INTRODUCTION

Recurrent pregnancy loss is a disease defined by two or more failed pregnancies before 20 weeks of gestation ***(Practice Committee of the American Society for Reproductive Medicine, 2008; Zegers-Hochschild et al., 2009)***. Approximately 1-5% of women who conceive experience RPL and this proportion are even higher among women who are older than 35 years of age ***(Branch et al., 2010).***

Recurrent spontaneous miscarriage (RSM) is a major reproductive problem affecting 1–5% of otherwise healthy women ***(Sierra and Stephenson, 2006; Brown, 2008)***. These authors reported several factors contributing to RSM pathogenesis that included endocrinologic disorders, immune dysfunction, infectious diseases, Mullerian abnormalities, parental chromosomal abnormalities and environmental factors. Despite this, the underlying etiology of RSM remains unknown in most cases, up to 50%, but is likely to be multi-factorial ***(Christiansen et al., 2008).***

Established causes of recurrent miscarriage include the presence of antiphospholipid antibodies (aPLs), uterine anomalies and abnormal chromosomes, particularly translocations, in either partner ***(Farquharson et al., 1984; Sugiura-Ogasawara et al., 2004; Sugiura-Ogasawara et al., 2010),*** a majority of miscarriages that occur before 10 weeks of

gestation are due to chromosomal aneuploidies arising from new nondisjunctional events, such events being more frequent in very early ***miscarriages (Sierra and Stephenson, 2006)***. A recent review recommended chromosomal analysis of the products of conception in addition to the conventional tests in the evaluation of women with recurrent miscarriage ***(Branch et al., 2010).***

The immunological causes of recurrent miscarriage are now being thoroughly investigated. It is believed that pregnancy is a unique physiological process of immune tolerance of a fetal allograft in the mother and thus the developing embryo and trophoblasts are immune targets owing to their paternally inherited genetic products ***(Lachapelle et al., 1996; Kruse et al., 2003),*** The proposed mechanisms include both alloimmune rejection and autoimmune disruption of the pregnancy ***(Roque et al., 2004).***

In the past decade, Considerable effort has been made to identify cellular constituents and processes putatively underlying immune-based RPL, Natural killer cells have been the cells most extensively studied, primarily because they constitute the predominant leukocyte population present in the endometrium at the time of implantation and in early pregnancy ***(Bulmer et al., 1991; Matteo et al., 2007).***

During normal menstrual cycle, there is a significant increase in the numbers of uterine NK cells in through the

secretory phase with dramatic increase between day 7 and day 9 after LH surge and also in early pregnancy, Uterine NK cells comprise > 70% of endometrial leukocyte in the first trimester deciduas ***(Bulmer et al., 1991; Matteo et al., 2007).***

Uterine NK cells are a distinct leucocyte population found in both non-pregnant endometrium and pregnant decidua. Uterine NK cells are thought to play a significant role in the establishment and maintenance of early pregnancy, although their precise function remains unknown ***(Moffett-king, 2002; Bulmer and Lash, 2005; Hanna et al., 2006; Le Bouteiller and Tabiasco, 2006).***

The role of NK cells in human reproduction has been exploited in both normal and abnormal pregnancies. Natural killer cells are the most abundant lymphocyte population in decidua and have physiologic roles during normal implantation process ***(Ntrivalas et al., 2001).***

Decidual NK cells are closely associated with placental cells and can regulate trophoblast invasion into deciduas, they may also contribute in the control of local infections as well as process of angiogenesis through cytokine secretion ***(Trundley, 2005).***

The NK cells regulate their actions through a balance of activating and inhibitory receptors that are expressed in their and can recognize specific ligands on target cells. Number of

NK cell receptors which contribute to the function of NK cells as cytotoxic cells have been described ***(Moretta et al., 2001; Ntrivalas et al., 2005).***

The majority of uterine NK cells (90 %) express high level of CD56 but they are CD16 –ve and CD3 –ve, The remaining (10 %) of uterine NK cells resembles peripheral blood NK cells and they are CD16 +ve ***(Cooper et al., 2001).***

Several studies have demonstrated that NK cells play an important role in success of pregnancy, It has been shown that peripheral NK cells are down-regulated in successful pregnancy whereas women with history of RPL demonstrated NK levels (uterine and peripheral) higher than fertile controls ***(Coulam et al., 1995; Kwak et al., 1995; Quenby et al., 1999; Vaquero et al., 2006; Tukerman et al., 2007; King et al., 2010).***

Lymphocyte immune therapy or IV immunoglobulin therapy was reported to down-regulate NK cell cytotoxicity and dramatically improve reproductive performance in women with elevated NK cell numbers ***(Kwak et al., 1995; Ntrivalas et al., 2005).***

AIM OF THE WORK

This study aims to detect the prevalence of uterine natural killer cells (CD56) in women with history of recurrent miscarriage compared to women with no history of recurrent miscarriage and if there is any significance denoting a possible role of NK cells in pathogenesis of recurrent miscarriage.

RECURRENT PREGNANCY LOSS

Miscarriage is the commonest complication of pregnancy. The generally accepted definition stipulates that the fetus or embryo should weigh 500 gm or less, a stage corresponding to a gestational age of 20 week, according to the world health organization ***(WHO, 1997).***

A preclinical miscarriage is defined as a demise which occurred before 6 weeks of gestation. Clinical miscarriage can be divided into embryonic or fetal: embryonic miscarriage is defined as an embryo with crown rump length of more than or equal to 5 mm; without cardiac activity. A fetal miscarriage is defined as a fetus of 10-20 weeks size without cardiac activity ***(Rai and Regan, 2006; Stephenson and Kutteh, 2007).***

Spontaneous pregnancy loss is a relatively common phenomenon, with 10–15% of clinically recognized pregnancies ending in miscarriage ***(Macklon et al., 2002; Branch et al., 2010)***. Recurrent pregnancy loss (RPL) or recurrent miscarriage is a disease defined by two or more failed pregnancies ***(Practice Committee of the American Society for Reproductive Medicine, 2008; Zegers-Hochschild et al., 2009)***. Approximately 5% of women who conceive experience RPL and this proportion are even higher among women who are older than 35 years of age ***(Branch et al., 2010).***

The number of miscarriage has been a debate, according to the Royal college of obstetricians and gynecologists, the

definition is three or more consecutive losses ***(RCOG guidelines, 2003)***, but according to American college of obstetricians and gynecologists ***(ACOG, 2001)***, and American society for reproductive medicine ***(ASRM, 2005),*** the definition is two or more consecutive losses. The definition variation from three consecutive losses to two consecutive losses made an increase in the scale of the problem from 1% to 5% of all couples trying to conceive. It is part of a range of reproductive disorders sharing a common underlying cause ***(Greenwood and Jauniaux, 2002).***

INCIDENCE

Recurrent miscarriage (RM) is a major reproductive problem affecting 1–5% of otherwise healthy women ***(Sierra and Stephenson, 2006; Brown, 2008).*** These authors reported several factors contributing to RM pathogenesis that included endocrinologic disorders, immune dysfunction, infectious diseases, Mullerian abnormalities, parental chromosomal abnormalities and environmental factors. Despite this, the underlying etiology of RSM remains unknown in most cases, up to 50%, but is likely to be multi-factorial ***(Christiansen et al., 2008).***

Women with a history of one miscarriage carry a 24% risk of miscarriage in the next pregnancy, while women with a history of previous two miscarriages carry a 26% risk and those with history of previous three miscarriages carry a 32% risk of recurrence and thus women who had miscarried two or more

consecutive pregnancies deserve an evaluation to look for a cause which sometimes can be treated ***(Carson and Branch, 2001; Kiwi, 2006).***

AETIOLOGY

Historically, recurrent miscarriage has been attributed to the following causes:

1- Endocrinal abnormalities:

Endocrinal abnormalities constitute about 48.71% of the causes of recurrent miscarriage ***(Bricker and Farquharson, 2000)***. These abnormalities include anovulation and oligomenorrhea which comprise 26.57%, luteal phase defect, diabetes mellitus and thyroid disorders ***(Wang et al., 2001).***

a) Oligo-ovulation and oligomenorrhea:

Women with oligoovulation are believed to have a discordant endometrial to follicular maturity accounting for suboptimal implantation. Also, de novo chromosomal abnormalities may be more common in oocytes with discordant maturity time. Hence optimizing ovulation will lead to a better prognosis ***(Lee and Silver, 2000).***

b) Diabetes mellitus:

It is believed that poorly controlled diabetes increases the risk of miscarriage ***(ASRM, 2005)***. However, well controlled diabetes is proved not to be a cause of recurrent miscarriage ***(Rosenn et al., 1994; Mills et al., 1998; ROCG guideline, 2003).***

c) Thyroid abnormalities:

Although untreated thyroid abnormalities are well known cause of miscarriage, well controlled thyroid diseases are no longer considered a cause of recurrent miscarriage ***(ROCG guidelines 2003; ASRM, 2005).***

2- Anatomical factors:

This group constitutes about 10% of causes including cervical incompetence, fibroids and mullerian fusion defects ***(Bricker and Farquharson, 2002).***

The impact of uterine fibroids and septae in recurrent miscarriage is uncertain and the effect of surgical treatment for these abnormalities is poorly documented ***(Urman, 2005).***

3- Infections of the reproductive tract:

According to both the American college of obstetrics and gynecology and the Royal College of obstetrics and gynecology, there is no role for infections in the pathogenesis of recurrent miscarriage ***(ACOG 2002; ROCG guidelines 2003).***

4- Thrombophilicdisorders:

Data on the frequency of inherited thrombophilia are compromised by the small size of individual studies, stratification bias, and poor matching of cases and control due to racial heterogeneity. Nonetheless, two meta-analysis studies

have confirmed an association between recurrent miscarriage and gene mutations in factor V Leiden and Prothrombin ***(Kovalevsky et al., 2004; Rey et al., 2003).***

5- Genetic causes:

Genetic factors including chromosomal disorders, single gene defects, and multifactorial factors account for 3.5 – 5 % of the causes of recurrent miscarriage ***(Egozcue et al., 2000)***. Fetal aneuploidy is the most important cause of miscarriage before 10 weeks gestation ***(Jascobs and Hassold, 1987).*** At least 50-60% of all miscarriages are associated with cytogenetic abnormalities, the most frequent being trisomy followed by polyploidy and monosomy X ***(Stephenson et al., 2002).***

In about 4 % of couples with recurrent miscarriage, one partner carries either a balanced reciprocal translocation. In which there is an exchange of two terminal segments from different chromosomes, or robertsonian translocation, in which there is centric fusion of two acrocenteric chromosomes ***(Clifford et al., 1994).*** Carriers of a balanced reciprocal translocation are phenotypically normal, but 50-70% of their gametes and hence embryos are unbalanced, because of abnormal segregation at meiosis. The reproductive risk conferred by chromosomal rearrangement is dependant on the type of rearrangement and whether it is carried by the woman or her male partner ***(Murne et al., 2000).***

6- Immunological causes:

During the last 20 years, it has become clear that a large proportion of unexplained recurrent miscarriage (possibly more than 80%) may be due to immunological causes ***(McIntyre et al., 1989)***. Immunological causes include alloimmune factors and autoimmune factors especially antiphospholipid antibody syndrome, which constitute about 20-50% of the causes ***(Wilcox et al., 1988)***.

Nowadays, immunomediated abortions are known to be characterized by either autoimmune or alloimmune disturbance. In autoimmune abortions, the development of the placenta and the embryo is affected by maternal auto antibodies and auto reactive cells, which target decidual and trophoblastic molecules. In alloimmune abortions, the maternal immune systems react against the embryo and damages trophoblast through allogenic rejection-type reactions ***(Stern and Coulam, 1993; Murphy et al., 2004)***.

Approximately 30% of women with unexplained recurrent miscarriage have increased serum levels of auto antibodies, with antiphospholipid antibodies predominating (***Rai and Regan, 2006).*** The main cause of fetal loss in the presence of antiphospholipid antibodies is hypoxia to the placenta because of uteroplacental blood supply insufficiency resulting form multiple intervillous thromboses, intervillous infarctions and decidual vasculopathy. Additionally to thrombosis, antiphospholipid antibodies directly target

trophoblastic cells and may affect pregnancy by inhibiting normal phospholipid function related to trophoblastic cell division intertrophoblastic fusion, hormone secretion and trophoblast invasion ***(Emoto et al., 1996; Palomo et al., 2007).***

There are auto antibodies other than antiphospholipid antibodies that are related to recurrent miscarriage as the antinuclear antibodies. Antinuclear antibodies and antibodies against single and double stranded DNA appear to be increased in about 35% of women with recurrent miscarriage while their percentage is less than 10% in fertile women with no abortion history.

7- Miscellaneous factors:

Other miscellaneous factors as altered uterine receptivity (integrins, adhesion molecules), environmental toxins, illicit drugs, cigarettes, caffeine and placental abnormalities may account for 10 % of cases ***(Stephenson, 1996),*** Male factor including azoospermia, oligoasthenoteratopyo-zoospermia constitute for about 8.39 % of the causes ***(Lee and Silver, 2000; Enson, 1996),*** However, even after a thorough evaluation, the potential cause remains unexplained in about one third to one half of the cases ***(Lee and Silver, 2000)***.

Recently, parental chromosomal abnormalities and thrombotic complications of the antiphospholipid antibody syndrome are the only undisputed causes of recurrent miscarriage. However, these abnormalities collectively account for 10-15 % of cases of recurrent miscarriage ***(Kutteh, 1999).***

NATURAL KILLER CELLS IN NORMAL PREGNANCY

Natural killer (NK) cells, which form part of the innate immune system, are found in peripheral blood and in the endometrium of humans. The characteristics and possible functions of peripheral blood and endometrial NK cells are very different. The majority (90%) of the peripheral blood NK cells express the characteristic NK cell markers, CD56, CD16 and CD3. In contrast, the majority (90%) of NK cells found in the endometrium (uNK cells) express high levels of CD56, but is CD16 and CD3 negative. The remaining 10% of uNK cells resemble peripheral blood NK cells and are CD16+. Expression of NK cell receptors also differs between peripheral blood and uNK cells within any one individual. The numbers of uNK cells in the endometrium increase markedly during the secretory phase of the menstrual cycle and remain high during the first trimester of pregnancy ***(Bulmar et al., 1991; Laird et al., 2003).***

NK cell biology

The name 'natural killer' is evocative. It derives from an in-vitro assay used to detect these cells. NK cells only kill trophoblast in vitro if activated by interleukin (IL)-2, which is not present in the endometrium at the time of implantation ***(King et al., 1995; Abadia-Molina et al., 1996).***

NK cells are lymphocytes that are part of the innate immune system. They express the cell surface antigens CD16 and CD56. CD16 is a low affinity receptor for IgG complexes and is expressed on the majority of NK cells. It is the receptor responsible for NK-mediated, antibody dependent cellular cytotoxicity. Based on the intensity of CD56 expression, NK cells may be divided into two populations: $CD56^{dim}$ and $CD56^{bright}$. NK cells that are $CD56^{dim}$ are cytotoxic in vitro. In contrast, those that are $CD56^{bright}$ have little cytotoxic ability, but produce immunoregulatory cytokines such as interferon-γ and tumour necrosis factor-α (TNF-α). NK cell function is tightly regulated by a network of specific activating and inhibitory receptors. In particular, CD69 is one of the earliest cell surface activation markers expressed by NK cells. NK cells also express a variety of killer inhibitory receptors (KIRs) and killing activating receptors (KARs), which recognize HLA-G expressed on extravillous trophoblast ***(Moffett-King, 2002).*** NK cells are found in both peripheral blood and the uterine mucosa. However, there are important phenotypic and functional differences between NK cells present at the two sites. The majority (90%) of peripheral blood NK cells is $CD56^{dim}$ and expresses high levels of CD16; these levels do not fluctuate during the menstrual cycle. During pregnancy, peripheral NK cell numbers and functional activity are suppressed ***(Sacks et al., 1999; Yovel et al., 2001)***.

In contrast, NK cells are the predominant leucocyte population in the endometrium, particularly in the decidua basalis at the implantation site.The number of uterine NK (uNK) cells varies during the menstrual cycle. They are sparse during the proliferative phase, increase significantly throughout the secretory phase, remain in high numbers during early gestation, decrease after 20 weeks gestation and are absent in term decidua ***(Bulmer and Sunderland, 1984; Bulmer et al., 1991; Trundley and Moffett, 2004).***

Role of natural killer cells in maintenance of pregnancy:

The endocrine system and the immune system interact closely during implantation and maintenance of pregnancy. One of the most striking examples of this communication is at the level of the decidua. Here, under the influence of sex steroids, there is a dramatic increase of a unique population of lymphocytes, the uterine natural killer cells, in early pregnancy ***(King et al., 1997)***. These cells derive predominantly from a subset of peripheral blood natural killer cells, which under hormonal influences gets recruited to the uterus. In mice, uterine natural killer cells play an important role in the development of placental vasculature. The role of these cells in human pregnancy is still not definitively established; however, they are believed to promote placental and trophoblast growth and provide immunomodulation at the maternal – fetal interface ***(Clifford et al., 1994)***.