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DEPARTMENT OF OBSTETRICS & GYNAECOLOGY

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Title of Research Project: **EFFECT OF GRANULOCYTE-COLONY STIMULATING FACTOR ON UNRESPONSIVE THIN ENDOMETRIUM**

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TITLE: - Effect of Granulocyte-Colony Stimulating Factor on Unresponsive thin Endometrium

INTRODUCTION

Infertility is defined as failure to conceive after one year of regular unprotected intercourse with the same partner. Childlessness is a life crisis and impaired fertility has been reported to affect 10-15% of couples [1].There is about 10-15 percent of reproductive age population concerned with infertility due to various explained and unexplained reasons. The World Health Organization (WHO) estimates that 60 to 80 million couples worldwide currently suffer from infertility. [2] The overall prevalence of primary infertility in India estimates to be between 3.9% - 16.8%. [3]

Causes of female infertility may be due to various etiologies. It may be due to congenital defects or acquired. Delayed age, tobacco smoking, body weight, eating disorders also play an important role in etiology. Defects in anatomical factors may involve ovarian, tubal, uterine, cervical or vaginal abnormality or disorders in hypothalamic-pituitary axis leading to hormonal imbalance.

A good blood supply towards the endometrium is usually considered to be an essential requirement for better thickness of endometrium and finally for the implantation of embryo(s).

A good correlation between endometrial thickness and the prevalence of conception has been found. The optimal thickness for the endometrial lining is between 7 and 14 mm. It reaches its maximum thickness at the time of implantation at around day 21 of a woman's menstrual cycle [4].

There is no officially accepted definition of "thin lining", but a measurement of the endometrial lining less than 7 mm on the day of surge/positive ovulation predictor test is commonly accepted as meeting the criteria for thin endometrium. The incidence of thin endometrium in natural cycles has been reported to be 5%- 25% in women [5].

The growth of the endometrial lining is dependent upon the quality of blood flow to the uterus as well as the effect of estrogen in encouraging the lining to develop.

Several therapeutic approaches have been suggested to overcome the problem of thin endometrium like; low-dose aspirin [6], vaginal sildenafil [7], in addition to ovarian stimulation high-dose oral and vaginal estrogens [8].
Estradiol can be administered orally, transdermally, or intramuscularly. It is possible that one type of medication works better than another for certain patient. But ultra low doses of 17beta-estradiol, which might improve serum lipid levels, did not significantly change endometrial thickness or uterine diameter. [9]

The mechanism by which sildenafil improves the endometrial thickness is that it improves the uterine blood supply. It may have an effect on any of the cytokines that regulate endometrial development or implantation. Though, no significant difference has been seen in placebo and in viagra cycles [10]

Aspirin could improve circulation in the endometrium by its action on prostacyclin / thromboxane pathways, but its anti-inflammatory action could equally blunt the prerequisite inflammatory response necessary for implantation.
It's been seen that aspirin therapy did not enhance endometrial thickness, augment the ovarian response, or improve pregnancy rates [11]. Aspirin even inhibits prostaglandin synthesis and implantation could be compromised.

The purpose of these strategies is to increase the endometrial blood flow at implantation site
Recently, chronically thin endometrium can be expanded after uterine perfusion with G-CSF which is a cytokine which act like hormones & neurotransmitters and are involved in a variety of immunological, inflammatory, and infectious diseases. [12] The type of G-CSF which is given as treatment is a recombinant form (r G-CSF) and is made by genetic engineering to produce an identical substance which acts like the naturally occurring cytokine.

Its effect can be observed by measuring endometrial thickness by trans - vaginal sonography. In women with extremely thin endometrium, G-CSF effectively appears to reach minimal endometrial thickness of 7.0 mm within approximately 48 hours. [13]

Therefore, clinical research is necessary to improve the use of G-CSF in patients with thin endometrium which cannot be improved by other methods. In view of above, present study is designed to execute this prospective pilot study.

REVIEW OF LITERATURE

Infertility varies across regions of the world and is estimated to affect 8 to 12 % of couples worldwide [14, 15]. Overall prevalence of primary infertility in India estimates to be between 3.9% - 16.8% [3].

Causes of female infertility may be due to various aetiologies. It may be due to congenital defects or acquired. Defects in anatomical factors may involve ovarian, tubal, uterine, cervical or vaginal abnormality or disorders in hypothalamic-pituitary axis leading to hormonal imbalance. Delayed age, tobacco smoking, body weight, eating disorders also play an important role in etiology.

The endometrium is the innermost glandular layer and functions as a lining for the uterus, preventing adhesions between the opposed walls of the myometrium, thereby maintaining the patency of the uterine cavity. During the menstrual cycle the endometrium initially proliferates under the influence of estrogen. However, once ovulation occurs, in addition to estrogen, the ovary will also start to produce progesterone. Proliferate and secretary changes at the endometrial lining are the result of a complex intrauterine environment where sex steroid hormones and different local factors play an important role for endometrial thickening. Optimum endometrial thickness reflects an adequate maturation which is a key factor for embryo implantation [16].

This changes the proliferative pattern of the endometrium to a secretory lining. Eventually, the secretory lining provides a favourable environment for implantation of embryos.

Endometrial development is regulated by steroid hormones, various growth factors and cytokines. Sufficient uterine blood supply is required for these factors to reach the endometrium, especially to its functional layer [17].

Endometrial receptivity is essential for successful implantation and establishment of pregnancies. Transvaginal ultrasonography has been proposed as a tool in the assessment of endometrial receptivity. In a retrospective cohort study done by Shufaro et al, attempt was made to assess the endometrial receptivity and define uterine predictors of implantation and pregnancy by ultrasonographic endometrial features like thickness, echogenicity. Despite the existence of quite a few published studies, the prognostic value of ultrasonographic endometrial thickness measurements in predicting implantation and pregnancy rates remains controversial [18].

Several studies have tried to evaluate the association between the morphologic characteristics of the endometrium and pregnancy rates. Although the results are sometimes conflicting, most studies agree that the endometrium has to reach a certain thickness for successful pregnancy to occur [19].

Hence, the thickness of the endometrium is almost as important as the number of eggs that are developing during ovarian stimulation. During ovarian stimulation estrogen is secreted, either directly or indirectly increase the endometrial lining. Pregnancies did not occur when the endometrial thickness was less than 7 mm. [20]

Poor endometrial lining more commonly occurs when the basal germinal endometrium, from which the full endometrial layer develops, is compromised in its response to estrogen. This most commonly occurs when:

1. The basal endometrium is permanently damaged

    - Inflammation of the uterine lining and endometritis following septic delivery, abortion or miscarriage
    - Following repeated or over aggressive Dilatation & Curettage procedures
    - Following uterine surgery that causes excessive endometrial scarring

2. Endometrial resistance to estrogen

    - Overuse of clomiphene citrate

- Developmental: following prenatal and in utero exposure to diethylstilbestrol (DES)

3. Reduced blood flow to the basal endometrium

    - Multiple uterine fibroids (sub mucosal)
    - Following extensive uterine surgery
    - Adenomyosis

4. Over exposure to ovarian testosterone

    - Overgrowth of ovarian stroma that produces testosterone in response to LH

Therefore the treatment approach with thin endometrium is to increase blood flow to the reproductive organs, increase receptivity of the uterus to estrogen and balanced reproductive hormones.

To improve uterine vascularization:

Low dose aspirin

Low dose aspirin treatment significantly improves uterine and ovarian blood flow velocity, implantation and pregnancy rates by inhibiting the synthesis of thromboxane A2 without affecting the synthesis of prostacyclin. [21]

Hurst et al studied that Aspirin does not enhance endometrial thickness, augment the ovarian response, or improve pregnancy rates. [11]

L-arginine (Nitric oxide donor)

L-arginine supplementation improves the uterine blood flow, endometrial receptivity, implantation and pregnancy rates in comparison to a control group [22]. In addition, oral L-arginine improves endometrial thickness on the day of hCG administration.

Sildenafil (viagra)

Nitric oxide relaxes vascular smooth muscle through cGMP mediated pathway and nitric oxide isoforms have been identified in the uterus [23]. Sildenafil citrate is a newly developed,

type 5-specific phosphodiasterase inhibitor that prevents the breakdown of cGMP and potentiates the effect of nitric oxide on vascular smooth muscle. Vaginal sildenafil may be effective for improving uterine blood flow and endometrial receptivity.

Hoad et al used viagra in a double blind cross-over trial to determine its effect on endometrial and junction zone volume in both unexplained infertility and a control group of apparently fertile women and observed there was no difference in volumes between the placebo and viagra cycles. [10]

Check et al studied that neither vaginal E2 nor sildenafil significantly improved endometrial thickness or blood flow [24].

Granulocyte- Colony stimulating factor (G-CSF)

G-CSF is a cytokine normally produced by the human body itself. Cytokines are proteins and peptides that allow cells to communicate with one another. They act like hormones and neurotransmitters and are involved in a variety of immunological, inflammatory, and infectious diseases. The G-CSF is given in a recombinant form for the treatment of thin endometrium and is made by genetic engineering to produce an identical substance which acts like the naturally occurring cytokine.

G-CSF may have physiologic roles in the endometrium throughout the menstrual cycle. Endometrial G-CSF protein production is stimulated by interleukin-1β and that G-CSF may in part, mediate local actions of interleukin-1β and modulate trophoblast proliferation. Studies show that it also helps in implantation [25, 26].

Lucena et al found that uterine infusion of G-CSF quickly increased endometrial thickness resulting in a successful pregnancy. It also seems G-CSF is a factor that participates during endometrial remodelling enhancing the synchronisation between uterine environment and embryo development [16].

In the Prospective cohort study done by Norbert Gleicher et al found successful endometrial expansion to at least minimal thickness of 7 mm after uterine perfusion with G-CSF in patients previously resistant to treatment with estrogen and vasodilators [27].

However, study done by Yu Li et al, failed to demonstrate that G-CSF has the potential to improve embryo implantation and clinical pregnancy rates in women with thin endometrium [28].

LACUNAE

To the best of our knowledge there is paucity of research work for use of G-CSF in relation to the management of unresponsive thin endometrium in infertile women.

PERIOD OF THE PROPOSED STUDY

Expected duration of this proposed study is 03 years (2 year, 06 months period for clinical work and 06 months for Documentation and Statistical Analysis).

AIMS AND OBJECTIVES

1. To assess the response of intra-uterine perfusion of granulocyte–colony stimulating factor in women with thin endometrium (<7 mm) undergoing *in-vitro* fertilization (IVF) cycle.

2. Occurrence of pregnancy during the follow-up after instillation of G-CSF in these women, if any.

Primary outcome: Triple line and endometrial thickness of ≥7 mm ~48 hours after the first dose i.e intra-uterine perfusion of recombinant granulocyte–colony stimulating factor (G-CSF).

Secondary outcome: Occurrence of pregnancy during the follow-up after instillation of G-CSF in these women, if any

MATERIALS AND METHODS

Women with primary and secondary infertility will be recruited from infertility clinic at Department of Obstetrics and Gynaecology, Maulana Azad Medical College & associated Lok Nayak Hospital, New Delhi. All women with normal investigations specific to infertility, but having thin or not responding endometrium to stimulation protocol, will be enrolled. Patients with prior infertility management and failed due to thin endometrium irrespective of receiving one or more adjunct will be also noted.

General physical and local examinations will be performed to rule out any pelvic abnormality. Subsequently Trans-vaginal (TVS) sonography will be done on day 14 of spontaneous menstrual cycle to measure the thickness of the endometrium. If it is <7 mm, then these women will be put on tablet estradiol in titrating dose maximum of 12 mg. /day (Tablet Progynova,-----) for 10 to 14 days. On repeat transvaginal sonography, if the endometrium will be found <7 mm will be designated as *unresponsive endometrium*, will be recruited for IUI or IVF and/or ICSI cycles, as indicated for this proposed study.

Detailed informed consent will be taken from all the recruited patients. General physical and local examinations will be performed to rule out any abnormality in all these women.

Sample size:

A sample size of 80 is required for this proposed study as per 15 % prevalence of the infertility among the population, 5-25% incidence of patients with thin endometrium, 95% confidence level with 5% error (Adequacy of sample size in Health Studies, Stanley Lemeshow & Stephen K. Lwanga *et al.*) [29] To find out possible role of the use and effect of G-CSF infusion in endometrial lining response, implantation and clinical pregnancy in the selected patients.

Inclusion criteria:

1. Women with primary and secondary infertility with the normal investigations specific to infertility (Normal sperm count according to WHO 2010 criteria, bilateral patency of the fallopian tubes and normal ovulation).

2. All patients with Inadequate endometrial lining response/ thin endometrium (Endometrial thickness is less than 7 mm) in spite of receiving one or more adjuvant to improve endometrial lining.

3. Women with normal endocrinal function.

Exclusion criteria:

1. Women with other etiological factors of infertility
2. Women receiving infertility treatment for the first time.
3. History of haematology Disease
4. Uterine anomalies
5. Uncompensated heart diseases
6. Neutrophilia
7. Severe lower back pain
8. Liver dysfunction
9. Hyperuricemia
10. A recent febrile disease or pneumonia
11. Rheumatoid arthritis
12. Sickle cell disease

Infertility treatment:

1. Controlled ovarian stimulation with clomiphene and/or recombinant/urinary or human menopausal gonadotropins (hMG).
2. Follicular monitoring and measurement of endometrial lining.

Adjuvants to improve endometrial lining:

1. Estrogen
2. Sildinafil
3. Low dose aspirn

4. L-arginine

Methodology:

The women who recruited for *in-vitro* fertilization (IVF) procedures will be enrolled in this study. They will be called in early follicular phase, on Day 2 or Day 3 of the menstrual cycle. They will be examined for general physical examination, per speculum, per vaginal examination will be done. Trans- vaginal sonography will be done to rule out any endometrial or ovarian abnormality.

Ovarian stimulation will be achieved by providing clomiphene and/or gonadotropin hormones [recombinant/urinary follicle stimulating hormone (FSH)/ human menopausal gonadotropin (hMG)] from Day 2/3 of menstrual cycle for 10 to 11 days, depending upon the response.

On day 6, day 8 and day 10/11/14 of the menstrual cycle all women will be called for regular trans- vaginal sonography to assess the size of follicle and endometrial thickness.

All women will be followed up for follicle monitoring and endometrial thickness till 18mm or more of follicle size is achieved. If endometrial thickness remains less than 7 mm, intrauterine infusion of GCS-F (300mcg) will be done on the day of hCG trigger. Effect of G-CSF on endometrial thickness will be observed 34 to 36 hours after the trigger on the day of oocytes retrieval or 40 to 42 hrs after in the cases of IUI.

Method of instillation

Women will be asked to lie in dorso-lithotomy position. Cusco's speculum will be applied. Intra uterine catheter attached with pre filled 1 ml tuberculin syringe containing G-CSF, [Human Granulocyte Colony Stimulating Factor (Hu G-CSF) - 300 U/ml], will be gently introduced in the uterine cavity without touching the endometrium and is administered by slow intrauterine infusion under the Trans abdominal scan.

Composition of Injection hu G-CSF [Human Granulocyte Colony Stimulating Factor (Hu G-CSF) - 300 mcg/1.0 mL]

Contents	**Quantity**
r-Hu G-CSF	300 mcg
Glacial Acetate	0.60mg

Sorbitol	50 mg
Ploysorbate 80	0.04 mg
Sodium Hydrochloride	0.06 mg
Water for Injection	1.0 mL
pH	4.0

All women will be followed up during ovarian stimulation for follicle monitoring and endometrial thickness. If endometrial thickness remains less than 7mm, intrauterine infusion of GCS-F (300mcg; 1st dose) will be done on the day of hCG trigger. Trans vaginal sonography will be done 34 to 36 or up to 40 hours after the trigger. If no or low response observed on endometrium, 2nd dose of GCS-F will be instilled after oocytes retrieval or 06 hrs prior to IUI. The dose (s) of GCS-F (300mcg) will be administered as intrauterine infusion with the help of IUI Catheter. The drug will be stored at 2-8º C in refrigerator for maintaining cold chain and avoid shaking.

Adverse Events

No adverse events like toxicity and any kind of allergy are known so far as in literature. (Undertaking to take care of any side effect will be taken from each patient).

Follow-up:

Ovarian stimulation followed by intra-uterine insemination (IUI) and/or *in-vitro* fertilization-embryo transfer (IVF-ET) procedures will be put on micronized progesterone (400 mg twice daily vaginally, Susten, Sun Pharmaceuticals, Gujarat, India) for fifteen days. After 15 days all the patients will be called for βhCG estimation for the conformation of the pregnancy. Clinical pregnancy will be diagnosed two weeks after the βhCG estimation by the presence of the fetal cardiac activity.

β hCG more then 50 mIU will be diagnosed as successful pregnancy and less then this will be implantation failure.

DATA COLLECTION

Data will be collected after quantitative analysis and examine the effect of Human Granulocyte Colony Stimulating Factor (Hu - G-CSF).

STATISTICAL ANALYSIS

The data generated from the experiment will be analyzed by Student's t-test to observe the mean difference between the values of different Interleukins mentioned for this proposed study. Data will be expressed as mean ± SEM and significance will be taken at $P < 0.05$. Computer generated randomly divided patients will be taken as

1. Lining response after G-CSF infusion in case group and compared with the patients who under prior adjuvant therapy for thin endometrium.
2. Implantation (Successful pregnancy) and non-implantation (pregnancy failure) groups.

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UNDERTAKING

This is hereby declared that I, **Dr. Sudha Prasad**, Director Professor and IVF Coordinator, IVF and Reproductive Biology Centre, Department of Obstetrics & Gynaecology, MAM College & associated LN Hospital, will conduct the research project entitled **"Effect of Granulocyte-Colony Stimulating Factor on Unresponsive Thin Endometrium".** Proper precautions shall be taken so as to minimize risk and prevent irreversible side effects. The study involves the off label use of G-CSF in women diagnosed with thin endometrium any adverse event after use of the same drug will be taken care on priority and reported to the Institutional Ethical Committee.

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UNDERTAKING

1. I/ We care to abide by the ethical guidelines for biomedical research on human subject (as per ICMR guidelines) while conducting the research project being submitted for ethical committee consideration;

2. Project is considered absolutely essential for the advancement of knowledge and for the benefit of all.

3. Only subjects, who volunteer for the study, will be included. Their informed consent shall be obtained prior to commencement of the study & subjects will be kept fully appraised of all the consequences.

4. Privacy & confidentiality of the subjects shall be maintained & without the consent of the subject no disclosure will be made.

5. Proper precautions shall be taken so as to minimize risk and prevent irreversible side effects.

6. Study will be conducted by professionally competent persons.

7. Study will be conducted in a fair, honest, impartial & transparent manner. Researcher will be accountable for maintaining proper records.

8. Study will be conducted keeping in view the public interest at large.

9. Study reports, materials & data will be preserved.

10. Result of study will be made known through scientific publications.

11. Professional & moral responsibilities will be of the researchers, directly, or indirectly connected with research.

12. Only those drugs which are approved by the drug controller of India for a specific purpose will be used in the research.

13. The protocol has been discussed in my department & has been approved.

Principal Investigator : Dr. Sudha Prasad

Date:

Place:

INDEX

Introduction	10-11
Research question and Hypothesis	12
Review of literature	13-17
Aims and Objective	18
Materials and Methods	19-25
References	26-29
Proforma	30-34
Patient's information sheet	35-36
Patient's informant consent	37-41