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Recurrent early pregnancy loss (REPL) is usually defined as three consecutive first-trimester pregnancy loss. It is known to affect 1% of all women (Li, 1998). In case of a primary recurrent pregnancy loss there are no live born children, but in a secondary recurrent pregnancy loss there may be usually first a live born child followed by a series of pregnancy loss. Reported risk factors for the REPL are increased maternal age, previous miscarriages, genetic factors, anatomical factors, immunological factors, endocrine factors and environmental or life style factors such as smoking, caffeine intake, alcohol or drug intake and stress. Early efforts to evaluate the nature of genetic cause behind REPL have mostly been confined to chromosomal analyses of parents and abortuses (Bowé 1985; Eiben 1990). Karyotypic abnormalities are found in 50% of abortuses of first trimester primary REPL. On the contrary, estimated number of women with constitutive chromosomal defects, that may lead to aneuploid embryo and subsequent pregnancy loss, is much lower (approximately 3%). Thus, attempts were made to focus on the nonkaryotypic genetic aspects through the analysis of genetic polymorphisms in various candidate genes using the case control approach. Case control studies are well suited to investigate the underlying genetic defects in complex genetic disorders where the classical linkage analyses are not possible.

Distribution of etiological factors in 545 women with recurrent early pregnancy loss as per Kutteh & Carney 1999 is given in Table 1.1
Table 1.1 Distribution of various etiological factors in recurrent early pregnancy loss

<table>
<thead>
<tr>
<th>Factor</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic</td>
<td>3.1</td>
</tr>
<tr>
<td>Endocrinological</td>
<td>20.2</td>
</tr>
<tr>
<td>Anatomic</td>
<td>21.6</td>
</tr>
<tr>
<td>Immunological</td>
<td>25</td>
</tr>
<tr>
<td>Infectious</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Life style factors such as cigarette smoking, use of illicit drugs or even consumption of coffee or alcohol are shown to significantly affect a successful pregnancy outcome. All these factors were known to increase the oxidative stress through their activation and elimination by members of detoxification systems. An increased load of oxidative stress is especially damaging to the first trimester fetus as the fetus is less efficient in scavenging the reactive oxygen species. The importance of total maternal antioxidant status in idiopathic infertility is already reported (Perkins 2006). Metabolism of most of the pharmacologically active drugs as well as the environmental chemicals (xenobiotics) by human body is achieved through the coordinated action of a group of enzymes collectively known as detoxification system. They are highly complex, show a great amount of individual variability and are extremely responsive to an individual’s environment, lifestyle and genetic uniqueness. The phase I detoxification system, composed mainly of the cytochrome P450 super gene family of enzymes, is generally the first enzymatic defense against foreign compounds. In a typical phase I reaction, a CYP450 enzyme uses oxygen and NADH (NADPH) to transform the foreign compound into its hydroxy derivative.
The phase II conjugation reactions convert the xenobiotic into a water-soluble compound that can be excreted through urine or bile. The body practices several types of conjugation reactions and to name a few are glucuronidation, sulfation, acetylation, glutathione and amino acid conjugation. A balance between the phase I and II enzyme activities is not only necessary for the efficient elimination of xenobiotics but also for the maintenance of cellular redox potential.

The maternal circulation through the placenta is more restricted during first few weeks of gestation. However, the blood supply sharply rises to its full level at 10-12 weeks of gestation. This sudden release of blood is required for embryonic differentiation and development events. As an adequate placental vascular network is vitally important for the growing fetus, all abnormalities that diminish blood flow to the placenta may result in gestational pathologies. These abnormalities may influence the blood vessel formation or thrombosis.

Angiogenesis is rare in adults with an exception of female reproductive tract and in certain pathological conditions. The processes of implantation and placentation are associated with an extensive angiogenesis and this process is mediated by concerted action of soluble factors such as vascular endothelial growth factor, placental growth factor, and soluble vascular growth factor receptor. Reports indicate that maternal plasma VEGF levels are reduced in pre-eclampsia (Kupferminc 1997). The vectorial secretion of
VEGF by polarized maternal endometrium supports the importance of VEGF in establishment of a symbiotic relationship between the fetal and maternal compartments.

Apart from promoting endothelial cell proliferation, VEGF promotes vascular permeability and helps to maintain newly formed blood vessels. VEGF has been suggested as being the principal angiogenic factor secreted by endometrial cells and a stage-specific expression of VEGF isoforms during the menstrual cycle has been observed.

Nitric Oxide (NO) is an important bio-regulatory molecule responsible for endothelium dependant relaxation, activation of guanylate cyclase, neurotransmission in central and peripheral nervous systems and activated macrophage cytotoxicity. NO is synthesized from one of the guanidino nitrogen of L-arginine by an enzyme nitric oxide synthase (NOS). The three prototypical forms of NOS, neuronal, cytokine-inducible and endothelial NOS are derived from separate genes and are regulated by diverse signaling pathways. Endothelial NOS helps in maintenance of pregnancy by controlling the vascular tone and hCG release.

Pregnancy is a hypercoagulable state secondary to an increase in coagulation factors, a reduction in the naturally occurring anticoagulants and an impairment of fibrinolysis. The evolutionary advantage of these changes is thought to be reduction in post-partum blood loss.
The polymorphisms in coagulation proteins can lead to an increase in the coagulation process leading to thromboembolic events (Dahlback 1995). The severity of the condition is enhanced by the pregnancy-induced changes and can result in a pregnancy loss. *Factor V Leiden (FVL)* mutation is associated with an increased risk of venous thromboembolism. When combined with the hypercoagulable state that is characteristic of pregnancy, presence of the *FVL* mutation can increase the risk of pregnancy loss. Activated protein C cleaves the factor V thereby limiting the clot formation. The mutant protein is resistant to cleavage by protein C leading to the clot formation in circulation. The polymorphisms in the promoter region of prothrombin were shown to enhance the expression resulting in risk of excess clot formation (Poort 1996). Increased levels of homocysteine correlate with the risk of REPL. The excess of homocysteine result in neural tube defects, decreased folate levels, thrombosis—all these can lead to pregnancy loss. This can be due to an underlying mutation in *MTHFR* that can lead to hyperhomocysteinemia.

Estrogen and estradiol are responsible for the successful implantation and maintenance of pregnancy. Abnormally low maternal estradiol levels characterize most of the first trimester pregnancy loss (Witt 1990). Animal studies have shown that inhibitors of enzymes involved in estrogen biosynthesis led to termination of pregnancy. Estrogen and estradiol modulate the expression of progesterone required for successful maintenance of pregnancy. In addition, factors such as endometrial receptivity and successful implantation that are crucial for successful pregnancy outcome are regulated by estrogen.
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The estrogen contribution by the first trimester placenta is negligible. Life style habits such as smoking make the ovaries less sensitive to gonadotropins decreasing the production of estrogen and estradiol. Two members of the Cytochrome P450 family of enzymes, CYP17 and CYP19 participate in estrogen biosynthesis and are implicated in a number of reproductive as well as malignant disorders. The elimination of estrogen is chiefly through its sulfation followed by excretion. The transfer of sulfate to estrogen is catalyzed by sulfotransferase 1E1 (SULT1E1). Conjugated estrogens are not appreciable ligands for estrogen receptor, hence cannot exhibit their effects once conjugated.

Androgen levels measured in terms of testosterone concentrations were found to be higher in complicated pregnancies such as preeclampsia (Laivuori 1998). An association between androgen levels and recurrent pregnancy loss, infertility, polycystic ovaries was reported from various populations (Gurbuz 2003, Carmina 2006). The effects of androgens are mediated by the androgen receptor, which is a highly polymorphic gene. A trinucleotide repeat in exon 1 of androgen receptor gene codes for a polyglutamine tract that determines the sensitivity and affinity of the androgen receptor towards its ligands. This polymorphism is responsible for clinical conditions such as bulbospinal neuropathy, known as Kennedy’s disease.

Among the various chromosome abnormalities reported in connection with REPL, X chromosome abnormalities alone contribute to about 10% (greater than any other individual chromosomal abnormalities) (Ljunger 2005). A number of genes present on X
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Chromosome abnormalities are involved in establishment and maintenance of pregnancy. In addition, X chromosome abnormalities can result in poor or inefficient ovarian reserves that can lead to REPL. A way to look at the functional status of an X chromosome is to check for the skewed X inactivation. In mammals, dosage differences in X linked genes between the male and female are compensated by inactivating one female X chromosome during the early stages of embryonic development. This process is known as X-chromosome inactivation (XCI) or lionization. Normally, XCI is a random process resulting in mosaicism for X linked gene expression. However, mutations in XIST locus, imprinted inactivation patterns (as is the case with paternally derived X in extra embryonic lineages in mouse), can lead to primary non-random XCI. On the other hand, selection against a chromosomal abnormality in X can result in secondary XCI. Non random XCI has also been implicated in an increased prevalence of autoimmune disorders noted in females. Apart from the cytogenetically detectable X chromosome abnormalities, nonrandom patterns of X inactivation may also result from single gene mutations as seen in X linked immunodeficiencies. In any case, a nonrandom X inactivation event indicates an underlying abnormality in the X chromosome.

Immunologically a successful pregnancy is a compromise by the maternal immune system towards the foreign fetus. The non-classic, less polymorphic histocompatibility antigen, HLA-G, mediates this immune tolerance towards the allogenic fetus. Under normal circumstances HLA-G protein has been detected only in trophoblast cells, monocytes, T-cells and in the thymus. HLA-G inhibits CTL response and NK functions.
Expression of HLA-G by TH cells directs them towards immuno suppression. Soluble HLA-G expression promotes CD8 cell apoptosis through Fas/FasL pathway. sHLA-G levels are found associated with pregnancy related complications such as preeclampsia, spontaneous miscarriage and abruptio placentae. Elevated TNF-α activity during pregnancy essentially results in fetal loss and HLA-G decreases the TNF-α level during pregnancy.

Endocannabinoids are an emerging class of lipid mediators, which mimic several effects of cannabinoids. Anandamide (arachidonylethanolamide) is a major endocannabinoid, which has been shown to impair pregnancy and embryo development. The activity of anandamide is controlled by cellular uptake through a specific transporter and intracellular degradation by the enzyme anandamide hydrolase (fatty acid amide hydrolase, FAAH). Physiological concentrations of progesterone stimulate the activity of the endocannabinoid-degrading enzyme anandamide hydrolase (fatty acid amide hydrolase, FAAH) in human lymphocytes (Gasperi 2005). Stimulation of FAAH occurs through up-regulation of gene expression at transcriptional and translational level. Th1 cytokines, which favor miscarriage, reduce FAAH expression. Clinical studies revealed low levels of FAAH in REPL while no change was observed in the endocannabinoid transporter suggesting FAAH to be the major regulatory switch.

Abnormalities in any of these enzyme activities are manifested as a potential threat to the survival of the pregnancy. Genetic variations in some or all of these factors can influence
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the success rate of a pregnancy. In an effort to gain insights into the causal genetic factors responsible for REPL in women from South India, polymorphisms in these candidate genes were analyzed using a well-defined matched case and control groups.

As majority of drug targets are proteins, another route to study genome is the proteome approach. This strategy offers not only clues to critical targets of cellular metabolism, but also provides a global and integrated view of the disease process. Though the concept to examine human proteome was first proposed by Anderson and Anderson in 1982, it found genuine applicability only recently with the availability of the immobilized pH gradients and development of highly sensitive mass spectrometric techniques. In pregnancy severe vascular adaptation occurs; platelets and platelet-derived products are very crucial in terms of their thrombotic and hypertensive activities. It is well known that oxidative stress leads to platelet aggregation (Naseem 1999). In addition, elevated homocysteine levels favor platelet aggregation and activation (Luo 2006). Use of aspirin was shown to decrease the platelet activation and recent reports indicate the positive effect of aspirin in decreasing the pregnancy loss rate (Tempfer 2006). Estradiol, whose levels are low in REPL, inhibits platelet activation (Bar 2000). Activated platelets release thromboxanes and other bioactive substances that result in vasoconstriction, decreased vascular permeability, thrombus formation. Reports indicate that in pre-eclampsia, a higher number of platelets expressing fibrinogen receptors exist in the circulation (Bharucha 1999). A similar condition can be envisaged in REPL (Anderson 1982).
Analysis of platelet proteome will reveal the differentially expressed proteins that characterize the REPL women. We carried out a proteomic analysis of platelets with the aim of identifying differentially expressed proteins diagnostic of the risk of REPL. Further, these proteins and the pathways in which they are involved can be exploited to develop neutralization strategies that counteract the platelet induced damage towards the pregnancy outcome.

1.1 Review of literature

Oxidative stress is defined as the imbalance between the activities that generate the reactive oxygen species and those that eliminate these species (antioxidant systems). Morphological features and physiological data suggest that the architecture of the human first trimester gestational sac is designed to limit fetal exposure to oxygen to that which is strictly necessary for its development. In vivo data suggests that the values for the intraplacental partial pressure of oxygen are two to three times lower at 8-10 weeks than after 12 weeks. There is a significant maternal to fetal oxygen gradient throughout pregnancy. The first trimester uterine \( O_2 \) gradient exerts a regulatory effect on placental tissue development and function. In particular it influences cytotrophoblast proliferation and differentiation along the invasive pathway and villous vasculogenesis. The physiological hypoxia of the first trimester gestational sac may protect the developing fetus against the deleterious and teratogenic effects of oxygen free radicals. Recent studies indicated that hypoxic conditions are necessary for maintenance of stem cells in a fully pluripotent state. At the end of the first trimester, a burst of oxidative stress is
evidenced in the early placenta. This leads to focal trophoblastic oxidative damage and progressive villous degeneration. These events trigger the formation of fetal membranes. The oxidative stress and rise in oxygenation is responsible for the synthesis of hCG, estrogens by the trophoblasts. Thus pregnancy loss can be considered as inability to tolerate or adapt to the dynamics of oxygen tension in the uteroplacental unit during the course of pregnancy especially in the first trimester where the changes are physiologically more significant. Concentrations of lipid peroxides have also been shown to increase in the villous and decidual tissues of women undergoing early pregnancy loss. Any factor causing abnormally high or rapidly fluctuating concentrations of $O_2$ will have a harmful and rapid effect on the early villous tissue. The involvement of reactive oxygen and nitrogen species in complicated pregnancies such as pre-eclampsia, maternal diabetes is confirmed by number of studies. In pre-eclamptic pregnancies there is an increased abundance of nitrated proteins in the placenta such as phospho p38MAPkinase, poly ADP-ribose polymerase, acetyl CoA transferase, P2X4 receptor suggesting the possible role played by oxidative and nitrative stress in pre-eclampsia. Recent reports suggested low levels of antioxidants such as glutathione are very crucial in male and female reproductive functioning. The GST family of enzymes that catalyze its conjugation to xenobiotics mainly regulates the dynamics of glutathione levels. Recently, the role played by environment and stress in pregnancy loss is gaining importance. Detoxification and reactive oxygen scavenging mechanisms are responsible for the effect of environment on an individual.
Clinical results obtained from IVF studies indicated that the oocyte competence decreases due to a decrease in aromatase activity. In addition, it was shown that the levels of CYP19 transcripts in the endometrium are the markers of endometrial receptivity for implantation, which is crucial for a successful pregnancy outcome. Polymorphisms in CYP17, which can influence the rates of estrogen biosynthesis showed a correlation with the risk of REPL in other populations.

The shift in immune responses from a Th1 to Th2 type seen during pregnancy is mainly due to the expression of HLA-G. In vitro, HLA-G expression on target cells may stimulate PBMCs to secrete anti-inflammatory IL-10 and inhibit the secretion of TNF-α and IFN-γ both of which are reported to be elevated in idiopathic REPL.

Anandamide produces its effects by acting on the G-protein coupled CB1 and CB2 cannabinoid receptors. Following cellular uptake of anandamide, termination of the signal is achieved by degradation of the anandamide by FAAH. A strong correlation between decreased maternal FAAH activity in PBMC and early pregnancy success was reported. FAAH activity is also found correlated with the success rate of IVF. Furthermore, FAAH levels are highest and anandamide levels are the lowest at implantation sites suggesting a role played by anandamide in blastocyst implantation. Recent studies suggested that the FAAH activity increases from 9wk, peaking between 10 and 11wk, followed by a decline after 12wk. These programmed changes are necessary to maintain anandamide at proper levels that are essential for pregnancy
maintenance. A Korean study reported five aberrantly expressed proteins in follicular fluid, complement component C3c chain E, fibrinogen g, antithrombin, angiotensinogen, and hemopexin precursor in recurrent spontaneous pregnancy loss. The levels of expression were found lower in cases than in controls. On the other hand, a number of proteomic studies were carried out in complex diseases and various hematological disorders. With the aim of finding out differentially expressed platelet proteins in spontaneous pregnancy loss as platelet activation, aggregation and oxidative stress are closely related.

1.2 National status of the present work

The initial studies on pregnancy loss from India dates back to 1959 where a report (Bose 1959) from Calcutta using 1217 cases stated the microbial infections and gynecological abnormalities to be the major etiological factors. Other studies, which came mainly from Northern and Eastern region of India (Kaur 1999, Asha Bai 1981, Kumar 2002a, Kumar 2002b, Ghosh 2006), concentrated on the dietary factors, use of specific drugs, consanguinity in family history, presence of anti cardiolipin antibodies etc. in relation to the risk of pregnancy loss. However, with the development in life style, today most of the dietary factors are taken care of. Chromosomal abnormalities that account for majority of pregnancy loss are routinely diagnosed even though the published literature is limited. However, there are no reports from the South Indian population relating the risk of idiopathic REPL and the genetic factors, which are not manifested at the chromosome level. Hence, this study is an attempt to shed light on various etiological factors at molecular level that augment the risk of REPL.