

Evaluation of Serum Vegf - A and Interleukin 6 as Predictors of Angiogenesis During Peri-Implantation Period Assessed by Transvaginal Doppler Ultrasonography Amongst Women of Prior Reproductive Failure.

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Study protocol

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Abstract

BACKGROUND

Infertility is a wide spectrum of disorder affecting many couples and is defined as the inability of a couple to achieve pregnancy. A series of investigations are required to know the reason behind infertility, yet a small percentage (08–37%) of couples exist, in which no obvious cause is delineated. Infertility causes enormous emotional, physiological, psychological, sexual, social and financial burden on family. Different hormones, growth factors and interleukins are responsible for successful ovulation, implantation and further growth of embryo. Endometrial receptivity is an important predictor of the outcome of implantation and further growth of embryo.

METHOD

The study would be conducted in a tertiary care centre over a period of two years from July 2022 till July 2024 on non-pregnant unexplained infertile women (63) who will undergo In Vitro Fertilisation and Embryo Transfer (IVF-ET) in the following cycle and the result will be compared with the fertile women (21) with same demographic features. The levels of serum Vascular Endothelial Growth factor – A, Interleukin- 6 will be measured on day 21 of the menstrual cycle in both the group, simultaneously, transvaginal ultrasonography and doppler findings will be measured in both groups to see for endometrial receptivity. The values will be compared for any significance.

RESULTS

The values of Serum VEGF-a, IL6 and Doppler findings will be compared between fertile and infertile group to see for significance difference. The main reason behind doing research on this topic is to find a more cost effective, non-invasive and less time-consuming technique to find Endometrial Receptivity as compared to invasive Endometrial Receptivity Analysis (ERA) Technique. Also, a new scoring system will be developed considering these 3 parameters of endometrial receptivity (PREDICGIO scoring system) for better analysis of endometrial receptivity and to determine the method to be used in patients of infertility for getting pregnancy without much financial burden.

Introduction

Every creature in this world has a desire to have their progeny.

Infertility is a wide spectrum of disorder affecting many couples and is defined as the inability of a couple to achieve pregnancy even after one year of marriage and not using any contraception, in spite of all the conventional investigations of both partner, women in reproductive age group less than 35 years and 6 months if women's age is more than 35 years. A series of investigations are required to know the reason

behind infertility, yet a small percentage (08–37%) of couples exist, in which no obvious cause is delineated ¹.

Infertility can be primary where the couple has not yet achieved pregnancy or secondary infertility where the couple who have been able to get pregnant at least once, but now are unable. ¹ WHO stated that most of the patients suffer from primary infertility. Currently, the prevalence of infertility among the Indian population is 17.9%, (NFHS-IV), higher in urban areas. ² WHO has stated the prevalence of primary infertility between 3.9–16.8% in India. ²

Infertility causes enormous emotional, physiological, psychological, sexual, social and financial burden on family. ³

Infertility is a relative state. The process is complex in both men and women. About 08–10% of couples are affected by infertility. ⁴

Evaluation of infertility requires simultaneous counselling of both partners so as to be time saving and simultaneous treatment of either partner can be started.

Successful pregnancy is characterised by many factors including cervical, tubal, ovarian, uterine, peritoneal and male factors. Even after having no abnormalities in any of these factors, couples have unexplained infertility.

After ovulation occurs, the successful implantation depends on proper tubal motility with appropriate development of embryo to the blastocyst stage, mobility of embryo to the uterine cavity and requirement of a receptive endometrium for further implantation and growth of embryo.

Implantation is a very intricate process which is governed by various growth factors, hormones and inhibitory and supportive cytokines. Combination and coordination of all the factors are required for implantation of the embryo on endometrial surface to achieve a successful pregnancy. The implantation process also requires coordinated effects of autocrine, paracrine and endocrinological factors. ⁵

Endometrial receptivity for a successful implantation is a series of events that take place at the embryo and endometrial junction which determines the outcome of pregnancy during implantation.

Appropriate and adequate angiogenesis and vasculogenesis are required for successful implantation and development of embryo for successful growth of pregnancy. ⁶ In the first few weeks of pregnancy (1–2 weeks), capillaries grow and covers the syncytiotrophoblastic lacunae. The stages of implantation take place in steps as follows: first apposition then adhesion followed by invasion of maternal decidua by the developing embryo. Initially, the capillaries surrounding the syncytiotrophoblast constitutes the vascular supply of the rapidly growing embryo. ⁷ VEGF forms one of the most essential pro-angiogenic factor. It is responsible for the early placental vascular changes. ⁸ It is produced by stromal and epithelial cells in the top layers of the uterine endometrial layers and the embryo and it is a soluble angiogenic factor. The

receptors of VEGF are found in the endothelium which regulates various functions of endothelium. VEGF has mitogenic action on the microvasculature and macrovasculature of endothelium derived from lymphatics and blood vessels. ^{9,10}

VEGF is responsible for physiological and pathological development of vessels. ¹¹ VEGF is amongst subcategory of growth factors. It is a glycoprotein which is homodimeric and of 45,000 Daltons. VEGF-A also called as VEGF and was the first of VEGF family to have been known. The VEGF class has five subtypes: VEGF-A, B, C, D and Placenta Growth Factor (PGF).^{10,12} Abnormalities in vascular endothelial growth factor can cause Utero-placental insufficiency as in cases of Growth Retardation in utero (IUGR), pre-eclampsia and in many cases of unexplained recurrent abortions. ¹³

Recurrent miscarriages or recurrent abortions are said when there are 2 or more continuous or recurrent pregnancy loss before 20 weeks of pregnancy or foetus weighing less than 500 grams from the date of last menstrual period as per American Society for Reproductive Medicine. ^{14,15} It affects at least 2–4% of couples who are trying to conceive. Many factors like anomalies, endocrinological, autoimmune, infectious, thrombophilic and chromosomal abnormalities have been found to be some of the causes of recurrent abortions. In over 50% cases, the causes of recurrent abortions are unexplained. ^{16,17}

Reduced levels of pro vasculogenic factors like VEGF-A and their receptors on the endometrium has been suggested as cause of spontaneous abortions, as it mainly affects the foetal and placental angiogenesis.

Women having infertility and recurrent abortions have been found to have low levels of VEGF. ^{18,19}

Some studies have found serum VEGF-A levels more in Reproductive failure group as compared to normal fertile group. (Atalay 2016)

Results have disparity in role of VEGF in reproductive failure.

While VEGF C is also a factor causing angiogenesis, its main role is in lymphangiogenesis in carcinomas of breast, endometrial, prostate, gastric and oesophageal. ²⁰

Another important factor postulated in infertility and recurrent abortions is serum Interleukin 6 (IL6). Interleukin 6 is vital in division and attachment of trophoblastic cells and is helpful in implantation and pregnancy. ^{5,21}

It comes under the Th2 immune response family and is shown to have an important effect on implantation, angiogenesis and pregnancy outcome. IL 6 is an important cytokine of Th2 immune response. ²¹

Elevated IL6 was found in patients of unexplained infertility, recurrent abortions, preeclampsia and preterm deliveries. ²²

Studies done in 2010, states that in normal pregnancy, a decrease in Th1 immune response and increase in Th2 immune response occurs whereas the opposite occurs in Recurrent pregnancy loss. ^{23,24.}

Result in different studies have got different responses.

Transvaginal ultrasonography with doppler flow plays an essential role in infertility and recurrent abortion management.

Ultrasonography helps in determining uterine abnormalities, cervical abnormalities, tubal anatomy, ovarian reserve and peritoneal adhesions. During perimenstrual period and during implantation window, it helps to determine the angiogenesis and vasculogenesis by assessing the sub endometrial blood flow, zones of endometrium, resistance index (RI) and pulsatility index (PI) of the uterine arteries. It can aid us in the cases of infertility and recurrent miscarriages so as to provide proper angiogenic factors to the suboptimal endometrium for better pregnancy outcome. Assessment of uterine and ovarian blood flow is important aspect of reproduction. Pulse doppler and colour doppler helps in determining uterine and ovarian blood flow which changes dynamically according to the hormonal changes during menses. Doppler study helps to determine the sub endometrial blood flow and helps in accessing uterine receptivity.²⁵

In the secretory phase, because of increased mucus and glycogen content within the glands of endometrium, the endometrium achieves a width between 8 and 16 mm and becomes echogenic with tortuous gland opening and tortuous vessels. The endometrium on an average, achieves its greatest thickness in the mid secretory phase of a spontaneous cycle, which measures up to 14 mm in width.

Endometrial and sub endometrial blood flow measurements act as indicators of uterine receptivity and outcome of treatment.^{26,27}

A study conducted by Tzafra Cohen, Dorit Nahari in 1996 Journal of Biological Chemistry, 28 studied the correlation of IL6 and VEGF-A in angiogenesis. They stated that as angiogenesis is regulated by growth factors and cytokines, they studied the effect of treatment of various cell lines with IL6 for 6–48 hours and to see the induction of VEGF mRNA. Pregnancy, physiologically is initially a relative hypoxic state. VEGF and IL6 are the factors which gets stimulated in response to hypoxia. So, in a hypoxic state, IL6 induction takes place which in turn promote the expression of VEGF leading to angiogenesis.²⁹

In the current study, reproductive failure will involve combination of both group of patients one who are dealing with failure to conceive conventionally or by various artificial reproductive techniques used or those women who have conceived but could not carry pregnancy beyond first trimester. All these patients will be subjected to IVF-ET by antagonist protocol and their outcome in the same cycle will be assessed in the form of pregnancy in same cycle with assessment of angiogenesis by quantitative measurement of their VEGF-A and IL6 levels and qualitative assessment in the form of TVS with doppler.

Several studies are being conducted to expand the knowledge on various factors and hormones on reproduction and newer advanced technologies are being used, the success rate of pregnancy after various procedures of ART remains 40–50%. Still a 50–60% remains unexplained even after various

determination of causes. Still a lot needs to be done to understand the pathophysiology behind actual cause of reproductive failure.

Certain articles have proven that VEGF levels are decreased in patients with reproductive failure but some contradictory findings are observed in various other studies mentioned in review of literature below. Similar findings are observed in the levels of Interleukin-6, some studies found no correlation of IL6 with reproductive failure while some studies found IL-6 levels lower in patients with reproductive failure. As the studies conducted till now are inconclusive regarding the exact role of VEGF and IL6 in reproductive failure, current study will throw light on the role of important growth factor VEGF-A and Cytokine IL6 correlation in reproductive failure and defect in angiogenesis because of their abnormal values and their effect on angiogenesis will be observed by transvaginal ultrasonography done during the implantation window period. The values of these levels will be compared with normal fertile women of same demographic characters, during the same menstrual phase and their values will be compared to see for the difference in the levels of VEGF, IL6 and angiogenesis in both the groups. The reproductive outcome of the study group, those who will undergo IVF will be seen for outcome in the form of pregnancy in the same cycle of conducting the tests.

ERA (Endometrial Receptivity Analysis), is a recent advance in infertile women with reproductive failure, which is mainly used in women who had 2 or more unsuccessful embryo transfer or who have a thin endometrial lining which is of concern, or who had unsuccessful implantation even with high quality embryos. It is done during the implantation window period. Endometrial biopsy is being taken to see the gene polymorphism. It is now considered the best diagnostic tool to find optimal time for embryo transfer in patients of infertility or with reproductive failure who will start on In Vitro Fertilisation. But the drawback of the tests is:

1. Genetic configuration of the cells can change in actual cycle of pregnancy or embryo transfer.
2. One extra cycle is wasted as after the test result, the embryo is transferred in next cycle.
3. It is invasive procedure.
4. It is costly.
5. The extra one cycle can cause more mental stress in women dealing with reproductive failure.

Novelty behind this study is to find a more cost effective, non-invasive and less time-consuming method to detect the endometrial receptivity by a combination of growth factor and cytokine level and their outcome in the form of angiogenesis by transvaginal doppler ultrasonography so that these women can be considered for giving drugs and immunomodulators to increase the endometrial receptivity in the same cycle. A more cost effective, less time-consuming scoring system will be generated with the 3 parameters as VEGF-A, IL6 and TVS Doppler for prediction of endometrial receptivity in the reproductive failure patients to predict their outcome.

Research Gap Analysis

Various research studies have been done to assess the VEGF-A, IL6 serum levels and their receptor gene polymorphism in infertility and recurrent abortions separately. Studies have also been done to see correlation between VEGF-A and transvaginal ultrasound during implantation window in cases of infertility, which all showed varied results.

Certain studies have shown that VEGF-A levels are decreased in patients with reproductive failure but some contradictory findings are observed in various other studies mentioned in review of literature above. Similar findings are observed in the levels of Interleukin- 6, some studies found no correlation of IL6 with reproductive failure while some studies found IL-6 levels lower in patients with reproductive failure.

As several studies are being conducted to expand the knowledge on various factors and hormones on reproduction and newer advanced technologies are being used, the success rate of pregnancy after various procedures of ART remains 40–50%. Still a 50–60% remains unexplained even after various determination of causes. Still a lot needs to be done to understand the pathophysiology behind actual cause of reproductive failure.

But, till date, to the maximum of my knowledge, **the evidences of relationship between the combined evaluation of VEGF-A and IL 6 in angiogenesis during the peri implantation window in reproductive failure patients and their outcome observed by transvaginal doppler ultrasonography has not been done. This area of infertility is still in the dark side with little knowledge about the effects of the values of the growth factors and cytokines on the angiogenesis factor in reproductive failure. As most of the studies didn't correlate inflammatory markers with angiogenesis. Any many of them have not associated reproductive outcome of study group. The actual role of these markers in angiogenesis will be studied and simultaneous assessment of these markers on endometrium will be seen by TVS doppler. The data collected from these patients will be used to prepare a new scoring system for angiogenesis considering all 3 parameters (VEGF-A, IL6 and TVS Doppler) according to which prediction of angiogenesis will be done and outcome will be assessed in the form of pregnancy in same cycle. Scoring system can be used to put patients on immunomodulators in later cycles.**

A more cost effective, non invasive, less time consuming method to detect angiogenesis and outcome will be studied, which can be considered in place of costly techniques like Endometrial Receptivity Analysis for patients of reproductive failure.

Aim Of Study

Aim of the study is to evaluate the levels of serum VEGF-A and IL6 during peri-implantation period and to assess them as predictors of angiogenesis by transvaginal ultrasonography with doppler and to also assess the reproductive outcome of study group in the same cycle undergoing IVF-ET and the comparison of serum levels with normal fertile women during the same menstrual phase. Study also aims to develop a new scoring system based on the values of the 3 parameters in study so as to assess the endometrial receptivity by a cost effective, non-invasive and less time-consuming method as compared ERA technique of endometrial receptivity on the basis of existing literature.

OBJECTIVES:

1. To assess IL6 and VEGF-A serum level in relation to angiogenesis by Transvaginal ultrasonography in Reproductive failure group.
2. To assess IL6 and VEGF-A serum levels correlation with angiogenesis by Transvaginal ultrasonography in fertile control group.
3. Comparison of IL6, VEGF-A and angiogenesis in between women of fertile group and women of reproductive failure group.
4. To assess the reproductive outcome of the women of study group in the same cycle.
5. To assess the potential of combined values of VEGF-A, IL6 and Transvaginal doppler ultrasonography as a marker for endometrial receptivity.
6. To compare the new scoring system generated out of 3 parameters (Serum VEGF-A, IL6 and TVS Doppler – PREDICGIO) with ERA on the basis of existing evidences in literature.

Material And Methodology:

The study would be conducted on non-pregnant women attending the Outpatient Department or admitted ward patients in the Gynaecology and Obstetrics department of Datta Meghe Institute of Medical Sciences and Jawahar Lal Nehru Medical College, Sawangi, Wardha, over a period of 2 years.

Study design

Observational, Analytical Cross-Sectional, Hospital based study.

Sample size

Calculated using Epi- Info statistical software

Two-sided confidence interval = 95%

Power (percentage chance of detecting): 80%

Ratio (unexposed: exposed) 0.33

Percentage outcome in unexposed group: 50%

Least extreme Risk ratio to be detected: 0.31

Least extreme Odds ratio to be detected: 0.18343

Percentage outcome in exposed group: 15.5%

N = 84 (63: exposed and 21: unexposed)

Inclusion criteria:

1. Non pregnant women of age group between 20–40 years.
2. Primary or secondary infertility patients having unexplained infertility with previous stimulated or unstimulated cycles.
3. Patients with history of one or more abortions.
4. Willing to give consent.

Exclusion criteria:

1. Patients not giving consent.
2. Patients with anatomical (uterine, tubal or cervical abnormalities diagnosed with ultrasonography or hysterosalpingography).
3. Patients with chromosomal abnormalities diagnosed by chromosomal study will be excluded.
4. Patients with autoimmune or endocrinological disorders diagnosed by blood tests or clinical features.
5. Patients with chronic illness or infective aetiology which could be a cause of infertility or abortion.

Controls will be of same demographic characters, non-pregnant women attending OPD of Obstetrics and Gynaecology for contraception or for gynaecological treatment other than that of fertility, or non-pregnant women attending OPD of paediatrics for follow up of their kids, during the same menstrual phase, who have at least one full term live child with no previous history of any abortions, still births or intra uterine demise and no other significant medical, anatomical, chromosomal, autoimmune, endocrinological or infective history in the past will be consented for their participation in research study and will be explained regarding the benefits of study to the population. They will be called during the implantation window phase for the collection of samples for VEGF-A and IL6 serum level estimation and their transvaginal ultrasonography will also be performed on the same day to assess the angiogenesis visually.

After taking prior informed consent for their inclusion in research study and explaining them details of the purpose of research, cases will be taken up for the study and worked up. History will be taken according to the proforma which will include age of both partners, demographic history, social history, duration of marriage, use of contraception, menstrual history, details of obstetrics history including previous history of abortions, still births, live births and intra uterine demise, whether histopathology of the specimen/chromosomal study of abortus was performed in the past pregnancy, how was the pregnancy terminated, personal history, medical or surgical history including hysteroscopy or laparoscopy for infertility in past, sexual history and relevant family history.

All the cases will undergo In Vitro Fertilisation, and Embryo Transfer by Antagonist protocol. Details regarding their previous course of treatment, medications and various modalities of Artificial Reproductive Techniques used, hysteroscopy in past, number of previous cycles of undergoing IVF and ET will be asked.

Anthropometry: The anthropometric parameters to be measured will be weight, height, body mass index (BMI). BMI will be used to classify patients into different classes of obesity, and subjects will be classified into 4 groups based on World Health Organization classification as underweight ($< 18.5 \text{ kg/m}^2$), normal ($18.5\text{--}24.9 \text{ kg/m}^2$), overweight ($25\text{--}29.9 \text{ kg/m}^2$), and obese ($\geq 30 \text{ kg/m}^2$).

General examination will include vitals like pulse, blood pressure, temperature, respiration, oedema feet, clubbing, cyanosis, lymphadenopathy, followed by thyroid and breast examination, systemic examination, abdomen examination, speculum examination and vaginal examination will be done.

Lab investigations: Blood and urine tests which includes complete blood counts, blood grouping, HIV, VDRL, HBsAg, sickling, urine routine and microscopy will be done in cases and control.

Special investigations for patients with previous abortion including blood sugar Fasting/Post meal, HbA1c level (if required), serum fasting insulin (if required), serum prolactin, Serum freeT3,fT4, TSH (fasting sample), coagulation profile (Prothrombin Time (PT)/activated Partial Thromboplastin Time (aPTT)), Antiphospholipid antibodies: Lupus Anti-coagulant (LAC), anticardiolipin antibody (ACL), b2glycoprotein, vaginal swab for culture, sensitivity and anatomical study of uterine structure and adnexa by ultrasonography if not already done will be done. HSG and karyotyping of partners (if required) will be done.

Special investigations for infertile patients will include blood sugar Fasting/ Post Prandial, HbA1c level (if required), serum free T3, T4, TSH levels, serum prolactin, sr. fasting insulin (if required), vaginal swab culture, sensitivity, ultrasonography on day 2/3 of cycle for antral follicular counts and anatomical structure of uterus, cervix and adnexa, hysterosalpingography between day 7–9 of menstrual cycle, husband's semen analysis (after abstinence of 2–3 days) if not already done will be done. Serum AMH, LH, FSH and Sr. oestradiol will be subjected to individual cases.

Patients in both subtypes who will have any anatomic, infective, autoimmune, chromosomal or endocrinological abnormalities will be excluded from the study. Those participants with unexplained primary or secondary infertility with no known cause and history of abortion with no underlying factors will be taken up for further analysis in the research study.

The participants of control group will be of the same age group non pregnant women who have no history of abortions, intrauterine demise or still births and have one live child. Participants with medical problems like autoimmune disorders on medications, bleeding diathesis, endocrinological diseases like thyroid dysfunction, diabetes mellitus, uterine, cervical or any other congenital anomaly will be excluded. The consent form will be given for their better understanding and an informed written consent will be taken from all the participants for enrolment in the research study. The blood sample will be collected from cases of both group between day 21–23 of the menstrual cycle or during the day of Embryo Transfer in patients undergoing IVF-ET that is during peri implantation period for assessing the levels of serum VEGF-A and IL6 levels. The VEGF-A and IL 6 levels will be assessed through Enzyme-Linked Immuno sorbent assay (ELISA) technique -Human VEGF-A BIOLISA KIT and Human Interleukin 6 ELISA

KIT. On the same day of sample collection, the cases will undergo transvaginal ultrasonography and doppler study to see for the endometrial thickness, pattern of endometrium, sub endometrial blood flow and the doppler indices of uterine arteries will be studied by using modified Applebaum scoring system which will help in visualising angiogenesis without invasive technique in the study group as well as control group.

Initially, study will be conducted on 10 patients to evaluate the levels of VEGF-A and IL6 based on which a new scoring system based on 3 factors as VEGF-A, IL6 and TVS doppler will be developed on which the patients of reproductive failure will be classified. According to the score, the reproductive outcome of patients undergoing IVF-ET in the same cycle will be noted. The patients who have lower score of who don't achieve pregnancy in the same cycle will be considered for immunomodulator drugs in later cycles.

Datta Meghe Institute of Medical Sciences, Sawangi, Wardha, Institutional Ethical Committee clearance obtained on 29/9/2021.

Re- regd no: ECR/440/Inst/MH/2013/RR-2019

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Declarations

CONFLICTS OF INTEREST: None

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