sonographic findings. Interpretation of pregnancy outcome data for any variable in early pregnancy is hampered by significant differences in study design and entry criteria as described above.

Crown Rump Length

Measurement of the CRL has long been the mainstay of ultrasound estimation of gestational age in the first trimester[299]. More recently,

with the development of transvaginal sonography, charts have been developed for the period of gestation less than seven weeks[300]. If an embryo has developed up to 5mm in length, the risk of subsequent miscarriage is 7.2%[298]. Pregnancy loss rates drop to 3.3% for embryos of 6-10mm and to 0.5% for embryos over 10mm[298]. There is conflicting evidence for an association between early growth restriction, as defined by a deficit between the CRL and that predicted by the LMP, and karyotypic abnormalities[301-305]. A smaller than expected CRL has however been associated with subsequent miscarriage[305;306].

An association has also been made between first trimester growth discrepancies and subsequent low birth weight, both related to growth restriction and pre-term labour[307;308] but again results between studies is variable[305]. Mean gestational sac diameter to crown-rump length ratios have been used to predict pregnancy outcome with varying degrees of accuracy[174;288;289;309]. Unfortunately this technique, as for GSD measurements is of limited clinical usefulness in isolation (Table 4.1).

4.2 Objectives

The aim of this study was to prospectively follow a cohort of women with threatened miscarriage in order to determine whether bleeding in the first trimester had a significant impact on both the short and long term outcome of pregnancy, when compared with a low risk

population of women with no first trimester bleeding. It was also intended to determine whether the presence or absence, size and location of intrauterine haematomas significantly increased the risk of adverse pregnancy outcome and whether there were any ultrasound predictors after threatened miscarriage that could identify those women who were more likely to have an adverse pregnancy outcome.

4.3 Pilot Study

Before embarking on a prospective study of women with threatened miscarriage, we performed a retrospective analysis of the cases that had passed through the Early Pregnancy Unit[131]. This was firstly to give us an idea of the scope of the project and secondly to enable us to calculate an adequate sample size for a prospective study.

4.3.1 Pilot Study Methods and Study Participants

A retrospective case-control study was conducted. Pregnant women presenting with vaginal bleeding and/or lower abdominal pain at less than 12 completed weeks of pregnancy are routinely offered an ultrasound scan in the early pregnancy unit as part of their medical assessment. This study group consisted of 144 consecutive women referred to the early pregnancy unit, from the accident and

emergency department or their GP, with threatened miscarriage and an ongoing pregnancy on ultrasound over a twelve month (Sept 2000- Aug 2001) period whose continuing pregnancy was managed within the same institution.

Data on the ultrasound findings at the time of presentation together with ultrasound findings at 11-14 weeks and 20 weeks gestation (routinely scheduled appointments within our department) were obtained from the ultrasound department's computerised database. These were matched with the data regarding the outcome of the index pregnancy from the hospital's maternity services database. Further outcome information was collected by reviewing the patients' medical records.

The control group included 144 consecutive asymptomatic women attending the routine obstetric ultrasound department for a dating scan at 11-14 weeks gestation. The controls were selected from the ultrasound department database, using 'routine dating scan' as the search word and were scanned over the same time scale as the subjects. They were matched with study cases for maternal age and parity. Multiple pregnancies were excluded from analysis, as were women who had previously attended the early pregnancy unit in the index pregnancy or those who reported bleeding earlier in pregnancy. Women referred from other hospitals for nuchal translucency scans in the fetal medicine unit were also excluded.

Pregnancies were dated on the basis of maternal history of the last menstrual period (LMP) and corrected for cycle length,

unless the initial ultrasound findings (measurement of crown-rump length) showed a disparity in excess of seven days when the pregnancy was re-dated by ultrasound. The presence or absence of an intrauterine hematoma, defined as a crescent shaped echo free area between the chorionic membrane and the myometrium (Figure 4.1), was recorded.

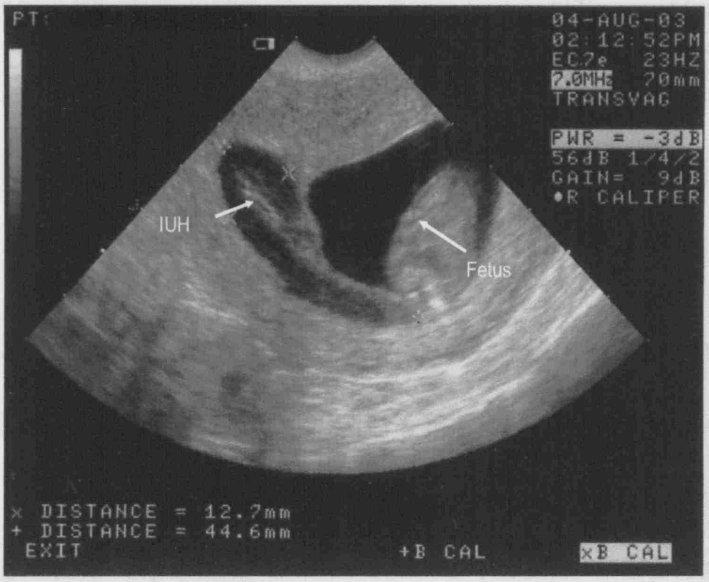


Figure 4.1: Transvaginal image of an intrauterine haematoma (IUH) adjacent to a viable 9 week fetus

An adverse pregnancy outcome was defined as any serious complication of pregnancy resulting in hospital attendance or admission, requiring treatment or delivery, or resulting in admission to the neonatal unit or treatment of the newborn infant. These adverse outcomes were classified into groups including PIH, defined as arterial blood pressure of at least 140mmHg systolic and 90mmHg diastolic on two occasions 24 hours apart after 20 weeks of

pregnancy; FGR, defined as birth weight <10th percentile (data was not available for serial scan findings to determine a reduction in growth velocity and therefore the above criteria was used); placental abruption; PPROM and PTL. The birth weight centiles for both cases and controls were calculated using maternal age, weight, ethnicity, gestation at delivery and the sex of the baby, using a centile calculator available online from the Perinatal Institute for Maternal and Child Health (Gardosi J, Francis A. www.gestation.net/centiles/).

4.3.2 Pilot Study Statistical Analysis

The maternal age distributions of the two groups were compared using the Mann-Whitney Rank Sum Test. Relative frequencies of adverse pregnancy outcome in the study and control groups were compared using the Fisher's Exact Test. Results were considered significant when $p < 0.05$. Relative risks and confidence intervals for adverse pregnancy outcome were also established. Statistical analysis was performed with The Statistics Package, Confidence Interval Analysis (CIA 1.1, 1991, Martin Gardener, BMJ Publications, London, United Kingdom).

4.3.3 Pilot Study Results

One hundred and forty four women had an ultrasound examination

after presenting with symptoms of threatened miscarriage before 12 completed weeks of pregnancy. These were matched to 144 control pregnancies on the basis of maternal age and parity. Baseline characteristics of the study and control population are provided in Table 4.2. The median maternal ages were 31 (range 17-41) and 32 (range 16-40) years in the study and control groups respectively and there was no significant difference in the age distribution between these groups (p=0.68). The median gestational age at the time of the first ultrasound examination was eight weeks and 5 days (range 4 to14 weeks) in the study group and 11 weeks and 5 days (range 5 to16 weeks) in the controls. Within four weeks of presentation, 15 women in the study group and one woman in the control group opted to terminate the pregnancy for psychosocial reasons and were excluded from the final analysis. Ten (7.8%) women in the study group miscarried before 14 weeks of gestation. The adverse pregnancy outcomes affecting the remainder of the study and control groups are shown in Table 4.3. The prevalence of adverse pregnancy outcome was significantly higher in women with a history of first trimester threatened miscarriage than in the controls (p=0.02). The relative risk of an adverse pregnancy outcome for women in the study group was 2.22 (95% CI 1.12; 4.39) compared to the control group. Although there was no significant difference in the incidence of PIH or FGR between the two groups, the relative risk of delivering a baby of less than 1000g was 4.43 (95% CI 0.50;39.2) in women with first trimester threatened miscarriage. There was also a non-

significant increase in the incidence of pre-term labour in the study group, with a relative risk of 3.05 (95% CI 0.99; 9.34).

There was no significant difference in the distribution or total number of adverse pregnancy outcomes between women who had evidence of a haematoma on ultrasound and those who did not. A comparison of adverse pregnancy outcomes between women with and without a hematoma on ultrasound examination is shown in Table 4.4.

Table 4.2 Maternal demographics in the study and control populations

Characteristic	Threatened Miscarriage n = 144	Routine Booking Scan n = 144	Haematoma on ultrasound n = 51	No haematoma on ultrasound n = 78
Mean Maternal Age (years)	30.5	30.1	32.3	32.3
Median Maternal Age (years)	31	32	31	31
Primigravid (%)	49.3%	44%	37%	37%
Multigravid (%)	50.7%	56%	63%	63%
Mean Gestation (wks + days)	8 + 5	11 + 5	8 + 5	8 + 5
Gestational Age Range (wks)	4-14	5-16	5 – 13	5 -14

Table 4.3 Adverse pregnancy outcome in 129 women presenting with threatened miscarriage compared to 143 normal controls.

Adverse Outcome	**Study group n (%)**	**Controls n (%)**	**Fisher exact test (p)**	**Relative Risk (95%CI)**
Pregnancy induced hypertension	3 (2.3)	4 (2.8)	1.00	0.83 (0.19;3.64)
Fetal growth restriction	3 (2.3)	2 (1.4)	0.67	1.66 (0.28;9.79)
Placental abruption (and IUD)	2 (1.6)	0	0.22	
Preterm prelabor rupture of membranes	3 (2.3)	1 (0.7)	**0.35**	**3.55 (0.37;33.6)**
Preterm labour	11 (8.5)	4 (2.8)	**0.06**	**3.05 (0.99;9.34)**
Total	22 (17.1)	11 (7.6)	**0.02**	**2.22 (1.12;4.39)**

Table 4.4: Comparison of adverse pregnancy outcome in women who presented with threatened miscarriage categorised by the presence (n=51) or absence (n=78) of a haematoma on initial ultrasound examination.

Adverse Outcome	Hematoma n (%)	No hematoma n (%)	Fisher exact test (p)	Relative Risk (95%CI)
Pregnancy induced hypertension	1 (2.0)	2 (2.6)	1.00	0.75 (0.07;8.22)
Fetal growth restriction	2 (3.9)	1 (1.3)	0.56	1.21 (0.29;32.9)
Placental abruption	1(1.7)	2(2.3)	1.00	
Preterm prelabor rupture of membranes	1 (2.0)	2 (2.6)	1.00	0.77 (0.07;8.22)
Preterm labour	5 (9.8)	6 (7.7)	0.75	1.27 (0.41;3.96)
Total	9 (17.6)	11 (14.1)	0.8	1.25 (0.56;2.80)

4.4 Sample Size Calculation

The overall incidence of adverse pregnancy outcome after threatened miscarriage in this study was 17.1% compared with 7.6% in the control group (RR 2.22). The sample size required for the prospective study was calculated at a 5% level of significance, with 80% power using the proportions 0.171 and 0.076. The sample size required to detect a difference was calculated as 376, 188 women in the threatened miscarriage group and 188 controls (Stata 8, Stata Statistical Software, StataCorp LP Texas USA).

4.5 Prospective Study

4.5.1 Participants

The sample population was taken from the Early Pregnancy Unit (EPU) at the Elizabeth Garrett Anderson Hospital, Huntley St, London. The EPU is a clinic designed to assess and manage women with early pregnancy problems. It sees and assesses women who have pain and/or bleeding in the first trimester of pregnancy, and women who have a history of pregnancy problems such as previous ectopic pregnancies or recurrent pregnancy loss. Referrals to the unit come from local GP's, the accident and emergency department and other hospital departments such as the reproductive medicine unit,

local family planning services and the main ultrasound department. Of the women referred to the EPU, 70% have an ongoing pregnancy. Sixty percent of these present with threatened miscarriage, resulting in approximately 1000 women passing through the unit with threatened miscarriage and an ongoing pregnancy per annum (departmental audit statistics).

4.5.2 Recruitment of Subjects

Threatened miscarriage was defined as a history of vaginal bleeding in an ongoing pregnancy of less than 14 completed weeks of gestation. Women who reported 'spotting' only of a short duration were not included in the study. After informed written consent, women were recruited into the study. Exclusion criteria included the presence of a multiple pregnancy, the finding of a congenital uterine anomaly, large fibroids distorting the uterine cavity or a known thrombophillia. We also excluded women presenting to the unit from outside our catchment area. Women who subsequently booked at a hospital other than UCLH were asked to consent to be contacted by telephone to determine the outcome of the pregnancy.

The control group consisted of women who booked for antenatal care in our hospital over the same time period. They were identified from the obstetric ultrasound database as having attended for routine first trimester screening between 10 and 14 weeks gestation as before. Control cases were excluded if they had attended the Early

Pregnancy Unit in the index pregnancy with threatened miscarriage in the first trimester, or if they gave any history of first trimester bleeding. Other exclusion criteria were the same as those listed above for the study population.

4.5.3 Data collection

Outcome Data

We followed up all women from both groups prospectively from their first appointment until delivery. Pregnancy outcome data collected included gestation at delivery, birth weight and the incidence of adverse pregnancy outcomes. Demographic data were examined including maternal age, gestation at recruitment, previous obstetric history and smoking habits. First trimester miscarriage was defined as miscarriage before 14 completed weeks of gestation, late miscarriage as pregnancy loss from 14 to 22 weeks and 6 days gestation. Pre-term labour was defined as delivery between 23 and 36+6 weeks gestation and PPROM as rupture of the fetal membranes prior to 37 weeks gestation before the onset of labour. Pregnancies delivering from 23 weeks gestation are considered viable and are actively resuscitated in our unit, and therefore 23 weeks was used as a cut off rather than 24 weeks gestation. Adverse pregnancy outcomes were classified into groups and included PET, defined as an arterial blood pressure of 140mmHg systolic and

90mmHg diastolic or above on two or more occasions 24 hours apart after 20 weeks gestation with persistent proteinuria. Fetal growth restriction was defined as a birth weight less than the 10th percentile for gestation or a documented decrease in growth velocity on serial ultrasound, with or without abnormal uterine artery Doppler parameters. Other adverse outcomes recorded included placental abruption, congenital abnormalities, placenta previa and neonatal complications requiring admission to the neonatal unit or treatment of the newborn infant.

Ultrasound data

Ultrasound data collected included the CRL, GSD, the presence of fetal cardiac activity and the presence or absence of an intrauterine haematoma. If a haematoma was identified, its location with respect to the pregnancy sac, and its diameter in three planes was recorded on each subject and the maximum haematoma volume in millilitres calculated for each. Measurements of the maximal haematoma diameter were made in 3 planes (Figure 4.1) and haematoma volume was calculated using the formula:

Length X Width X Depth X 0.5 = Volume

Pregnancies were only re-dated using ultrasound if there was a discrepancy of greater than seven days between the ultrasound findings and the date of the LMP. All scans on women in the threatened miscarriage group were performed by the same operator using an Acuson 128/XP with a 7-MHz transvaginal probe (Siemens, Mountain View, CA). After recruitment into the study, women were not routinely re-scanned unless there were persistent clinical symptoms in order to reduce any bias this may produce on the clinical outcome of the pregnancy.

4.5.4 Statistical Analysis

Statistical analysis was performed using SPSS 12.0.1 for Windows and Statgraphics Plus Version 5.1. Pregnancy outcomes, ultrasound parameters and gestational ages were compared using Fishers Exact Test and students' t-test, and birth weights using one way ANOVA.

4.5.5 Results

Demographic Data

The study included 214 women with threatened miscarriage and 214 control cases over a two year period from April 2002 to March 2004. A

larger number of women were recruited into the two groups to account for women who may have subsequently dropped out of the study or might be lost to follow up. Demographic data and overall outcome for the two groups are presented in Table 4.5. The mean maternal ages for the threatened miscarriage and control groups were 32 and 31.5 years respectively (non-significant). There was no significant difference in the smoking habits between the two groups (p=0.55).

Miscarriage and Live Birth Rate

The overall first trimester miscarriage rate for the study group, after a viable pregnancy was diagnosed on ultrasound was 9.3%. Women in the control group were recruited later in the first trimester than the cases (eight weeks compared with 11 weeks) and as a result there were no first trimester miscarriages in the control group. Once first trimester miscarriages and terminations of pregnancy were excluded, the live birth rate over 23 weeks in the threatened miscarriage group was significantly lower than controls; 95% compared with 99.5% respectively (p=0.004). Of the first trimester miscarriages, we were able to obtain tissue for karyotype in six cases. Four of these had a normal karyotype; one was trisomy 16 and one trisomy 21.

Table 4.5: Characteristics and outcomes in 214 women with threatened miscarriage compared with 214 asymptomatic controls

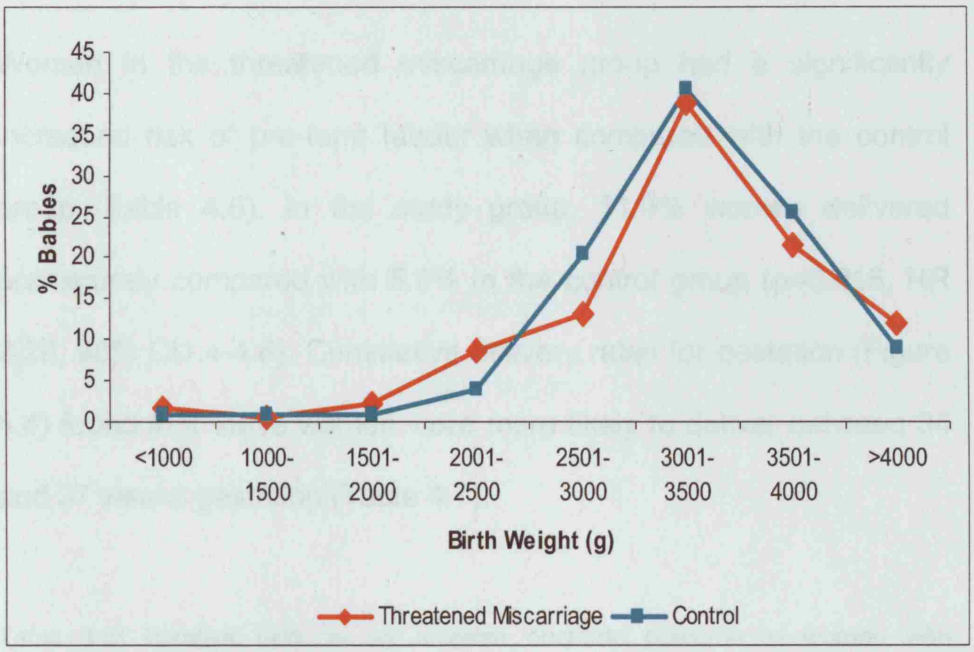
	Study Group	Control Group
Mean Maternal Age (y)	32	31.5
Primigravidas	52%	48.6%
Mean Gestation at Presentation (w)	8	11
Range (w)	5-14	10-14
Mean Gestation at Delivery (w)	38.6	39
Mean Birth Weight (g)	3215.6	3336
Live Births (*)	182 (95%)	212 (99.5%)
First Trimester Miscarriages	20 (9.3%)	0
Late Miscarriages (14-22+6 weeks)	7 (3.3%)	1 (0.46%)
Stillbirths	3 (1.4%)	0
Termination of Pregnancy	2 (0.9%)	1 (0.46%)

(*) after exclusion of first trimester miscarriages

Birth Weight

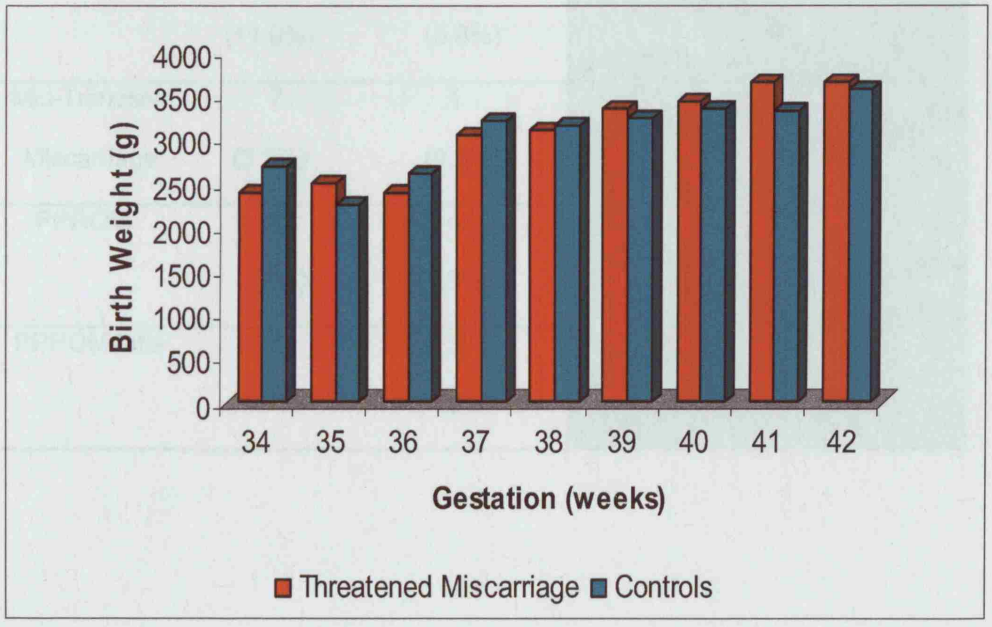
There was no significant difference in the overall mean birth weight between the two groups (mean weight 3265g and 3337g in the cases and controls respectively p=0.078) but women in the threatened miscarriage group were more likely to deliver babies between 1501 and 2000g (p=0.04) (Figure 4.2).

Figure 4.2 Percentage of babies born at each birth weight in women with threatened miscarriage compared with controls



This difference appeared to be related to premature delivery because a comparison of birth weights by gestation showed no difference between the groups (Figure 4.3) and the incidence of documented FGR between the two groups was also non-significant (p=0.1).

Figure 4.3 Comparison of birth weight by gestational age at delivery for cases of threatened miscarriage compared with controls



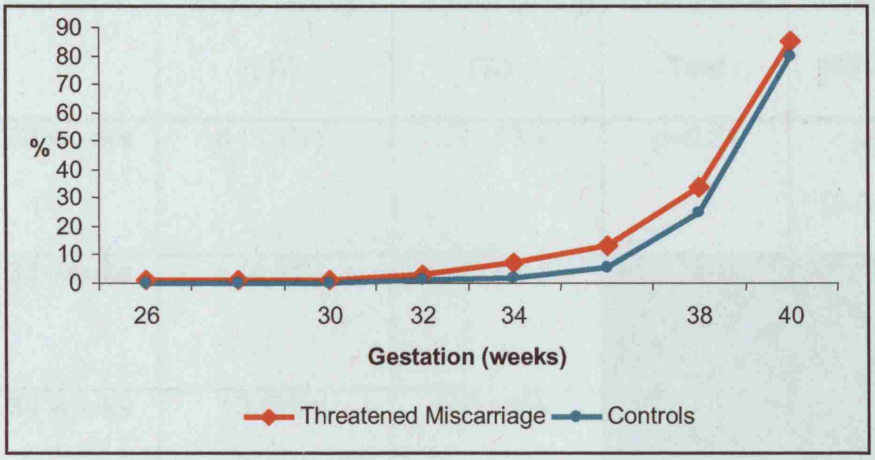
Risk of Pre-term labour and PPROM

Women in the threatened miscarriage group had a significantly increased risk of pre-term labour when compared with the control group (Table 4.6). In the study group, 11.9% women delivered prematurely compared with 5.6% in the control group (p=0.018, RR 2.29, 95% CI1.4-4.6). Cumulative delivery rates for gestation (Figure 4.4) found that these women were more likely to deliver between 34 and 37 weeks gestation (Table 4.7).

Table 4.6: Relative risks of an adverse obstetric outcome in women with threatened miscarriage compared with controls PPROM=pre-term pre-labour rupture of the membranes.

Outcome	Study Group	Control Group	Relative Risk (95% CI)	Fishers Exact Test
Pre-term Labour	22 (11.9%)	11 (5.6%)	**2.29 (1.4-4.6)**	**p=0.018**
Mid-Trimester Miscarriage	7 (3.3%)	1 (0.5%)	**6.9 (0.86-56)**	**p=0.068**
PPROM	13 (7%)	4 (1.9%)	**3.72 (1.2-11.2)**	**p=0.01**
PPROM total	17 (7.9%)	4 (1.8%)	**4.2 (1.4-12.3)**	**p=0.006**

Figure 4.4: Distribution of gestational age at delivery. Cumulative percentage is calculated from the number of women delivered by each gestational age group as a percentage of the total number for each group.



The incidence of PPROM was also significantly increased (Table 4.6) with a relative risk of 3.72 (p=0.01, 95% CI 1.2-11.2), increasing to a relative risk of 4.2 if mid-trimester membrane rupture is also taken in to account (p=0.006, 95% CI 1.4-12.3). The incidence of mid-trimester loss was increased but this finding was not significant (p=0.068, RR 6.9, 95% CI 0.86-56). When smokers were compared with non-smokers, within the threatened miscarriage group, there were no differences in the incidence of pre-term labour or PPROM.

Table 4.7 Distribution of gestational age at delivery in women with threatened miscarriage compared with controls

Gestation	Study Group (%)	Control Group (%)	Fishers Exact Test	Relative Risk (95% CI)
≤ 32 weeks	6 (3.2%)	3 (1.4%)	p=0.31	**2.0 (0.6-7.2)**
≤ 33 weeks	9 (4.9%)	3 (1.4%)	**p=0.07**	**3.0 (0.9-10.2)**
≤ 34 weeks	13 (7%)	4 (1.9%)	**p=0.01**	**3.72 (1.2-11.2)**
≤ 35 weeks	18 (9.7%)	5 (2.4%)	**p=0.002**	**4.1 (1.6-10.9)**
≤ 36 weeks	24 (13%)	11 (5.2%)	**p=0.008**	**2.5 (1.3-5.0)**
≤ 37 weeks	31 (16.8%)	20 (9.4%)	**p=0.04**	**1.78 (1.0-3.0)**
≤ 38 weeks	**63 (34%)**	**52 (24.5%)**	**p=0.05**	**1.4 (1.0-1.9)**

Pregnancy outcome, gestation at presentation & ultrasound parameters

The ultrasound parameters measured included the crown-rump length (CRL), the gestational sac diameter (GSD) and the presence or absence of an intrauterine haematoma (IUH) on each subject.

Haematoma size and location was recorded at each scan appointment. Interestingly the size and location of the IUH often varied from scan to scan getting bigger or smaller with gestation. The vast majority of IUH's (95%) could not be identified after 14 weeks gestation, with only the larger later bleeds still visible after this time. Haematomas were noted to migrate in the direction of the cervix as they resolved and supracervical bleeds appeared to be more often associated with continued or recent vaginal bleeding. The gestation at presentation with vaginal bleeding and an intrauterine haematoma were compared between the groups. Pregnancies that ended in a first trimester miscarriage presented at a significantly earlier gestation than those that continued on to a pre-term or term birth ($p=0.004$ and $p=0.003$ respectively). When gestation at maximum haematoma volumes for each pregnancy were compared, those that ended with first trimester miscarriage reached maximum volume significantly earlier than those resulting in term births ($p=0.001$). Pregnancies ending in pre-term birth reached maximum haematoma volume significantly later than the term births ($p=0.001$).

Intrauterine haematoma volumes were divided into five groups: ≤1ml, 1-5mls, 5-10mls, 10-50mls and >50mls. There were no significant differences between the groups in terms of the live birth, PTL, PPROM or late miscarriage rates.

Ultrasound data was compared to determine whether there was any correlation between initial ultrasound findings and pregnancy outcome. In the 192 cases which ended in either a live

birth or a first trimester miscarriage, there were 170 pregnancies where both a certain LMP and complete ultrasound data could be compared (88.5%). In 73% of these cases, the LMP and CRL were within seven days of each other and the pregnancy was not re-dated, they are subsequently classified as the 'normal' subgroup. In 18.8% (32 cases), the CRL measured greater than seven days smaller than the predicted date from the LMP (mean 14.9 days, range 8 to 62 days) and are subsequently classified as the 'small-for-dates' subgroup. In 8.2% (14 cases), the CRL was measured greater than seven days larger than that expected from the LMP (mean 14.9 days, range 8 to 35 days) and these were classified as 'large-for-dates'. When pregnancies that were small for dates were compared with those which were the expected size, no statistically significant differences could be identified in either the number of miscarriages ($p=0.77$) or the number of live births ($p=0.4$). There were no differences between outcomes when the GSD: CRL ratios or the GSD-CRL measurements were compared between groups.

The birth weights of the live births were compared between the 'small-for-dates' and the 'normal' subgroups using the student's t-test. The mean weights were 3207.5 grams (SEM 115.79) and 3308 grams (SEM 67.28) respectively. There was no statistically significant difference between the birth weights between these groups ($p=0.5$) and there was no correlation with delivery at less than 37 weeks gestation ($p=0.4$).

Other Adverse Outcomes

1. Obstetric Outcomes

The incidence of PET was similar between the cases and controls (p=0.47), as was abruption (p=0.22) and placenta previa (p=0.1). There were no differences in the Apgar scores at one and five minutes between the groups (p=0.68 and 0.37 respectively).

2. Congenital Anomalies and Neonatal Outcomes

The number and type of congenital abnormalities between the groups are shown in Table 4.8, the differences between the groups was non-significant (p=0.1). In the threatened miscarriage group, four babies were admitted to the neonatal unit with neonatal seizures. In two cases, a diagnosis of antenatal cerebral infarction was made. Both mothers subsequently underwent thrombophillia screens that were negative and both babies made a full recovery. One baby was admitted with mild hypoxic ischaemic encephalopathy (HIE) and made a full recovery and one baby was diagnosed with cerebral palsy of unknown aetiology. There were three still births (SB) within the study population, one at 23 weeks after PPROM, one at 27 weeks with severe IUGR and PET and an unexplained SB at 39 weeks. In the control group, one baby was admitted with moderate HIE that made a full recovery.

Table 4.8 Congenital abnormalities diagnosed in cases of threatened miscarriage and controls

	Abnormality	**No of Cases**
Cases	CCAM*	1
	Cleft Palate	1
	Hydronephrosis	2
	Renal Agenesis	1
	Anencephaly	1
	Gastroschisis	1
	Extra Digit	1
Controls	Cleft Palate	1
	Hydronephrosis	1

*CCAM – Congenital cystic adenomatoid malformation of the lung

Chapter 5

Oxidative Stress and Antioxidant Defences in Early Pregnancy and Threatened Miscarriage

5.1 Introduction

The evidence discussed in chapter three suggests that the oxygen tension in the early placenta is tightly controlled and that the normal physiological rise in oxygen tension that occurs towards the end of the first trimester is integral to the differentiation of the cytotrophoblast from a proliferative to an invasive phenotype. The trophoblast plugs that are evident within the spiral arteries appear to limit the amount of communication between the maternal and fetal circulations, suggesting that the early embryo is supplied with essential nutrients via another pathway, namely the uterine glands, the exocoelomic fluid and the secondary yolk sac (Figure 5.1). Levels of the principal intracellular antioxidant enzymes Copper/Zinc superoxide dismutase and catalase are low in the syncitiotrophoblast of early pregnancy[28;44;45], only increasing towards the end of the first trimester, coinciding with the increase in oxygen tension. Evidence suggests that disruption to this tightly controlled protection system

might be involved in the pathophysiology of early pregnancy failure. In cases of threatened miscarriage, it is possible that early and overwhelming influx of oxygenated maternal blood into the intervillous space, or around the fetal membranes may impair this process resulting in either immediate miscarriage, or longer term pregnancy complications as a result of chronic oxidative stress.

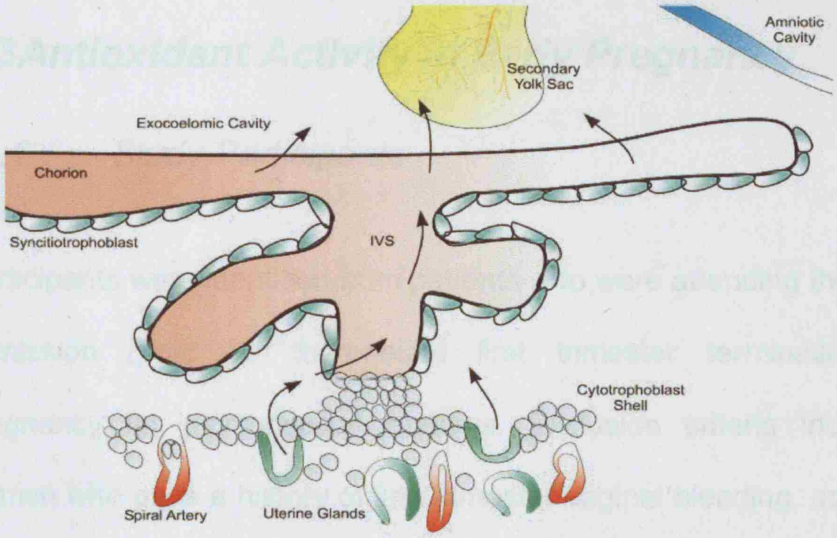


Figure 5.1: Diagrammatic representation of early embryonic nutrition. Secretions from the maternal uterine glands are delivered into the intervillous space (IVS), which are then taken up by the syncitiotrophoblast and secondary yolk sac via the exocoelomic fluid.

5.2 Aims and Objectives

The aims of this study were (i) to further investigate the presence and activity of antioxidant substances in early pregnancy by measuring a panel of antioxidant substances in both maternal serum, and fetal fluids and (ii) to compare antioxidant levels in the serum of women

with threatened miscarriage with control pregnancies without threatened miscarriage. The original aim was to measure the same substances as for part (i) of this study; however technical difficulties with the ascorbic acid measurements (described below) made this impossible. In view of this, an evaluation of a new 'total antioxidant power' assay was evaluated in the second part of the study.

5.3Antioxidant Activity in Early Pregnancy

5.3.1 Study Participants

Participants were identified from patients who were attending the pre-admission clinic for therapeutic first trimester termination of pregnancy for psychosocial reasons. Exclusion criteria included women who gave a history of first trimester vaginal bleeding, multiple gestations and pregnancies that were being terminated for fetal abnormality or maternal disease. After written informed consent, participants were recruited into the study and demographic (including smoking habits) and current and past pregnancy details were recorded. Gestations were confirmed and accurately dated using transvaginal ultrasound during the procedure and measuring the CRL.

5.3.2 Sample collection, preparation and storage

All samples were collected whilst the subject was under general anaesthetic. Twelve millilitres (mls) of blood was collected from the

antecubital fossa, 6mls was allowed to clot in a plain tube and 6mls was transferred immediately into a tube containing lithium heparin (BD Vacutainer ®, BD UK) and wrapped in silver foil to prevent oxidation. The samples were kept cool and centrifuged within one hour at 3000 rpm and stored in aliquots of 500 microlitres, wrapped in foil at -80˚C until analysis. Fetal fluid samples (amniotic and coelomic) were collected from the same subjects under transvaginal ultrasound guidance (Figure 5.2) using 30cm, 20 gauge purpose made needles (Cook ® O'Halloran Road, National Technology Park, Limerick, IRELAND) as previously described[58]. The first 200 microlitres of each fluid sample was discarded to avoid contamination with maternal blood. The fluids were also aliquoted into 500 microlitre samples and immediately snap frozen in liquid nitrogen and stored at -80˚C until analysis.

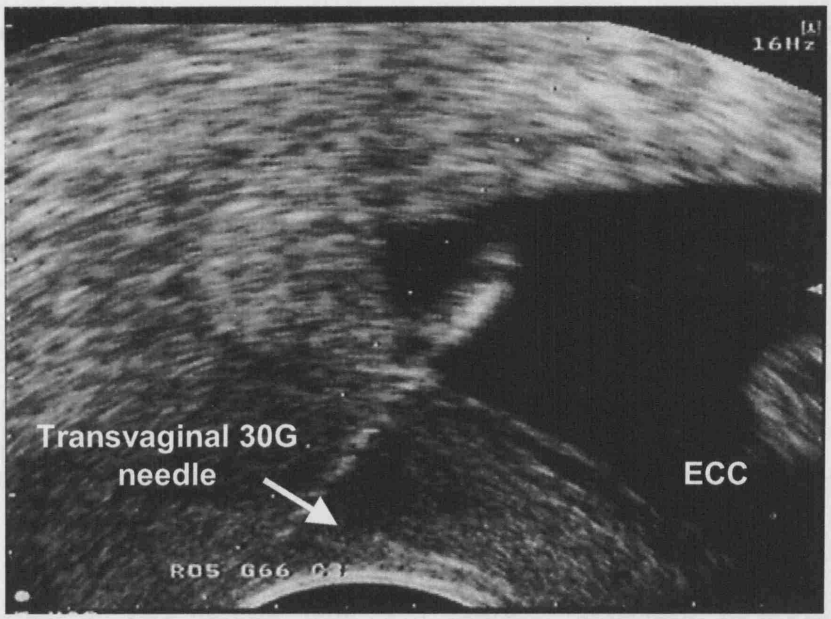


Figure 5.2: Transvaginal ultrasound image of coelocentesis. The fluid is collected with a 30 cm 20G needle from the exocoelomic (ECC) and amniotic compartments.

5.3.3 *Fluid assays*

Glutathione, α- and γ-tocopherol, uric acid and ascorbic acid assays were performed in the laboratory at the School of Health and Life Sciences, Kings College, London, under the direction and supervision of Ms Christina Dunster.

Glutathione

Total glutathione was measured using an enzyme recycling method based on previously reported methodology[310]. Samples were defrosted and mixed thoroughly. Fifty µl of fluid samples and external standard were aliquoted in duplicate into a 96 well ELISA plate and 100µl of substrate (0.3mM nicotinamide adenine dinucleotide phosphate reduced, 0.22 mM 5,5' dithiobis-2-2-nitrobenzoic acid and 19U glutathione reductase in 100mM phosphate buffer (pH 7.5)) was added. Total glutathione was detected as an increase in tetrazolium nitroblue colour development over two minutes at 30°C on a MRX 2 Microplate Reader (Dynex Technologies, Chantilly, VA, USA) at 290nm.

Alpha- and γ-tocopherol in maternal plasma

α- and γ-tocopherol were measured in plasma using high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection. 100µl of sample was mixed with 5µl internal standard (α-tocopherol acetate) in 100µl ethanol to remove proteins. Based on previous work, it is assumed that recovery of α-tocopherol reflects the

recovery of the other tocopherol isomers[311]. 400µl ice cold HPLC grade hexane was added to the plasma and the samples vortexed twice for 40 seconds. The samples were centrifuged at 3000 rpm for five minutes at 4°C and the hexane layer carefully removed and transferred into an 0.8ml HPLC vial. The sample was evaporated to dryness under a stream of nitrogen and then re-constituted into 400µl HPLC grade methanol and vortexed twice for 40 seconds. Aliquots of 100µl were injected onto the HPLC with UV detection. Concentrations were calculated with external standards and adjusted for recoveries with the internal vitamin E standards[311].

Alpha- and γ-tocopherol in fetal fluids

One hundred picomoles (pmol) deuterated α-tocopherol internal standard was added to a 1ml sample and mixed thoroughly. 50% sodium dodecyl sulphate was added 1:1, mixed and 3ml of ethanol was added. The tocopherols were extracted into hexane as above. The samples were centrifuged at 3000 rpm at 10°C for 10 minutes and the hexane layer transferred into a glass vial and evaporated under a stream of nitrogen to dryness. The residue was silylated in the presence of 100µl anhydrous pyridine and 50µl bistrimethylsilytrifluoroacetatamide containing 1% trimethylchlorosilane for one hour at 65°C. Vials were allowed to cool and evaporated to dryness under a stream of nitrogen. Once dry, 100µl gas chromatography (GC) grade octane was added to the sample, which was then capped and vortexed twice for 40 seconds

over a 20 minute period. The octane was transferred to GC vials and 1µl was injected onto GC with a mass spectrometry detector. Final concentrations for α- and γ-tocopherol were calculated with external standards and adjusted for recoveries with the internal deuterated vitamin E standard.

Ascorbic Acid

Plasma samples were acidified 1:1 with ice cold 10% metaphosphoric acid, mixed and diluted 2:3 with ice cold 5% metaphosphoric acid, resulting in a final dilution of 1:5. The fetal fluid samples were diluted 2:3 and 100µl HPLC grade heptane was added and the samples vortexed for 40 seconds. The samples were centrifuged at 13000 rpm for 5 minutes and the lower aqueous layer removed and re-treated with heptane until the supernatant was clear. This was transferred into 0.8ml HPLC vials. Twenty µl aliquots were injected onto a HPLC with an electrochemical detector working electrode set at 400 mV, with a sensitivity of 1µamp. Final concentrations of ascorbic acid were calculated with external standards. Dehydroascorbic acid (DHA) was calculated by measuring the total vitamin C in samples incubated with dithioreitol before acidification.

5.3.4 Statistical analysis

Data was analysed using Statgraphics (Manugistics, Rockville, MD).

Differences in maternal and fetal fluid samples were testing using ANOVA, the multiple range test and Fishers least significant difference procedure. Linear regression analysis between fetal fluids and gestational ages and fetal fluid and maternal plasma were calculated by the least squares method and the slopes tested for significance by the F ratio test. Multiple linear regression analysis was used to evaluate the relationship between coelomic fluid and maternal plasma and gestational age.

5.3.5 Results

Thirty four matched samples were used for analysis. The mean age of the participants was 27.7 years (range 16-45, median 27.5 years), the mean gestation was nine weeks (range 5-12, median 9 weeks). The results of the fluid and plasma analysis are represented in table 5.1 and figure 5.3. The mean level of GSH in CF was significantly lower than maternal plasma levels ($p<0.001$) and GSH was not detectable in AF. Maternal plasma levels of α-tocopherol were significantly higher than CF ($p<0.001$) which in turn were significantly higher than AF levels ($p<0.001$). γ-tocopherol levels in CF were significantly lower than maternal plasma ($p<0.001$) and were undetectable in AF. Ascorbic acid was higher in CF than AF ($p=0.05$) but levels were similar between CF and maternal plasma. DHA levels were similar between CF and AF but CF levels were significantly lower than maternal plasma ($p<0.001$).

Table 5.1: Comparison of the mean (SEM) values of antioxidant molecules in maternal plasma, coelomic fluid (CF) and amniotic fluid (AF).

Antioxidant (μmol/l)	Maternal Plasma	Coelomic Fluid	Amniotic Fluid
Glutathione	17.1 (2.3)[a]	0.10 (0.02)	ND
α-tocopherol	22.3 (1.1)[a]	0.60 (0.07)[b]	0.09 (0.03)
γ-tocopherol	1.69 (0.13)[a]	0.02 (0.01)	ND
Ascorbic Acid	37.6 (4.7)	31.9 (1.6)[b]	10.0 (3.5)
Dehydroascorbic acid	5.0 (0.9)[a]	3.0 (0.5)	1.7 (0.6)
Uric acid	171.8 (10.4)	162.8 (10.5)[b]	66.3 (7.4)

ND = not detectable
[a]Significant difference between maternal plasma and CF
[b]Significant difference between CF and AF

A significant positive linear relationship was present between gestational age and α-tocopherol concentrations in the CF (R=-0.50; SEM=0.27; F=5.4; p=0.033). There was no such relationship for AF or maternal plasma. A significant linear-positive relationship was present between CF and maternal plasma γ-tocopherol levels (R=0.59; SEM=0.01; F=8.74; p=0.009) and there were also significant linear-positive relationships between AF and CF for ascorbic acid (R=0.55; SEM=12.8; F=5.9; p=0.03) and DHA (R=0.60; SEM=1.9; F=6.1; p=0.03).

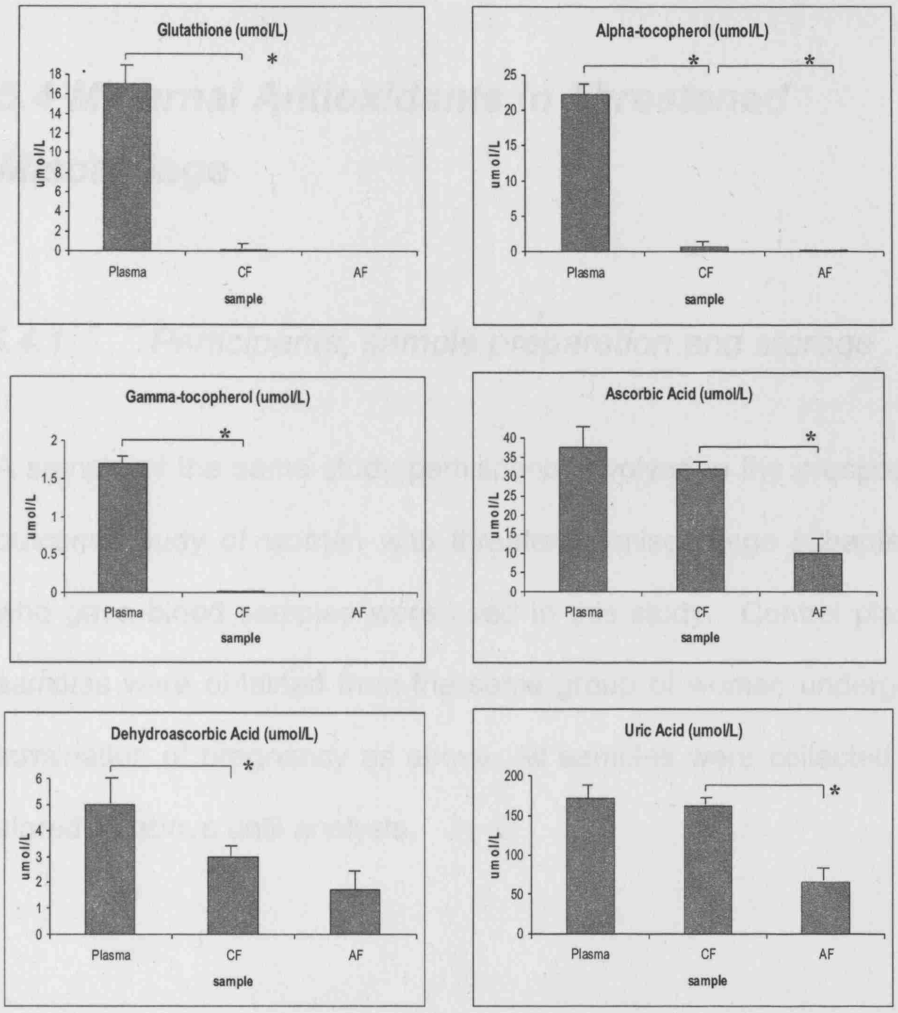


Figure 5.3: Comparison of the mean antioxidant levels between maternal plasma, coelomic fluid (CF) and amniotic fluid (AF). *=significant result

5.4 Maternal Antioxidants in Threatened Miscarriage

5.4.1 Participants, sample preparation and storage

A sample of the same study participants involved in the prospective outcome study of women with threatened miscarriage (Chapter 4) who gave blood samples were used in this study. Control plasma samples were obtained from the same group of women undergoing termination of pregnancy as above. All samples were collected and stored as above until analysis.

5.4.2 Antioxidant Assays

Alpha- and γ-tocopherol

Samples were analysed as above using HPLC with UV detection.

Ascorbic acid

Threatened miscarriage plasma samples were treated in the same way as the control samples above. Ascorbic acid was undetectable in all of the threatened miscarriage samples, despite no obvious difference in sample handling and storage. The samples were then spiked with known concentrations of ascorbic acid and these spikes

were detectable by the assay. As a result, no further ascorbic acid assays were performed. Further analysis was performed using the 'Total Antioxidant Power' assay.

Total Antioxidant Power

Total antioxidant power (TAP) was measured using a commercial colourimetric microplate assay kit which detects reduced copper (Cu^{+}) (Oxford Biomedical Research, P.O. Box 522, Oxford MI 48371, USA). The principal of the assay is that the combined antioxidant activity present in the sample or standard will reduce Cu^{++} to Cu^{+}. The reduced copper forms a 2:1 complex with the chromogenic agent which is detectable at 490nm on a microplate reader. A reference curve is created using a known concentration of uric acid and results are expressed in micromolar (mM) copper reducing equivalents for each assay. Uric acid standards were prepared at the following concentrations:

2.0mM	**1.0mM**	**0.5mM**	**0.25mM**
	0.125mM	**0mM**	

All buffers and solutions were allowed to equilibrate to room temperature for 30 minutes prior to running the assay and mixed well. All samples and standards were diluted 1:40 using the provided dilution buffer. Two hundred microlitres (µl) of diluted samples and

standards were placed in duplicates onto the assay plate and the plate was read for a reference measurement (OD reference) on a MRX 2 Microplate Reader (Dynex Technologies, Chantilly, VA, USA). Fifty µl of Cu^{++} solution was added to each well and the plate incubated for three minutes at room temperature, then 50µl stop solution (Appendix 5.1) was added to each well. The plate was read again at 490nm on the microplate reader (OD final).

The results are calculated by calculating the net absorbance (OD reference-OD final) and plotting the net absorbance against the uric acid concentration. The total antioxidant power is calculated by using the formula:

Y=mX+b where: Y = OD reading (Y axis)

X = uric acid concentration

(or Cu reducing equivalent)

m = slope of the graph

b = Y axis intercept

1.0mM = 2189 µM copper reducing equivalents.

5.4.3 Statistical Analysis

Differences in maternal serum results between cases and controls

were tested using ANOVA, the multiple range test and Fishers least significant difference procedure. Linear regression analysis was also performed between maternal serum alpha-tocopherol and TAP results by the least squares method and the slopes tested for significance by the F ratio test.

Results

Forty-five cases of threatened miscarriage were compared with 34 control cases, which were matched for gestation. Alpha-tocopherol levels were available in 21 cases of threatened miscarriage only. The basic demographic data for these groups can be seen in Table 5.2. Again, maternal ages between the two groups were significantly different. Table 5.3 represents the mean values (SD) for alpha-tocopherol and TAP.

Table 5.2: Demographic details and pregnancy outcomes of 45 cases presenting with threatened miscarriage and 34 controls.

	Cases (n=45)	**Controls (n=34)**
Mean Maternal Age (y)	33.3*	27.7
Mean Gestation (w)	9	8.7
Total Live Births	37 (82.2%)	NA
Term Births	29 (64.4%)	NA
Pre-term Births	8 (17.8%)	NA
First Trimester Miscarriages	8 (17.8%)	NA

NA = not applicable

Table 5.3: Mean (SD) values of alpha-tocopherol and Total Antioxidant Power (TAP) for cases of threatened miscarriage and controls. The TAP group were further subdivided into pregnancy outcomes.

	Term Birth n=29	PTL n=8	Miscarriage n=8	Cases (All)	Controls n=34
Alpha-tocopherol (µmol/L)	23.8 (4.0)	NA	NA	24.2* (3.6) n=21	21.4 (5.1)
Total Antioxidant Power (µmol)	829.7 (170.6)	879.9 (165.1)	813.0 (151.9)	844.6 (168.1) n=45	896.6 (190.1)

NA = not available – 1 case only
*statistically significant result

Alpha-tocopherol

Alpha-tocopherol levels were significantly higher in the threatened miscarriage cases compared with the controls (p=0.0335), Figure 5.4. Subgroup analysis by pregnancy outcome was not possible, as there was only one miscarriage and one case of pre-term labour in this initial pilot group.

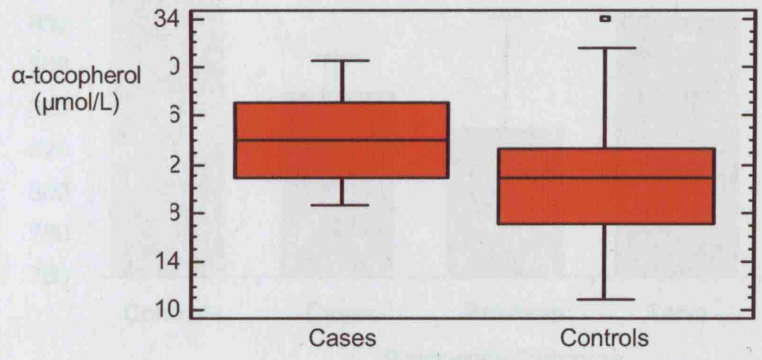


Figure 5.4: Alpha-tocopherol levels in cases of threatened miscarriage (n=21) compared with controls (n=34). The mean levels of alpha-tocopherol were 24.2 and 21.39 µmol/L for cases and controls respectively.

Total Antioxidant Power (TAP)

Overall, the mean TAP level was higher in the control group than in the cases of threatened miscarriage (Figure 5.5), the levels being lowest in cases of threatened miscarriage that resulted in first trimester miscarriage. These differences were not statistically significant, either between cases and controls, or between the different subgroups.

Linear regression analysis comparing alpha-tocopherol and TAP showed no relationship between the two in either the cases (term births compared) or the controls. A comparison of TAP between smokers (n=4) and non-smokers (n=42) in the control group showed no significant difference between the groups (p=0.13), although TAP levels were lower in the smoking group (mean 767.6 (SD 166.9), 930.9 (SD 185.7) compared with the non-smokers.

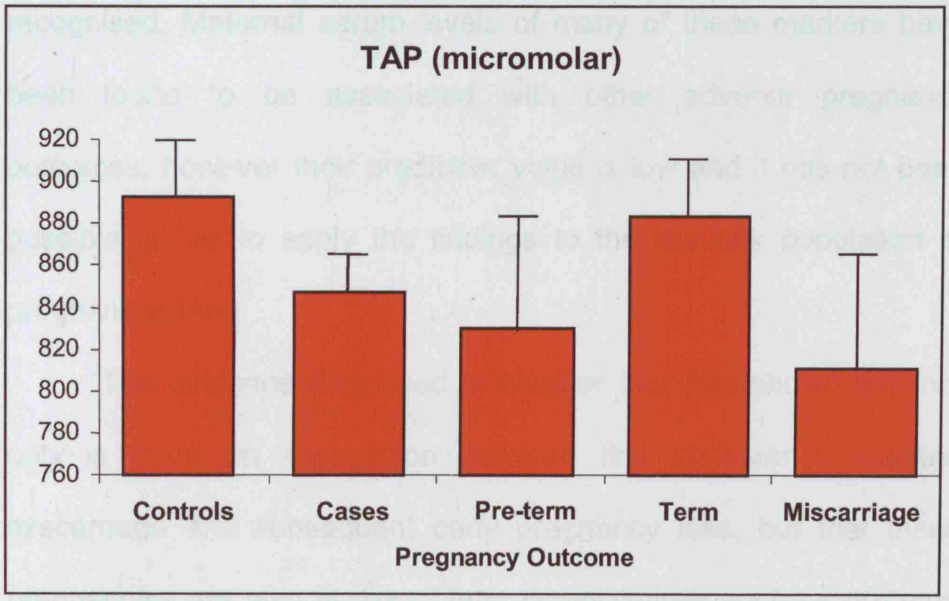


Figure 5.5: Total Antioxidant Power (TAP) in micromolar equivalents for cases and controls by pregnancy outcome means and SD's.

Chapter 6

Markers of Placental Function in Threatened Miscarriage

6.1 Introduction

In chapter three, the role of maternal serum markers in the assessment of placental function were discussed in detail. Variations in maternal serum levels of many hormones produced by the placenta have been used successfully in screening for chromosomal aneuploidies, and the role of hCG and more recently progesterone in the prediction of pregnancy outcome in early pregnancy is well recognised. Maternal serum levels of many of these markers have been found to be associated with other adverse pregnancy outcomes, however their predictive value is low and it has not been possible so far to apply the findings to the low risk population of pregnant women.

The evidence discussed in chapter four has shown that not only is there an association between first trimester threatened miscarriage and subsequent early pregnancy loss, but that these pregnancies are also at risk of later complications such as pre-term labour (PTL) and pre-term pre-labour rupture of the membranes (PPROM). After threatened miscarriage, alterations in the levels of

hormones produced by the placenta may either reflect damage to the placenta and fetal membranes, or compensatory mechanisms that occur as a result of this disruption to placental development at the time of the bleeding. It is also possible that measuring them in women presenting in the first trimester with threatened miscarriage will not only help to predict the likelihood of early miscarriage, but may help identify those women at risk of the later pregnancy complications described above.

In this prospective study, maternal levels of inhibin A, activin A, follistatin, hCG, PAPP-A, progesterone and E2 were measured in women presenting to the early pregnancy unit with threatened miscarriage. The study group consisted of the cohort of women from chapter four who presented with first trimester threatened miscarriage and an ongoing pregnancy and the blood results were related to the overall outcome of the pregnancy. The results were also compared with gestation matched control pregnancies with no history of first trimester bleeding, which underwent therapeutic termination of pregnancy for psychosocial reasons within the same time frame.

6.2Aims and Objectives

The objectives of this study were to investigate (i) if the serum levels if inhibin A, activin A, follistatin, hCG, PAPP-A, oestradiol and progesterone in women with first trimester threatened miscarriage

are related to the outcome of the pregnancy and (ii) to study the relationship between these hormones in first trimester threatened miscarriage patients compared to gestation matched controls pregnancies with no history of first trimester bleeding.

6.3Methodology

6.3.1 *Sample collection, preparation and storage*

After informed consent, women recruited into the prospective threatened miscarriage study were asked to provide a blood sample. Blood was taken at the first visit when the subject presented with threatened miscarriage, or as close to this time as possible. Twelve millilitres (mls) of blood were collected from the antecubital fossa of each subject, six mls into a plain tube where blood was allowed to clot and six mls into a tube containing lithium heparin (BD Vacutainers ®, BD UK). The lithium heparin samples were immediately wrapped in tin foil to prevent oxidation (see chapter 5) and kept cool until centrifuged. Within two hours of collection, the samples were centrifuged at 3000rpm for 10 minutes and serum/plasma was separated into one ml aliquots and immediately frozen at -80° Celsius, where they were stored until analysis.

6.3.2 *Inhibin-A, Activin-A and Follistatin ELISAs*

Inhibin-A, Activin-A and Follistatin were all measured using the Oxford Brookes Enzyme Linked Immunosorbent Assay[312] (ELISA) using monoclonal antibodies as the capture and detection antibodies. The assays are two-site 'sandwich' assays that use an ultra sensitive detection system with alkaline phosphatase as the label (Figure 6.1).

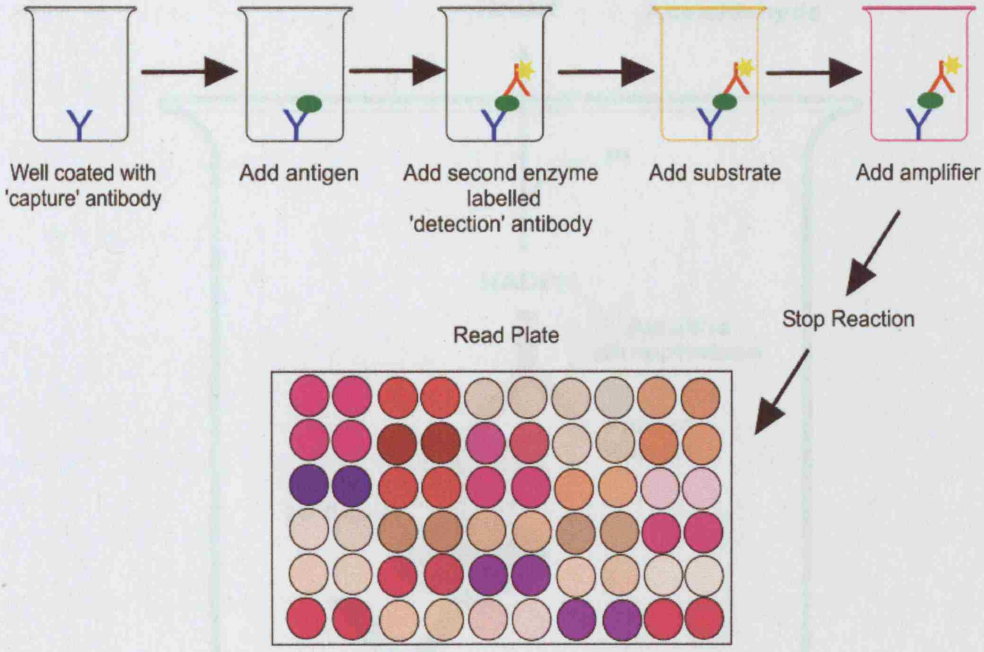


Figure 6.1: Diagrammatic representation of a 'sandwich' ELISA assay used for measuring inhibin A, activin A and follistatin

Inhibin A ELISA

Inhibin A was measured using a two-site enzyme linked immunosorbent in-house assay (ELISA) previously validated for use

in human serum[313]. The ELISA uses monoclonal antibodies against the βA subunit of inhibin (E4)[314] as the capture antibody and the Fab' fragment of a monoclonal antibody against the α-subunit (R1)[315] conjugated to alkaline phosphatase as the second detection antibody and is performed over two days (Figure 6.2).

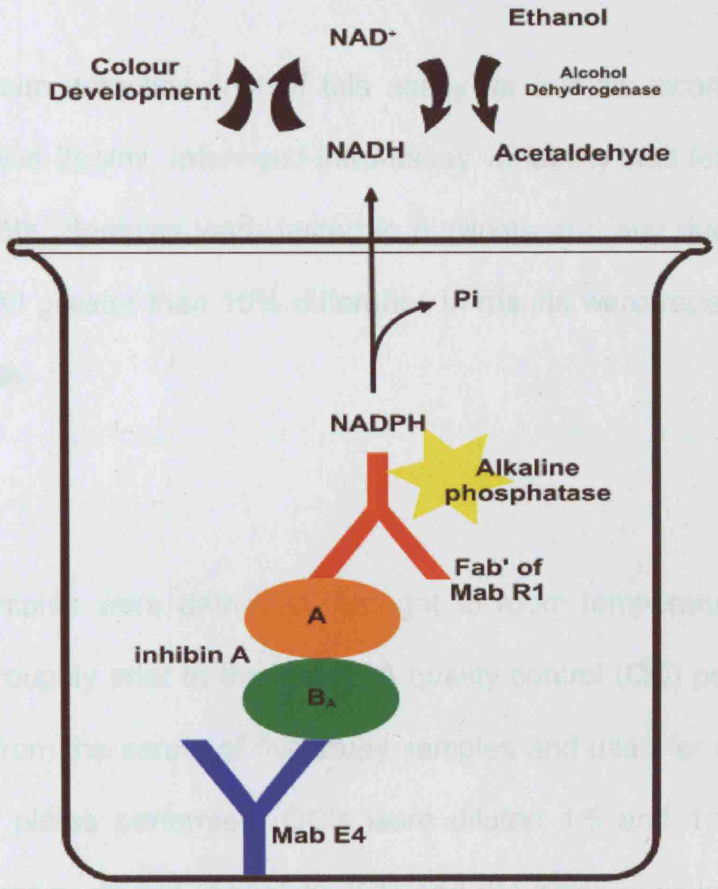


Figure 6.2: Diagrammatic representation of the inhibin A ELISA as described above

Sample standards were prepared in inhibin A assay diluent (Appendix 6.1) using human recombinant inhibin A (National Institute

for Biological Standards, Potters Bar, Herts UK) in the following concentrations:

500pg/ml: 250pg/ml: 125pg/ml: 62.5pg/ml: 31.3pg/ml: 15.6pg/ml: 7.8pg/ml: 3.6pg/ml

The minimum detection limit of this assay for human recombinant inhibin A was 2pg/ml. Inter- and intra-assay variability was less than 10% for both. Samples were tested in duplicate and any duplicates that showed greater than 10% difference in results were repeated in a new assay.

Day 1

Serum samples were defrosted, brought to room temperature and mixed thoroughly prior to the assay. A quality control (QC) pool was prepared from the serum of five study samples and used for each of the assay plates performed. QC's were diluted 1:5 and 1:10 with inhibin A assay diluent (Appendix 6.1) and the blank samples were inhibin A assay diluent. Initially a test assay was performed with different dilutions of the samples to obtain the samples on the standard curve. One hundred and twenty five microlitres (µls) of standards, samples, blanks and controls were pipetted into labelled tubes and 125µls inhibin A assay diluent was added to each sample tube. 125µls fetal calf serum was added to the standards and the

blanks (to overcome the matrix effect) and this was followed by 50µls of 10% hydrogen peroxide (H_2O_2) into all wells. Samples were mixed well and incubated for 15-30 minutes. 100µls blanks, samples, standards and controls were transferred into duplicate wells on an ELISA E4 coated plate. The plate was then covered, placed in a moist box and incubated overnight at 4° Celsius.

Day 2

Any unbound sample was discarded and the plates washed ten times with ELISA wash buffer (Appendix 6.2) using a Wellwash 4 Mk II plate washer (Thermo Electron Corp, Bioscience Technologies, Basingstoke, UK) and bang dried on paper towelling. Fifty microlitres of anti-α subunit antibody conjugated to alkaline phosphatase (R1-alk phos) was added to each well and the plate incubated for two hours at room temperature in a moist box. Any unbound antibody was then discarded and the plate washed 15 times on a plate washer and two manual washes were carried out using manual wash buffer (Appendix 6.3).

50µls substrate (ELISA Amplification System, Invitrogen Life Technologies) was added to each well and the plate incubated for one hour at room temperature. After one hour, 50µls amplifier was added to each well in the same sequence and timing as the substrate. The reaction was stopped with 50µls per well of Stop Solution (Appendix 5.1) when the blanks started to develop colour (after approximately 10 minutes) and the plate was shaken for a few

minutes. The plate was then read at 490nm on a MRX 2 Microplate Reader (Dynex Technologies, Chantilly, VA, USA).

Activin A ELISA

The activin A ELISA is also a two-site assay specific for 'total' activin A (follistatin-bound and unbound activin A)[210]. The assay uses the E4 monoclonal antibody as both the capture and detection antibody. The E4 used for detection is biotinylated and extravidin conjugated to alkaline phosphatase is incorporated into the assay as E4 cannot make a FAb' alkaline phosphate conjugate (Figure 6.3). An SDS/heat treatment is added which denatures follistatin to allow the measurement of total activin A and not just the free form. The detection limit of this assay for human recombinant activin A (Genetech, San Francisco, CA, USA) was 50pg/ml. Intra- and inter-assay variations were less than 10% respectively. Any duplicate samples that showed greater than 10% variability were repeated.

An affinity purified human follicular fluid stock standardised against human recombinant activin A was used as standard. Standards were made up to the following concentrations using PBS + 10% BSA solution (Appendix 6.4):

10000pg/ml: 5000pg/ml: 2500pg/ml: 1250pg/ml: 626pg/ml: 312pg/ml: 156pg/well: 78pg/well

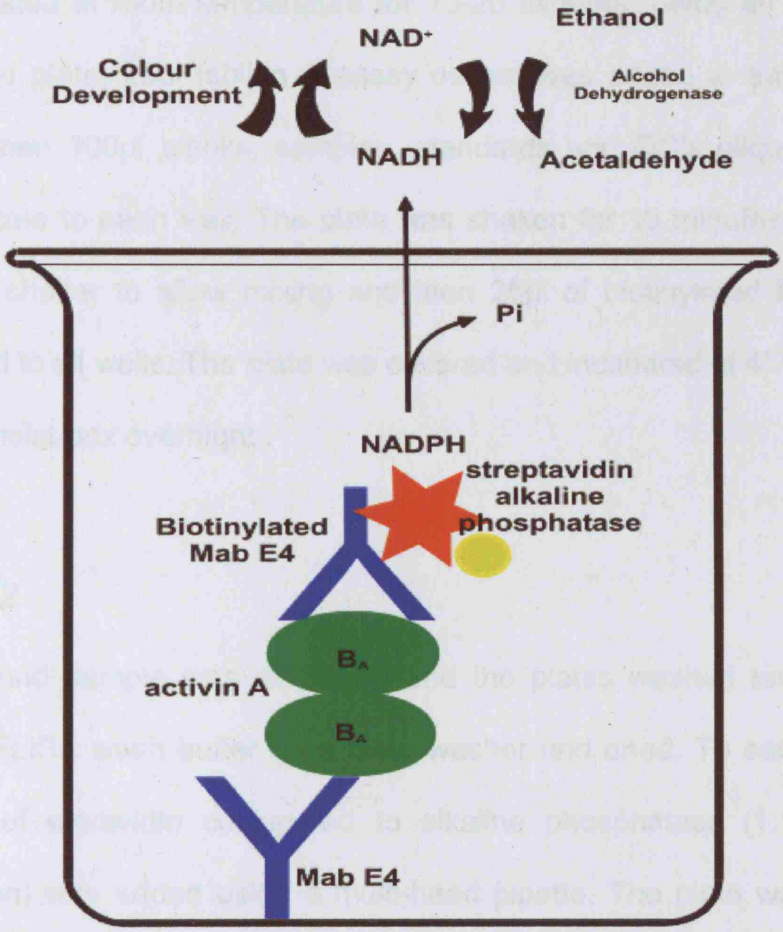


Figure 6.3: Diagrammatic representation of the actvin A ELISA

Day 1

Samples were defrosted as above and the same quality control samples were used. The blank samples were PBS + 10% BSA. 175µl of standards, samples, blanks and controls were added to labelled eppendorf tubes, 175µl 15% SDS added to each tube and the tubes mixed by inversion. The tubes were then boiled at 85-95° Celsius for 10 minutes. Samples were left to cool for a few minutes, 30µl of 30% H_2O_2 was added and then samples were mixed and

incubated at room temperature for 10-20 minutes. Using an E4 dry coated plate, 25µl inhibin A assay diluent was added to each well and then 100µl blanks, samples, standards and QC's aliquoted in duplicate to each well. The plate was shaken for 10 minutes on the plate shaker to allow mixing and then 25µl of biotinylated E4 was added to all wells. The plate was covered and incubated at 4° Celsius in a moist box overnight.

Day 2

Unbound sample was discarded and the plates washed ten times with ELISA wash buffer on a plate washer and dried. To each well, 50µl of extravidin conjugated to alkaline phosphatase (1:10 000 dilution) was added using a multi-head pipette. The plate was then incubated for two hours in a moist box on a plate shaker.

After incubation, the plate was washed 15 times on a plate washer, dried and then washed twice using manual wash buffer. 250µl manual wash buffer was added to each well and the plate was incubated on the plate shaker for 10 minutes. This process was repeated and the buffer discarded.

Fifty microlitres per well of substrate (ELISA Amplification System, Invitrogen Life Technologies) was added and the plate incubated for one hour at room temperature in a moist box. After an hour 50µl of amplifier was added to each well, once colour started to develop in the blank wells the reaction was stopped with 50µl per well of Stop Solution and the plate shaken for a few minutes to mix

the reagents. The plate was read at 490nm.

Follistatin ELISA

The follistatin ELISA uses the anti-follistatin monoclonal antibody (29/9) as the capture antibody and the Fab' fragment of another anti-follistatin monoclonal antibody (17/2) for detection[316] (Figure 6.4). Both samples and standards are diluted in a dissociating solution to disrupt activin-follistatin complexes enabling the measurement of 'total' follistatin.

The standard is immunopurified human follistatin which is freeze dried. It is reconstituted in 10.3mls dissociating solution giving a top standard of 2500pg/ml. Further dilutions are made to provide the following standard dilutions:

2500pg/ml:	**1250pg/ml:**	**625pg/ml:**	**313pg/ml:**
156pg/ml:	**78pg/ml:**	**39pg/ml:**	**19.5pg/ml**

The same QC samples were used for each plate as above. The sensitivity of this assay was 20pg/ml. Intra- and inter-assay variations were less than 10% respectively.

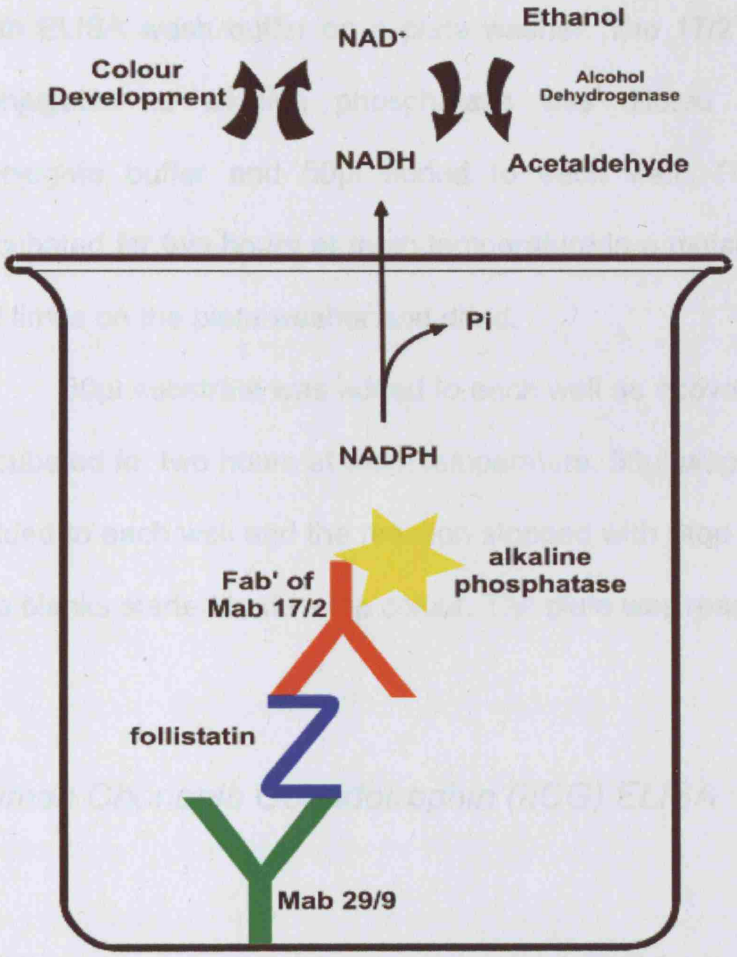


Figure 6.4: Diagrammatic representation of the follistatin ELISA

Day 1

Serum samples and QC's were brought to room temperature and diluted 1:3 in dissociating solution. Duplicate samples of blanks (dissociating solution), samples, standards and QC's were added to the anti-follistatin coated plate which was covered and incubated overnight at room temperature in a moist box.

Day 2

Unbound samples were discarded and the plate washed 10 times

with ELISA wash buffer on a plate washer. The 17/2 anti-follistatin conjugated to alkaline phosphatase was diluted 1:40 in Tris conjugate buffer and 50µl added to each well. The plate was incubated for two hours at room temperature in a moist box, washed 10 times on the plate washer and dried.

50µl substrate was added to each well as above and the plate incubated for two hours at room temperature. 50µl amplifier was then added to each well and the reaction stopped with Stop Solution once the blanks started to develop colour. The plate was read at 490nm.

Human Chorionic Gonadotrophin (hCG) ELISA

HCG was measured using a commercial ELISA kit which detects both the whole hCG molecule and the free β-subunit (DRG Instruments GmbH, Germany). The plate is coated with an anti-hCG monoclonal antibody. The samples are incubated on the plate with an anti-βhCG antiserum conjugated with horseradish peroxidase (Enzyme Conjugate). Standards were supplied at the following concentrations:

5mIU/ml: 25mIU/ml: 50mIU/ml: 100mIU/ml: 200mIU/ml

The assay has a sensitivity of 5mIU/ml. Intra- and inter-assay

variations were less than 10% respectively.

Assay Procedure

Samples were diluted 1:1000 with Specimen Diluent to obtain them on the standard curve. 25µl samples, controls, standards and zero standard were added to duplicate wells. 100µl Enzyme-Conjugate was added to each well and the plate mixed on a plate shaker. The plate was incubated for one hour at room temperature. After an hour the specimens were discarded and the plate washed five times on a plate washer and dried. 100µl substrate was added to each well at timed intervals and the plate incubated for 15 minutes at room temperature. The reaction was stopped with 50 µl Stop Solution at the same time intervals as above and the plate read at 450nm.

Pregnancy Associated Plasma Protein-A (PAPP-A) ELISA

PAPP-A was also measured using a commercial ELISA kit (IBL Hamburg, Germany). The plates were coated with a polyclonal anti PAPP-A antibody and an anti-PAPP-A antibody-horseradish peroxidase conjugate used as the detection antibody. Standards were provided in the following concentrations:

0µg/ml:	**1µg/ml:**	**2.5µg/ml:**	**5µg/ml:**	
	15µg/ml:	**30µg/ml**		

Intra- and inter-assay variations were less than 10% respectively. The sensitivity of the test was 1µg/ml.

Assay Procedure

Samples were defrosted as above and mixed well. 10µl samples, standard and controls were added to duplicate wells and 100µl Sample Buffer was added to each well. The plate was incubated at room temperature for 30 minutes. The unbound samples were then discarded, rinsed with diluted wash solution and dried. 100µl enzyme conjugate was added to each well and the plate incubated at room temperature for a further 30 minutes. The contents of the plate were again discarded and the plate washed three times with wash solution and dried. 100µl substrate was added at timed intervals and the plate incubated for 15 minutes. The reaction was stopped with 50µl Stop Solution and the plate read at 450nm.

Oestradiol (E2)

Oestradiol was measured using a commercial 17 β-Estradiol ELISA kit (IBL Hamburg, Germany). The assay is a competitive ELISA, with a known amount of horseradish peroxidase labelled antigen competing with the serum antigen for antibody coated onto the wells. Standards were provided in the following concentrations:

25pg/ml	**100pg/ml**	**250pg/ml**	**500pg/ml**	**1000pg/ml**
		2000pg/ml		

Intra- and inter-assay variations were less than 10%; the sensitivity of the assay was 4.6pg/ml.

Assay Procedure

Twenty five microlitres of standards, controls and samples were added to duplicate wells and incubated for five minutes before 200µl of Enzyme-Conjugate were added to each well at timed intervals and shaken on a plate shaker. The plates were then incubated for two hours at room temperature. The samples were then discarded and the plates washed three times on the plate washer. 100µl of substrate was added to each well and the plates incubated for 15 minutes at room temperature. The reaction was stopped by adding 50µl Stop Solution and the plates read at 450nm.

Progesterone (P)

Progesterone was measured using a commercial ELISA assay kit (IBL Hamburg, Germany). This assay also works on the competition principle as above using antigen labelled with horseradish peroxidase. The standard concentrations were provided as follows:

0.3ng/ml	**1.25ng/ml**	**2.5ng/ml**	**5ng/ml**	**15ng/ml**
		40ng/ml		

Inter- and intra-assay variations were less than 10% and the sensitivity of the test was 0.05ng/ml. Samples were diluted 1:2 with Standard Solution as levels were expected to be greater than the top standard in pregnancy.

Assay Procedure

Samples were defrosted, mixed and diluted, and then 25µl samples, controls and standards were transferred into duplicate wells. The plates were shaken on the plate shaker and incubated for five minutes at room temperature. 200µl Enzyme-Conjugate were then dispensed into each well, the plates were shaken and incubated at room temperature for one hour. The samples were discarded and the plates washed three times on the plate washer and banged dry to remove remaining liquid. 200µl Substrate Solution was added to each well at timed intervals and the plates incubated for 15 minutes at room temperature. The reaction was stopped with 50µl Stop Solution and the plates shaken and read at 450 nm.

6.4 Results

6.4.1 Statistical Analysis

Students' t-tests were performed using GraphPad Prism Version 4 and Stata Version 8 was used for logistic regression analysis and predictive testing. Maternal serum hormone levels were log-transformed to obtain normal distribution and compared using the

student's t-test. Maternal ages and gestations were compared using the student's t-test. To control for gestation related differences in hormone levels found in the first trimester, the logistic regression analysis was performed on multiples of the median (MoM's) for inhibin A and hCG using median values for our obstetric population at UCLH. Multiples of the median were calculated using the formula:

MoM = Study Value/Median Value for given Gestation

Subject demographics

One hundred and twenty two women with first trimester threatened miscarriage and 33 controls were recruited into the study and provided blood samples for analysis. Table 6.1 represents demographic details and the outcomes of the threatened miscarriage group. Outcomes in the threatened miscarriage group were sub-divided into three groups for analysis including first trimester miscarriage, pre-term and term labour.

There were no significant differences in the gestational ages between cases and controls (p=0.9), or between different outcome groups (p=0.07). The mean maternal ages between the cases and controls were significantly different (p=<0.001) with the cases having a mean age of 31.8 years and the controls 23.9 years. The live birth

rate in the study group was 91%, of these 12 (9.8%) delivered prematurely. The first trimester miscarriage rate was 6.6% in this group of women.

Table 6.1 Demographic details and pregnancy outcomes of 120 cases presenting with threatened miscarriage and 33 controls (where applicable).

	Cases (n=122)	**Controls (n=33)**
Mean Maternal Age (y)	31.8*	23.9
Median Gestation (days)	84.5	77
SD (days)	16.9	15.7
Total Live Births	111 (91%)	NA
Term Births	99 (81%)	NA
Pre-term Births	12 (9.8%)	NA
First Trimester Miscarriages	8 (6.6%)	NA
Second Trimester Miscarriages	3 (2.5%)	NA

NA = not applicable

*statistically significant (p=<0.0001)

6.4.2 Hormone Assay Results

Table 6.2 represents the mean values (SD) for each of the hormones measured. Because the results were not normally distributed, the values were log transformed to obtain normal distribution and further

statistical analysis was performed on this data. Figures 6.6 and 6.7 represent the results of each assay for each outcome group.

Table 6.2: Mean (SD) maternal serum levels of inhibin A, activin A, hCG, PAPP-A, follistatin, oestradiol and progesterone for cases presenting with threatened miscarriage by outcome and control samples.

	Term Birth	Pre-term Birth	First Trimester Miscarriage	Controls
Inhibin A (pg/ml)	252.2 (161.1)	227.4 (92.8)	148.1 (153.1)	211.8 (105.2)
Activin A (pg/ml)	339.6 (571.0)	377.8 (432.1)	26.2 (23.8)	356.5 (359.2)
hCG (mIU/ml)	157617.4 (117323.3)	94826.4 (30318.9)	57218.75 (53432.7)	74491.3 (22459.3)
PAPP-A (µg/ml)	8.2 (11.3)	8.4 (17.1)	2.5 (4.7)	8.4 (13.6)
Follistatin (pg/ml)	822.4 (1090.8)	1661.9 (3408.7)	256.7 (153.3)	820.5 (577.6)
Oestradiol (pg/ml)	2484.0 (1043.9)	2525.4 (894.6)	1475.0 (1179.9)	2403.2 (1450.2)
Progesterone (ng/ml)	62.7 (45.3)	38.3 (17.1)	35.5 (23.2)	54.6 (20.1)

Inhibin A & Activin A

Inhibin A levels were found to be significantly lower in cases of threatened miscarriage that ended in first trimester miscarriage, when compared with both pre-term (34% lower) and term labours (41% lower; p=0.04 and p=0.0007 respectively). The levels were also

30% lower in the miscarriage cases than the controls who had not had bleeding in the first trimester (p=0.02). There was no difference in the inhibin A levels between those pregnancies that ended in pre-term and those that ended in term delivery (p=0.97). Activin A levels were up to 93% lower in the cases that ended with first trimester miscarriage than the pre-term labour sub-group (p=0.018), term labour sub-group (NS) and the control cases (p=0.012).

Follistatin

Follistatin levels were 69% lower in cases resulting in first trimester miscarriages than the term labour sub-group (p=0.016) and 69% lower than the control cases (p=0.003). There were no differences in the Activin A: Follistatin ratios between any of the study groups.

Human chorionic gonadotrophin & PAPP-A

The hCG levels were positively correlated with the inhibin A levels. The levels were significantly lower in cases of threatened miscarriage that subsequently ended in a first trimester miscarriage when compared with the pre-term labour sub-group, where they were 40% lower (p=0.017), and the term labour sub-group (p=0.0001) where they were 64% lower.

hCG levels were significantly higher (195% higher) when all cases of threatened miscarriage were combined and compared with the control pregnancies (p=0.0009) with the pre-term labour sub-group (127% increase) and the term labour sub-group (212%

increase) having significantly higher hCG levels than the control pregnancies individually (p=0.032 and p=0.0001 respectively). There were no differences between hCG levels in pregnancies ending in pre-term labour when compared with term labour.

PAPP-A levels in the threatened miscarriage group were 70% lower in pregnancies ending in first trimester miscarriage (p=0.033) when compared with the term labour sub-group.

There was a significant correlation between the levels of Inhibin A, hCG, PAPP-A and Activin A for each of the outcome groups, in particular between hCG and inhibin A (r=0.6194 p=<0.001).

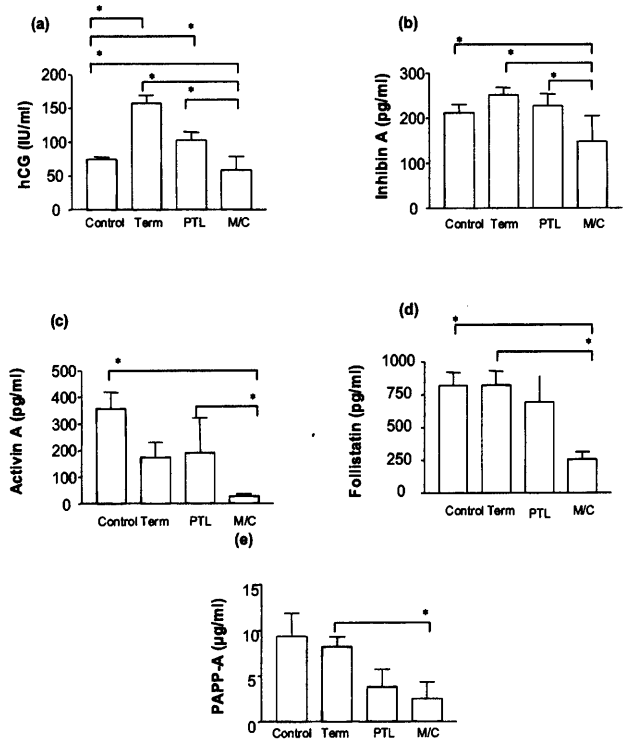


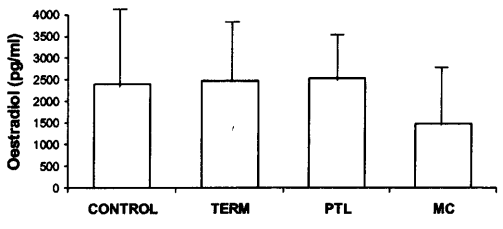
Figure 6.5: Mean (and SD) serum inhibin A, activin A, follistatin, hCG and PAPP-A levels for each outcome group, (TERM=term labour, PTL=pre-term labour, MC=first trimester miscarriage) *p<0.05

Oestradiol and Progesterone

Overall, oestradiol levels were 39% lower in the threatened miscarriage group when compared with the controls (p=0.16). The levels were 42% and 41% lower than the pre-term labour and term labour sub-groups respectively, but these findings were not statistically significant (p=0.07 and p=0.16 respectively). Progesterone levels were 48% lower in the cases of threatened miscarriage that went on to miscarry when compared with pregnancies that delivered at term (p=0.03). There were no other statistically significant differences in progesterone levels between the sub-groups. In cases that went on to miscarry, oestradiol and progesterone were positively correlated (r=0.932, p=0.007).

Haematoma Volume

There was no correlation between bleed volume and hormone levels for any outcome measure.



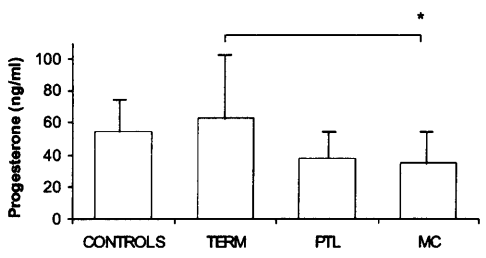


Figure 6.6: Mean (and SD) serum oestradiol and progesterone levels for each outcome group. (TERM=term labour, PTL=pre-term labour, MC=miscarriage. *p<0.05

*=significant result

Predicting Outcome

The above results suggests that maternal serum hormone levels were most useful for predicting first trimester miscarriage; and that the two hormones most likely to be useful for predictive testing for first trimester miscarriage are inhibin A and hCG. Logistic regression analysis of inhibin A and hCG MoM's found that inhibin A in isolation provided the best predictor for miscarriage in the first trimester miscarriage after threatened miscarriage with an area under the receiver operator curve (ROC) of 0.6916 and 0.6088 respectively (Table 6.3). In fact the addition of hCG weakened the predictive power of the inhibin A results.

Table 6.3: Logistic regression analysis Log_{10} hCG and inhibin A (MoM's) for predicting the likelihood of first trimester miscarriage after threatened miscarriage

Predictor	**Area under ROC curve**
Inhibin A MoM	0.6916
hCG MoM	0.6088
Inhibin A/hCG MoM combined	0.6810

Chapter 7

Discussion

Threatened miscarriage is an extremely common complication of early pregnancy. Not only has it been associated with an increased incidence of subsequent miscarriage, it has also been linked to a number of other adverse outcomes later in pregnancy. The mechanisms leading up to these complications are now becoming clearer.

It can be seen that the early human embryo is protected from external, potentially damaging environmental factors, including high or 'normal' oxygen tensions by a trophoblastic shell which limits the supply of oxygenated maternal blood, and contains high levels of antioxidants such as tocopherol. There is some evidence that a disruption to this system, for example after threatened miscarriage, with premature influx of oxygenated blood, the presence of substances which form free radicals, or a reduction in local antioxidant levels could result in a spectrum of adverse outcomes ranging from early pregnancy loss to pre-eclampsia and pre-term labour and PPROM. Further work is still required to examine first trimester nutrition and antioxidant capacity under these

circumstances. This study confirms an association between threatened miscarriage and adverse pregnancy outcome, and provides potential markers of placental damage or stress in these cases to add to a growing body of research elucidating the role of early oxidative stress in the developing placenta and later pregnancy complications.

The results of the adverse pregnancy outcome study indicate that women who present to the Early Pregnancy Unit with bleeding in the first trimester are not only at increased risk of first trimester miscarriage but also of later pregnancy complications; in particular PPROM and PTL. The incidence of severe pregnancy complications such as pre-eclampsia, FGR and abruption was not increased in this group, suggesting that the pre-term deliveries that did occur were spontaneous events i.e. non iatrogenic deliveries and are therefore potentially preventable.

Several studies have reported an association between first trimester bleeding and abnormal pregnancy outcome including preterm deliveries[9;12;13;15;131], FGR[9;12;13;15] and low birth weight (LBW)[9;12;13;15], but the majority of these reports, are retrospective, are uncontrolled, or rely on patient recall recruiting later in the pregnancy. Although it is recognised that unexplained antepartum haemorrhage later in pregnancy is associated with adverse pregnancy outcome[13;144]; threatened miscarriage in the first trimester is largely disregarded, if identified at all in the antenatal booking history. A recent retrospective study by Mulik at al[13] also reported that the only

risk factor in their study group to be independently associated with pre-term delivery in women with threatened miscarriage was unexplained second or third trimester bleeding, suggesting a common mechanism.

The largest study of threatened miscarriage was conducted by Weiss et al[9] and concluded that first trimester bleeding was an independent risk factor for adverse obstetric outcome. The investigators recruited women at 10-14 weeks, and divided women into subsets based on whether there was a history of bleeding in the four weeks prior to enrolment. The spontaneous pregnancy loss rates before 24 weeks in both the cases and controls were extremely low in their study and may be explained by the later recruitment of the cases, when many of the early miscarriages will have already occurred. In addition, no ultrasound parameters, in particular the presence or absence of an intrauterine haematoma were recorded in this study. The literature on early pregnancy loss rates after threatened miscarriage is variable. The Weiss study had a 2% loss rate, which was particularly low as mentioned above, other reports suggest that the rate is approximately 5%-8%[7;10] after vaginal bleeding, increasing to 20% in the presence of a haematoma[7]. In the present study, where the mean gestation at presentation was eight weeks, the early loss rate was 9.3%, which is similar to that reported before eight weeks gestation[10]. Studies conducted later in the first trimester have reported considerably lower loss rates, particularly after 10 weeks gestation[7;9;317]. This finding is in keeping with a recent

retrospective study of intrauterine haematoma in the first trimester which found that the risk of spontaneous miscarriage was significantly increased if the haematoma was diagnosed before 9 weeks[11]. Once the early miscarriages were excluded in our population, the loss rates were comparable to these studies. All cases in this study presenting with bleeding were also found to have a haematoma on ultrasound, however analysis of outcome according to haematoma volume did not show any correlation, suggesting that it is the bleeding which affects the pregnancy outcome, rather than a direct pressure-volume or physical disruption effect. It was found however that pregnancies ending in a first trimester miscarriage presented at a significantly earlier gestation than those that continued on to a pre-term or term birth, suggesting that early bleeding may result in an overwhelming insult to the early placenta and pregnancy failure in these cases. When gestation at maximum haematoma volume for each pregnancy were compared, those that ended with first trimester miscarriage reached maximum volume significantly earlier than term births and haematoma volume in relation to gestational sac volume or placental volume might have given a better insight into the mechanism of pregnancy loss in these cases, with a larger percentage surface area of the early trophoblast being damaged by the presence of haematoma. Unfortunately, these parameters were not measured routinely in all cases. The use of other early ultrasound parameters in this study (such as crown-rump length: gestational sac diameter ratios) to predict both short and long

term pregnancy outcomes did not appear to provide any clinically useful information and confirmed previous work showing no real benefit in recording this data. There did not appear to be an association between early growth discrepancies and small for gestation pregnancies (SGA) and FGR although the number of documented cases of SGA and FGR in this study was small as discussed in detail below.

The long term pregnancy outcomes in this study have confirmed the findings of our pilot study and in addition confirm the literature suggesting that the mechanism of pre-term labour in many of these cases is PPROM. In this study, we found that the increased pre-term deliveries occurred mainly between 34 and 36 weeks of gestation. Delivery at this gestation is considered to carry significantly lower risk of neonatal mortality and morbidity than earlier in pregnancy. Babies born at this gestation rarely require neonatal intensive care but they still carry an increased risk of neonatal and infant mortality and morbidity compared to babies born after 37 weeks[318-320]. A large study examining the effect of birth weight on developmental delay estimates that the risk of delay is directly related to weight at birth[319]. The additional healthcare costs for the care of these infants, both in the neonatal period and in the first year of life are also significantly higher than their term counterparts[321;322] and it has been estimated that births between 34 and 37 weeks gestation contribute an excess cost of almost $50 million per annum in the US[322]. Analysis of birth weights, found that although there was

an increased incidence of babies being born with birth weights between 1501 and 2000g, this appeared to be directly related to premature delivery rather than FGR as the incidence of FGR was the same in each group and a comparison of birth weights for gestation were also the same between the groups. This finding is comparable with other studies[12;13;16] and would suggest some mechanism other than impairment of placental development in the aetiology of pre-term birth in these cases, such as PPROM or chronic inflammation.

Evidence for a role for increased free radical production in the placental membranes in PPROM is emerging[142;143]. Smoking is associated with an increased risk of PPROM[144] and there is also good in-vitro evidence for increased oxidative stress in PPROM with vitamin C being involved in several biochemical processes in collagen synthesis and maintenance, which is vital to the strength of the placental membranes[153]. Vitamin E is an essential lipid soluble antioxidant and works as a chain breaker, halting ROS induced lipid peroxidation. It is concentrated in the syncitiotrophoblast brush border in early pregnancy[74] and appears to play an active role in early pregnancy development[323]. Recently it has been shown that culturing amnion cells and amnion in hydrogen peroxide induces apoptosis, cytokine and prostaglandin production[156] and that vitamins C and E can protect placental membranes from artificially induced oxidative damage[157]. Contradictory results have been published however, with some reports suggesting a possible dose related increased risk of membrane rupture with vitamin C alone[156].

The findings in Chapter 5 demonstrate the presence of antioxidant activity in the early first trimester pregnancy sac and in particular in the exocoelomic fluid, suggesting a role in the protection of the early embryo. Evidence discussed in Chapter 3 suggested that early embryonic nutrition is derived in part from the maternal uterine glands, which have been shown to deliver their secretions freely into the intervillous space in early pregnancy. These secretions are taken up by placental syncitotrophoblasts and also by the secondary yolk sac via the exocoelomic coelom (ECC). The fluid in the ECC (coelomic fluid) has been found to be an ultrafiltrate of maternal serum, and the above data adds to the growing list of nutrients that have been found in this fluid. It also suggests that the ECC acts as an important interface between the maternal and fetal environments, not only for nutrition, but also for protection against free radical damage.

Subchorionic bleeding will also result in an increase in the amount of free iron available, catalysing the generation of the extremely damaging hydroxyl radical and subsequent free radical damage to the membranes. Chorioamnionitis is a recognised risk factor for PPROM, the increased production of hypochlorous acid by neutrophils and macrophages as a response to infection may be the mechanism by which the placental membranes are weakened when infection is present. It has also been suggested that weakening of the membranes occurs due to thrombin generation and a subsequent proteolytic process lead to membrane weakness and eventual

premature rupture[324].

These findings and the data from this study suggest that bleeding between the chorionic membrane and the uterine wall can result in a spectrum of effects on pregnancy development and outcome. At one end, direct pressure and disruption of the placental bed results in miscarriage. At the other end of the spectrum is PPROM, where there is minimal or no disruption to uteroplacental development but a chronic inflammatory reaction within the decidua and placental membranes, with weakening and eventual rupture of the membranes. Cases that ended with pre-term labour in our series reached maximum haematoma volume significantly later than those that went on to a term birth and this could explain the chronic inflammation and PPROM with minimal disruption to placental development. When we examined the intrauterine haematoma volumes between cases, we were unable to find any difference between the groups for haematoma volume and this would suggest that vaginal bleeding, and the gestation at which it occurs is the most important factor determining the eventual outcome of these pregnancies, with the presence and volume of intrauterine haematomas playing a more minor role in pregnancy outcome.

A role for oxidative stress in disorders of reproduction and pregnancy have long been suspected, however studies have been hampered by the lack of reliable markers for oxidative stress in human systems. Vitamins C and E have been shown to work synergistically[70], and it is likely that an individuals overall oxidative

status is determined by a combination of factors. Vitamin C and E transfer across the human placenta has been studied extensively in later pregnancy and vitamin C appears to be transferred into trophoblast as DHA, possibly using a glucose transporter[325]. Vitamin E in all its forms appears to cross the placenta only slowly[66;326]. Fetal levels of vitamin E have consistently been found to be lower than maternal levels[327-331]. The correlations found between maternal and CF levels of γ-tocopherol in this study would suggest that there may be a role for maternal supplementation from conception for women at increased risk of oxidative stress, for example diabetic pregnancies which are known to have increased rates of congenital abnormalities [102] and miscarriage[103;104], thought to be associated with increased levels of oxidative stress[105;106].

Vitamin C concentrations are generally higher in the fetal than the maternal circulations however[325;331;332]. The finding of similar ascorbic acid and lower DHA levels in the fetal compartment in this study would suggest that transport of this antioxidant starts early in the first trimester of human pregnancy.

The results of the TAP assay were disappointing, with no significant findings between any of the groups or subgroups. TAP levels were lower but numbers were too small for analysis in this pilot study. The reasons for this could be many, including the small numbers for analysis, particularly within the subgroups.

The lack of any significant findings with the TAP assay may also reflect the inherent difficulty in assessing an individuals

antioxidant capabilities as discussed in Chapter 3. Table 3.3 demonstrated the difficulties in finding such a test by describing the multitude of assays currently under investigation. An individuals overall level of oxidative stress is determined by many different factors and can be affected by numerous external factors including smoking, drugs, genetics and diet. Any single measurement such as alpha-tocopherol or vitamin C is unlikely therefore to provide the total picture, hence the development of total antioxidant capacity assays. The findings above give the impression of a lower antioxidant capacity in those cases that were complicated by threatened miscarriage, suggesting either a pre-existing lower antioxidant capacity in these women or a subsequent reduction in capacity as a response to the threatened miscarriage and eventual partial or complete recovery. This pilot data requires further follow-up with a larger study examining TAP in women with threatened miscarriage and also to examine the possible effect on TAP of antioxidant supplementation in these cases.

The maternal serum hormone data was more revealing however and the results of these assays demonstrated that after threatened miscarriage in the first trimester, maternal serum hormone levels are altered in women who subsequently suffer a first trimester miscarriage. The study also demonstrated that changes in maternal serum hormone levels at this stage and in particular inhibin could be used to predict pregnancy outcomes in the second half of pregnancy. The differences in maternal ages between the cases and

controls clearly demonstrates the general demographics of our local population, however data from established international serum screening programmes suggests that there is little or negligible maternal age effect for the hormones measured[333].

Inhibin A, activin A and follistatin are present in high levels in maternal serum in normal human pregnancy[209;218]. Initially, in early pregnancy, inhibin A is produced by both the fetoplacental unit[213] and the corpus luteum[219], later the fetoplacental unit continues to be a source of inhibin A, activin A, follistatin and activin receptors throughout pregnancy[220-224]. The exact role of these proteins and their receptors remains unclear. Inhibin A has been shown to have autocrine and paracrine effects on placental hormone production and has been shown to reduce the secretion of hCG and progesterone from cultured placental trophoblasts[227-229]. Activin A has been shown to promote cytotrophoblast invasion[334] and follistatin to induce angiogenesis[335] in-vitro. Increased maternal levels of inhibin A and activin A can be found in women suffering from such pregnancy complications as PET[247-249] and FGR[254]. Alterations in gene and protein expression of inhibin/activin subunits has been demonstrated in placentas complicated by PET[256]; in miscarriages, there is a decrease in the expression of the hCG α and βA subunit gene in villous tissue of miscarriages compared with controls suggesting that down-regulation occurs in the hCG gene after embryonic demise. In the case of inhibin, activin and follistatin however, reduced levels of these proteins appears to be related to a decrease in trophoblastic

mass or reduced trophoblast secretion prior to embryonic demise rather than an alteration in gene expression[236].

hCG is widely used in the diagnosis and management of early pregnancy complications, including ectopic pregnancy, gestational trophoblastic disease and early pregnancy failure. A single serum hCG measurement has a sensitivity of 88% in determining between viable and non-viable pregnancies[188] (including ectopic pregnancies) and several studies have reported low initial hCG levels in non-viable pregnancies[173] but its use in predicting subsequent loss after the confirmation of a viable pregnancy is still being evaluated[181;189]. PAPP-A levels also decrease in miscarriage but they have a low predictive value for fetal demise[173;199-201]. Inhibin A levels have also been found to be lower in miscarriages when compared with gestation matched viable pregnancies[231;233;242].

The mechanism by which threatened miscarriage alters maternal serum hormone levels is uncertain. Vaginal bleeding in the first trimester has been associated with increased levels of free serum βhCG in maternal serum[336] and it has been suggested that this increase is related to an increase in hCG transfer into the maternal circulation due to disruption of the materno-fetal interface after vaginal bleeding[336]. It has also been suggested that weakening of the membranes occurs due to thrombin generation and a subsequent proteolytic process lead to membrane weakness and eventual premature rupture[324]. Subchorionic bleeding will result in an increase in the amount of free iron available, catalysing the

generation of the extremely damaging hydroxyl radical and subsequent free radical damage to the membranes causing disruption to the materno-fetal interface. During normal pregnancy development, two thirds of the developing placenta degenerates to form the chorion laeve. It has been shown that in the peripheral developing placenta, there is increased oxygen free-radical formation, correlating with the increase in maternal blood flow between 8 and 12 weeks gestation[25]; and it has been suggested that this is a normal physiological process required for villous regression and the formation of the chorion laeve. In threatened miscarriage, it is possible that maladaptation to this process results in free radical damage to the developing placenta and membranes, resulting in an increase in placental hormone production and subsequent release into the maternal circulation.

In a study of hCG concentrations in missed miscarriages, it has been shown that hCG levels are higher than normal controls in cases where fetal demise has been recent, whereas in cases with a larger discrepancy between expected and observed findings the hCG levels were lower than normal concentrations[337] and this could be explained by the trophoblastic regeneration that occurs after necrosis and apoptosis in cases of tissue damage[338] and oxidative stress[337]. In our study population, those pregnancies that went on to miscarry showed significantly lower levels of all pregnancy related hormones investigated, with significant correlation between many of the hormones. The most likely explanation for this is decreased placental

cell mass resulting from the excessive influx of oxygenated maternal blood into the developing placenta resulting in irreversible damage to the placenta and subsequent reduction in placental hormone production. If the damage is extensive and involves a significant proportion of the definitive placenta, the fall in progesterone, E2 and hCG would result in a complete miscarriage. If the placental damage is limited, and hCG gene expression is not affected, it is possible that increased hormone production and subsequent placental repair will ensue. A reduction in inhibin A levels, as a result of reduced cell mass, could result in an increase in hCG and progesterone production by the syncitiotrophoblast with resulting positive feedback on cytotrophoblast differentiation and hence hCG production and stimulation of the corpus luteum to produce further progesterone and E2 in an attempt to maintain the pregnancy. In our study population, the maternal serum levels of hCG were considerably higher than the control population. In those pregnancies that went on to deliver a live birth, the hCG levels were significantly higher, particularly in those pregnancies that resulted in a term birth. It is possible that after the initial insult, it was these pregnancies that were better able to adapt to the trophoblastic stress associated with threatened miscarriage, reflected in the higher hCG levels in these cases.

The lower levels of activin A and follistatin in those cases that subsequently miscarried, compared with the term labour group is consistent with the role of these hormones in early pregnancy suggested by in-vitro studies. Activin A has been shown to promote

cytotrophoblast invasion[334] in very early pregnancy and follistatin to induce angiogenesis[335]. It possible that a reduction in production of these hormones secondary to reduced placental mass could impair placental invasion and vascular development with resulting eventual placental failure and miscarriage.

It has been shown that inhibin A, in combination with other serum markers may be useful in determining those pregnancies presenting with threatened miscarriage that are destined to fail[238], however the predictive value for subsequent miscarriage is low under these circumstances[219]. The above results suggested that first trimester miscarriage was the most significant pregnancy outcome when examining maternal serum hormone levels in this study group and that the two hormones most likely to be useful for predictive testing for first trimester miscarriage are inhibin A and hCG. As discussed above, it was necessary to use MoM's to eliminate the gestation related changes in hormone levels that are found in the first trimester. In this study, inhibin A alone was found to be highly predictive of first trimester miscarriage, without the addition of other markers and its potential for use with other markers such as ultrasound parameters and demographic features requires further investigation. Inhibin A has been shown to clear from the maternal circulation more quickly than hCG, progesterone and oestradiol after termination of pregnancy[213], suggesting that it may be more sensitive than hCG in predicting the outcome of pregnancies in the first trimester.

Conclusions

Women with threatened miscarriage in the first trimester are at increased risk of premature delivery and this risk factor should be taken into consideration when deciding upon antenatal surveillance and management of their pregnancies. Development of interventions, such as progesterone and antioxidant supplementation require further investigation, however identification of women at risk would allow such interventions to be implemented from an early gestation. Current data would suggest that there may be a role for supplementing women with antioxidants after first trimester threatened miscarriage but a formal randomised controlled trial (RCT) would be required to confirm this. Identifying women who are 'high risk' for pre-term labour is imprecise and screening strategies have included both historical data and biophysical markers. Screening strategies using Doppler ultrasound of the uterine vasculature have suggested that they may be a useful non-invasive screening test for pregnancies that are likely to develop FGR, PTL and pre-eclampsia, but results are variable and more studies are needed to confirm this and evaluate the safety and sensitivity of such tests[339-342].

Serial or single cervical length measurements have shown that identification of a short cervix is a sensitive tool for identifying women at risk of pre-term labour [343;344], even in the low risk population, although intervention in these women with cervical

sutures has not been shown to reduce the incidence of pre-term labour[344;345]. Screening women for cervical or vaginal fetal fibronectin has also been found to be a useful predictor of pre-term delivery, both alone and in combination with cervical length measurements[346]. Although a history of vaginal infection, in particular with bacterial vaginosis[347;348], has been associated with both spontaneous miscarriage and pre-term labour, a meta-analysis of recent studies examining the role of screening for and treating vaginal infection showed no reduction in the rate of pre-term delivery or low birth weight in asymptomatic pregnant women unless there was a prior history of pre-term birth[349]. The more recent PREMET study[350] not only found that there was no reduction in pre-term birth in women at increased risk (history and a positive fetal fibronectin test) when treated with metronidazole; but there appeared to be an increased risk of pre-term birth associated with metronidazole use.

Other strategies designed to identify women at risk of pre-term delivery are of uncertain value and identification of those at risk depends heavily on a past history of pre-term birth[351], or concentrate on correct diagnosis of women who already present with symptoms of pre-term labour[351].

Little data is available from in-vivo studies on antioxidant intake and supplementation on the risk of PPROM and PTL. Several studies have reported an increased risk of PPROM in women with lower vitamin C levels[159;352;353].There is also some suggestion that diet alone may not be an adequate source of vitamins C and E in

pregnancy[142]. Vitamins C and E work synergistically with vitamin C providing vitamin E regeneration, and their use in combination may well reduce the risk of PPROM in high risk women[354]. Identification of women at risk would allow such interventions to be implemented from a much earlier gestation. This data is probably most important for its role in raising awareness amongst health care professionals and women alike, that they may be at increased risk of pre-term labour or PPROM. Increased antenatal surveillance, possibly with cervical length measurements or the use of fetal fibronectin tests might identify women within this group who are at increased risk. This would result in a higher index of suspicion in women presenting with symptoms later in pregnancy, enabling prompt identification of these complications should they occur. Knowledge of this increased risk may also facilitate decision making regarding management, for example, timely administration of antibiotics and corticosteroids or decisions regarding mode, place and timing of delivery, which will inevitably improve neonatal outcome[355].

Appendix 5.1

ELISA Stop Solution

Reagents:

Concentrated Sulphuric Acid (H_2SO_4)

Distilled H_2O

To make 1 Molar H2SO4 :

Under the fume hood take 946.74mls distilled H_2O in a glass bottle and measure 53.26mls concentrated H_2SO_4 with a glass pipette. Add the acid to the water and mix well.

To make 500mls 0.3M H_2SO_4 - ELISA Stop Solution:

Add 150.15ml of 1M H_2SO_4 to 349.85mls distilled H_2O.

Mix well and store at room temperature.

Appendix 6.1

Inhibin A Assay Diluent

Reagents:

- 10% BSA (protease free)
- 5% mouse serum
- 5% Triton X-100
- 0.15M NaCl
- 0.1% Sodium Azide
- 0.1M Tris HCl buffer (pH 7.5)

To make up 500ml:

- 50g BSA
- 25ml mouse serum
- 25ml Triton X-100
- 4.375g NaCl
- 0.5g Sodium Azide
- 45ml 1M Tris made up to 450ml (distilled H20)

Mix well (30-60 mins) and filter diluent through cotton wool.

Appendix 6.2

ELISA Wash Buffer (X 20 Stock)

Reagents:

- 1M Tris base – 0.05M (X20)
- 3M NaCl – 0.15M (X20)
- 1% Sodium Azide – 0.05% (X20)
- Tween20

To make up 2.5 litres stock buffer:

- Tris base – 302.75g
- NaCl – 175.32g
- Sodium Azide – 25g

Dissolve in 2 litres of distilled H_2O, neutralise pH using concentrated HCl to pH 7.5.

Make up volume to 2.5l using distilled H_2O.

Preparation of 10 l normal wash buffer from stock:

For 10 litres:

- 500ml stock buffer (pH 7.5)

- 9.5 litres distilled H_2O
- 5ml Tween20

  Stir well for 30 minutes and store at room temperature.

Appendix 6.3

ELISA Manual Wash Buffer

Reagents:

- 0.05M Tris HCl buffer
- 0.15M NaCl
- **To make up 500mls**
- 25mls of 1M Tris HCl buffer (pH 7.5)
- 475mls distilled H_2O
- 4.4g NaCl

Mix well and store at 4° Celsius.

Appendix 6.4

Sample Buffer for Activin A ELISA

Reagents:

- Phosphate Buffered Saline
- 10% BSA (protease free)
- 0.1% Sodium Azide

To make up 100mls:

- Dissolve 1 PBS tablet in 100 mls distilled H2O
- 10g BSA
- 100mg Sodium Azide

Mix well and store at 4° Celsius.

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Published papers arising from this thesis:

1. Maternal serum hormone concentrations for prediction of adverse outcome in threatened miscarriage. **Johns J**, Muttukrishna S, Lygnos M, Groome N, Jauniaux E. *RBM Online* 2007; 15(4): 413-421

2. Threatened Miscarriage as a Predictor of Obstetric Outcome. **Johns J**, Jauniaux E. *Obstet Gynecol* 2006; 107 (4): 845-850

3. Obstetric outcome after threatened miscarriage with and without a hematoma on ultrasound. **Johns J**, Hyett J, Jauniaux E. *Obstet Gynecol* 2003; 102(3): 483-7

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