Issue 4

Infertility and its management

Conundrums & solutions

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† Schindler AE. Progestational effects of dydrogesterone *in vitro, in vivo* and on the human endometrium. *Maturitas*. 2009;65(1):S3-S11. * Data on file. ‡ Internal calculations based on Quintiles IMS database, IMS Health Analytics Link MAT03 2017.

Abbreviated Prescribing Information. Dydrogesterone Tablets IP. Duphaston® Composition: Each white film-coated tablet contains: Dydrogesterone IP 10 mg. Excipients q.s. Colour: Titanium dioxide IP. **Indications:** Progesterone deficiencies, Treatment of progesterone deficiencies such as: • Treatment of dysmenorrhoea • Treatment of endometriosis • Treatment of secondary amenorrhoea • Treatment of irregular cycles • Treatment of dysfunctional uterine bleeding • Treatment of pre-menstrual syndrome • Treatment of threatened and habitual abortion • Treatment of infertility due to luteal insufficiency. Hormone replacement therapy - To counteract the effects of unopposed oestrogen on the endometrium in hormone replacement therapy for women with disorders due to natural or surgical induced menopause with an intact uterus. **Dosage and Administration:** Dosages, treatment schedule and duration of treatment may be adapted to the severity of the dysfunction and the clinical response. Dysmenorrhoea: 10 or 20 mg dydrogesterone per day from day 5 to day 25 of the menstrual cycle. Endometriosis: 10 to 30 mg dydrogesterone per day from day 5 to day 25 of the cycle or continuously. Dysfunctional uterine bleeding: When treatment is started to arrest a bleeding episode, 20 or 30 mg dydrogesterone per day is to be given for up to 10 days. For continuous treatment, 10 or 20 mg dydrogesterone per day should be given during the Second half of the menstrual cycle. The starting day and the number of treatment days will depend on the individual cycle length. Withdrawal bleeding occurs if the endometrium has been adequately primed with either endogenous or exogenous estrogen. Secondary amenorrhoea: 10 or 20 mg dydrogesterone per day, to be given daily for 14 days during the second half of the theoretical menstrual cycle to produce an optimum secretory transformation of an endometrium that has been adequately primed with either endogenous or exogenous estrogen. Pre-menstrual syndrome: 10 mg dydrogesterone twice daily starting with the second half of the menstrual cycle until the first day of the next cycle. The starting day and the number of treatment days will depend on the individual cycle length. Irregular cycles: 10 or 20 mg dydrogesterone per day starting with the second half of the menstrual cycle until the first day of the next cycle. The starting day and the number of treatment days will depend on the individual cycle length. Threatened abortion: An initial dose of up to 40 mg dydrogesterone may be given followed by 20 or 30mg per day until symptoms remit. Habitual abortion: 10 mg dydrogesterone twice daily until the twentieth week of pregnancy. Infertility due to luteal insufficiency: 10 or 20 mg dydrogesterone daily starting with the Second half of the menstrual cycle until the first day of the next cycle. Treatment should be maintained for at least three consecutive cycles. Hormone replacement therapy: Continuous sequential therapy: An estrogen is dosed continuously and one tablet of 10 mg dydrogesterone is added for the last 14 days of every 28 day cycle, in a sequential manner. Cyclic therapy: When an estrogen is dosed cyclically with a treatment-free interval, usually 21 days on and 7 days off. One tablet of 10 mg dydrogesterone is added for the last 12-14 days of estrogen therapy depending on the clinical response, the dosage can subsequently be adjusted to 20 mg dydrogesterone per day There is no relevant use of dydrogesterone before menarche. The safety and efficacy of dydrogesterone in adolescents aged 12-18 years has not been established. Currently available data are described in section 4.8 and 5.1, but no recommendation on a posology can be made.

Contraindications: Known hypersensitivity to the active substance or to any of the excipients. Known or suspected progestogen dependent neoplasms. Undiagnosed vaginal bleeding. Contraindications for the use of estrogens when used in combination with dydrogesterone. **Warnings and Precautions:** Before initiating dydrogesterone treatment for abnormal bleeding, the etiology for the bleeding should be clarified. Breakthrough bleeding and spotting may occur during the first months of treatment. If breakthrough bleeding or spotting appears after some time on therapy, or continues after treatment has been discontinued, the reason should be investigated, which may include endometrial biopsy to exclude endometrial malignancy. **Pregnancy and Lactation:** Pregnancy: It is estimated that more than 10 million pregnancies have been exposed to dydrogesterone. So far there were no indications of a harmful effect of dydrogesterone use during pregnancy. Some progestogens have been reported in the literature to be associated with an increased risk of hypospadias. However due to confounding factors during pregnancy, no definitive conclusion can be drawn regarding the contribution of progestogens to hypospadias. Clinical studies, where a limited number of women were treated with dydrogesterone early in pregnancy, have not shown any increase in risk. No other epidemiological data are hitherto available. Effects in non-clinical embryo-fetal and post-natal development studies were in line with the pharmacological profile. Untoward effects occurred only at exposures which exceeded the maximum human exposure considerably, indicating little relevance to clinical use. Dydrogesterone can be used during pregnancy if clearly indicated. Breastfeeding: No data exist on excretion of dydrogesterone in mother's milk. Experience with other progestogens indicates that progestogens and the metabolites pass to mother's milk in small quantities. Whether there is a risk to the child is not known. Therefore, dydrogesterone should not be used during the lactation period. Fertility: There is no evidence that dydrogesterone decreases fertility at therapeutic dose. **Adverse Reactions:** The most commonly reported adverse drug reactions of patients treated with dydrogesterone in clinical trials of indications without estrogen treatment are migraines/headache, nausea, menstrual disorders and breast pain/tenderness. Undesirable effects that are associated with an estrogen-progestogen treatment : Breast cancer, endometrial hyperplasia, endometrial carcinoma, ovarian cancer • Venous thromboembolism • Myocardial infarction, coronary artery disease, ischemic stroke. Issued on: 3/4/14. Source: Prepared based on full prescribing information (version 03) dated 13/03/2015.

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Preface

Infertility is a medical condition which is estimated to affect 8-12% of the couples in the reproductive age group. Apart from emotional challenges and stress it adds to interpersonal relationships, there is also social stigma that is attached to childless couples, particularly in the developing nations such as India. However, over the last couple of years the science of reproductive medicine has experienced a true revolution, thereby making it possible for several childless couples to realize their dream of parenthood.

"Infertility and its Management-Issue-4" is an educational initiative which has been developed to impart up-to-date information on various aspects related to the diagnosis and management of infertility in men and women. Genital tuberculosis is highly prevalent in infertile patients. However, the diagnosis of this curable disease is challenging. The first chapter of the input assesses the utility of Mycobacteria Growth Indicator Tube (MGIT) culture for the diagnosis of genital tuberculosis in women with infertility. The next chapter discusses how to best assess endometrial receptivity – an important determinant of IVF success – to improve fertility outcomes. Natural cycle IVF/M, i.e., the method of natural cycle IVF combined with in vitro maturation (IVM), has emerged as an attractive treatment for women with all types of infertility without recourse to ovarian stimulation, with acceptable pregnancy rate. This is being discussed in the next chapter. The selection criteria for natural and modified natural cycle IVF is highlighted in the subsequent chapter. The two promising therapeutic options for resistant "thin" endometrium in fertility treatment, i.e., granulocyte colony-stimulating factor and stem cell therapy, are described in the last chapter.

We sincerely hope that the valuable insights provided in this input will help stimulate new ideas and perspectives and assist fertility specialists in providing the best care possible to childless couples.

Happy reading!

Contents

The Role of MGIT 960 Culture Medium in Resolving the Diagnostic Dilemma for Genital Tuberculosis Patients Presenting with Infertility

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Abstract

The purpose of this study was to assess the utility of Mycobacteria Growth Indicator Tube (MGIT) 960 culture medium for the diagnosis of genital tuberculosis (GTB) in women presenting with infertility. The premenstrual endometrial biopsy samples in 300 women presenting with primary and secondary infertility were subjected to AFB smear method, histopathological examination and culture on LJ and MGIT 960 media. Detection rates were compared for diagnostic modalities and their combinations. In total, 30 cases were positive for genital tuberculosis by either of the four tests employed. The detection rates for AFB smear, MGIT culture, LJ culture and HPE were 50, 46.7, 3.3 and 33.3%, respectively. A combination of smear examination for AFB, MGIT 960 culture and histopathological examination was able to detect all the positive cases. A combination of MGIT and LJ media provided no added advantage over MGIT alone since the only case where LJ culture was positive had been detected by positive MGIT culture. In as many as five positive cases (16.7%), only MGIT culture was positive. The addition of MGIT 960 culture medium to routine battery of investigations in infertility patients significantly improves the diagnosis.

Keywords: Genital tuberculosis, Infertility, Culture, MGIT 960, Diagnosis

Introduction

Tuberculosis (TB) remains a major health concern in most of the developing countries including India. The World Health Organization estimated an incidence of 2.2 million cases of TB for India out of a global incidence of 9.6 million for the year 2015 [1]. Although the major type of prevalent TB is the pulmonary variety, extra-pulmonary types contribute to burden of the disease and present diagnostic and therapeutic challenges. Genital tuberculosis (GTB) a form of extra-pulmonary TB presents itself with myriads of symptoms and incurs significant morbidity by its short-

and long-term sequelae [2]. Infertility remains the most frequent clinical presentation of GTB, occurring in 43–74% of the cases [3]. Furthermore, a systematic review has revealed the prevalence of GTB among infertile patients to be as high as 24.2% [4].

Diagnosis of genital TB has profound implications for women seeking infertility treatment. Considering the high prevalence and its adverse effect on fertility, diagnosis and treatment of GTB should be one of the main priorities of health systems, at least in developing countries [4]. The diagnostic dilemma arises because of varied clinical presentations, diverse results on imaging and laparoscopy and a mixed bag of bacteriological and serological tests. Diagnosis depends upon collective evidence from imaging studies, direct visualization by laparoscopy and hysteroscopy, and histopathology of genital tract material, culture and serology [2, 5]. The techniques being used for the detection of *Mycobacterium tuberculosis* are time consuming and have low sensitivities and specificities. This results in lack of any conclusive evidence in the early course of the disease when disastrous consequences like infertility can be prevented by appropriate measures.

Culture remains the gold standard for diagnosis of GTB [6]. However, conventional culture media like LJ culture take an agonizing long time (6–8 weeks) for the growth of *Mycobacterium tuberculosis*. They frequently yield negative results in cases of paucibacillary disease, contributing to misdiagnosis in several cases. A culture medium which is rapid in identifying mycobacteria, cheap, easily available and confirmative would be an ideal one for diagnosis of GTB, facilitating early and accurate diagnosis.

Newer cultures like Mycobacteria Growth Indicator Tube (MGIT) 960 appear promising in this regard, seemingly fulfilling all the characteristics of an ideal culture medium. However, evidence regarding usefulness of MGIT in routine diagnostic battery and clinical practice is scanty. The purpose of this study, therefore, was to assess the utility of MGIT culture for the diagnosis of GTB in women presenting with infertility.

Materials and Methods

The study was conducted in the infertility clinic run by the Department of Obstetrics and Gynecology in a tertiary level institute. After approval of ethical committee, a prospective study was conducted on 300 women

being investigated for primary and secondary infertility in the age group of 20–40 years. After a detailed clinical history and thorough physical examination, all women were subjected to a battery of diagnostic tests (Fig. 1).

Endometrial biopsy was taken in premenstrual phase using paracervical block, after obtaining informed consent and duly explaining the procedure. The sample was processed for preparation of smear, histopathological examination and culture (conventional and rapid).

The MGIT 960 media contained 4 ml of Middlebrook 7H9 broth with an oxygen-sensitive fluorescent sensor embedded in silicon at the bottom of the tube which fluoresces under ultraviolet light when oxygen was depleted indicating mycobacterial growth. Uninoculated tubes served as negative control and tubes inoculated with H37Rv as positive control. The MGIT-positive cultures were then subjected to PNB (para-nitro benzoic acid) test to differentiate it from non-tubercular mycobacteria (NTM).

Statistical Analysis

Based on a *p* value of 0.05 and power of 90%, the minimum sample size for this study was calculated to be 300, considering the prevalence of GTB in infertility patients to be around 10% (by the most conservative estimates).

The endometrial biopsy samples positive by any of the four methods were labeled as positive. The baseline characteristics of the GTB and non-GTB groups were compared using the two-tailed Fisher's exact test. The diagnostic accuracy of various tests and their combinations was evaluated based on their sensitivity. The statistical analysis was carried out using SPSS version 19 (SPSS *Inc*., Chicago Illinois).

Results

The average age of 300 women in our series was 28.35 ± 4.29 years, and maximum patients of infertility were aged 26–30 years. In our series, 76% (228/300) had primary infertility, while 24% had conceived earlier.

Analysis of the social profile of women in our series revealed that the maximum cases of infertility (45.7%) belonged to lower-middle socioeconomic strata of the society. The mean Kuppuswamy socioeconomic score

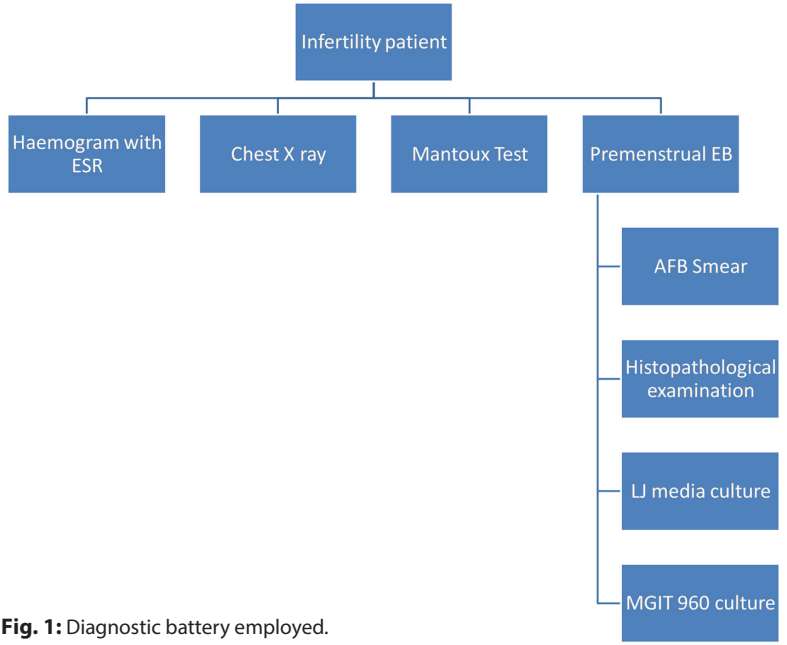

Fig. 1: Diagnostic battery employed.

was 15.26. Demographic data of the study population are summarized in Table 1.

The endometrial biopsy sample was positive for GTB in 30 cases by either of the four tests employed in the diagnostic battery. The detection rates for AFB smear, MGIT culture, LJ culture and HPE were 50, 46.7, 3.3 and 33.3%, respectively. The mean time for growth on MGIT 960 culture was 12.79 ± 4.6 days. In as high as 77% cases that were positive by MGIT 960 culture, the growth on the tube was evident within 15 days of inoculation. LJ culture was positive in only one case, and the growth in that case was evident after a time period of 29 days. In that case, MGIT had already clinched the diagnosis after 14 days of culture. A graphic comparison of the growth time of LJ and MGIT 960 culture is depicted in Fig. 2.

The higher detection rates afforded by MGIT medium over LJ culture came out to be significant. Since the individual methods were not able to detect more than 50% of the cases, sensitivities of combinations of tests were then evaluated (Fig. 3). It was found that a combination of smear and MGIT culture detected around 70% of the cases as positive. MGIT and

Table 1. Demographic profile of the study population.

	Number	Percentage
Age (years)		
20–25	77	25.7
26–30	148	49.3
31–35	47	15.7
36–40	20	6.7
>40	8	2.7
Type of infertility		
Primary	228	76
Secondary	72	24
Kuppuswamy's socioeconomic score		
<5	02	0.67
5–10	36	12
11–15	137	45.67
16–25	118	39.33
26–29	7	2.33

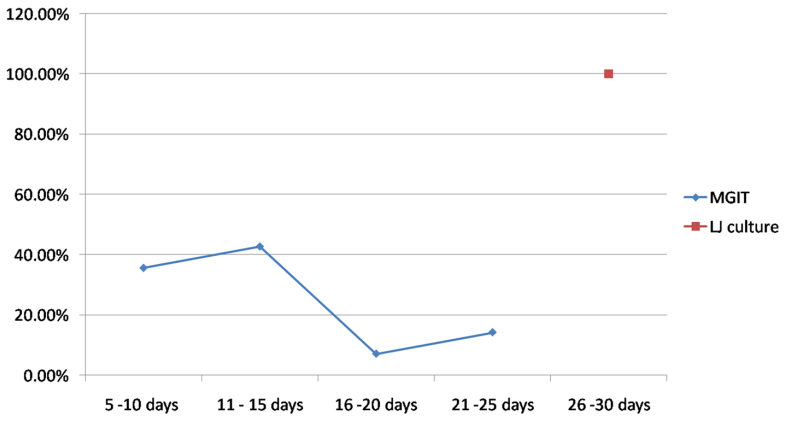

Fig. 2: Graphic comparison of the growth time of LJ and MGIT 960 culture.

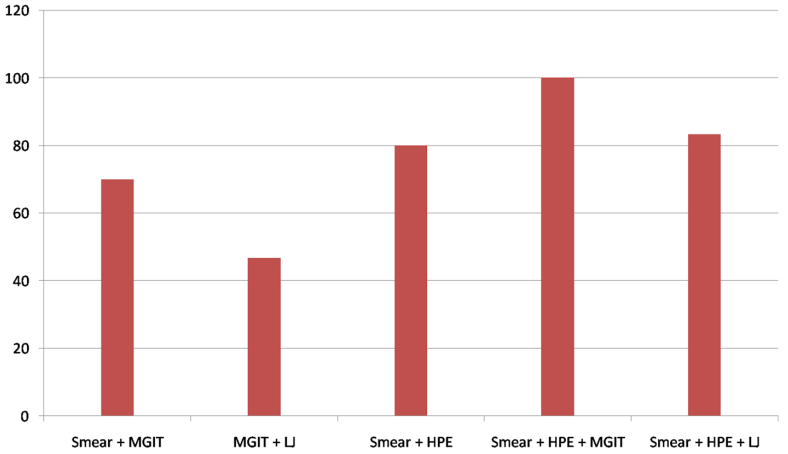

Fig. 3: Detection rates of combination of various methods (in percentage).

Table 2. Tabulated comparison of baseline characteristics of GTB infertile women from study cohort.

	Total infertility cohort ($n = 300$)	GTB cohort ($n = 30$)	Statistical analysis
Average age	28.39 ± 4.41 years	29.6 ± 3.67 years	–
Duration of infertility	58.8 months	87.2 months	$p < 0.001$
Primary infertility	76%	66%	–
Kuppuswamy's socioeconomic score	15.28 ± 4.45	13.3 ± 3.61	$p < 0.01$
Average menstrual cycle length	30.8 days	36.17 days	$p < 0.001$
Scanty periods	16.29%	56.67%	$p < 0.001$
Positive past history of TB	6.29%	20%	$p < 0.01$
Ectopic pregnancy history	14.51%	50%	$p < 0.02$
ESR (>15 mm)	35.5%	80%	$p < 0.001$

LJ media provided no added advantage over MGIT alone since the only case where LJ culture was positive had been detected by positive MGIT culture. A combination of smear examination for AFB, MGIT 960 and histopathological examination was able to detect all the positive cases.

Analyzing the diagnostic value of MGIT culture, it was found that in as many as five positive cases (16.7%) only MGIT culture was positive. In smear-negative cases (15/30), MGIT was positive in seven cases. Similarly in smear-positive cases, MGIT was positive in same number of cases. Thus, no difference in MGIT positivity was detected in smear-positive and smear-negative endometrial samplings.

A comparison of the symptomatology, demographics and clinical findings was undertaken between the GTB group of infertile women and the whole study population. The findings are summarized in Table 2.

Discussion

At present, the diagnosis of GTB is at best a collective one, employing multiple modalities like clinical picture, imaging, laparoscopy, bacteriological and serological tests.

Although culture remains the gold standard for laboratory diagnosis, it does not yield accurate and speedy results. The disease meanwhile continues to simmer inflicting more and more damage leading to complications, which can be avoided if the problem is diagnosed and treated in its early course.

In the current study, out of three hundred women screened for genital tuberculosis thirty women (10%) were diagnosed to have genital tuberculosis on the basis of diagnostic tests carried out on endometrial samples. In a review by Sharma, the incidence of GTB in infertility patients was reported to be between 3 and 16% [2]. A meta-analysis conducted by Chaman-Ara et al. [4] showed that the overall prevalence of GTB in infertile women is 24.2%. The differences in the findings of various studies stem from the differences in study populations since the prevalence of TB in general depends on several factors like the socioeconomic levels and the degree of congestion. Even in the meta-analysis mentioned above, the percentage prevalence of GTB in infertile women ranged from 2.9 to 75.6% in different studies.

In our study, the average duration of infertility in GTB patients was 87.2 months which was much longer than the mean infertile period of the whole cohort (58.8 months). This revelation underlines the chronic and persistent nature of genital tuberculosis and its contribution to unremitting infertility. Average age of patients with GTB was a little higher than

those in the whole study population. Around two thirds of entire GTB patients had age less than 30 years. In a review conducted by Neonakis et al. [7], it was found the disease inflicted younger age group in developing countries when compared to developed ones. Same findings of age trend in GTB patients have been quoted by Gupta et al. [8] and other studies [2] in the literature.

On evaluation of socioeconomic status with Kuppuswamy's socioeconomic status scale (SES) 2007 as a tool, we found out that around three fourth patients of GTB (73.3%) belonged to lower and lower-middle socioeconomic strata of the society. A study was conducted by Valsangkar et al. [9] in which 42.4% women belonged to lower socioeconomic scale. As a corollary, improvement in socioeconomic conditions of the society might help to decrease the prevalence of infertility secondary to genital tuberculosis. This is one among many reasons for the decreased infertility rates in developed nations.

Among the patients diagnosed with GTB, history of past affliction with tuberculosis was present in 20% of the cases diagnosed to have genital tuberculosis, pulmonary tuberculosis being the commonest one. Other studies [8] have also found similar figures in respect of past history of tuberculosis.

The menstrual profile found in GTB patients presented a stark contrast to the one found in total infertility cohort. In as many as 50% of the cases, the length of menstrual cycle was more than 35 days. More than half of GTB cases (56.7%) had scanty flow during their menstrual periods. The comparison of the menstrual duration and flow in GTB and total study population was statistically significant ($p < 0.001$). Also the incidence of hypomenorrhea among infertile women was found to be extremely high in GTB cases than in non-GTB ones. This finding underlines the pathogenesis of tubercular infertility by causing endometrial destruction.

Erythrocyte sedimentation rate was 23.1 ± 11.37 in positive cases and 15 ± 5.12 in negative cases, and the difference was highly significant ($p < 0.0001$). In other studies [9] also, ESR was found to be raised in as many as 90–98% cases of genital tuberculosis. Although a positive correlation between ESR and the presence of tuberculosis was found, the low specificity precludes its use as an indicator for start of antitubercular therapy in clinical usage.

Detection rates for endometrial biopsy AFB smear, MGIT culture, LJ culture and HPE were 50, 46.7, 3.3 and 33.3%, respectively. The detection rate of the conventional LJ medium was very poor as compared to other modalities. Sorlozano et al. [10] found out the sensitivity of MGIT 960 to be the best (86.5%) among a comparison of MGIT 960, MB/BacT ALERT 3D and LJ medium. They, however, found that LJ medium was best to detect non-tuberculous mycobacteria with a sensitivity of 76.2%. Rodrigues et al. [11] determined that MGIT culture was able to detect 97.9% of isolate containing *Mycobacterium tuberculosis,* while LJ medium was able to detect 57.4% of isolates. The mean growth time in culture for smear-positive cases was nine days for MGIT 960 and 38 days for LJ medium. For smear-negative cases, it was 16 days for MGIT versus 48 days for LJ medium. In our study, the mean time for growth was 12.79 ± 4.6 days. In as high as 77% cases positive by MGIT method, the growth on the tube was evident within 15 days of inoculation. LJ culture was positive in only one case and that too after a time period of 29 days.

The relative difference between the detection rates of MGIT and LJ medium encountered in our study as compared to those determined by various authors might be explained by the difference in clinical samples submitted for analysis. The studies described above have used all kinds of clinical samples for analysis. The samples ranged from respiratory to body fluids, biopsies, exudates from various sources. We conducted an extensive review of the literature and found that no study has taken endometrial biopsies from infertility patients as clinical samples for evaluation of detection rates of MGIT and LJ media. The low detection rates determined by the present study as compared to the literature reflect upon the relative difficulty to grow isolates from endometrial samples since the endometrium is shed off cyclically every month.

Since the pathological samples are subjected to a battery of tests in routine clinical practice, sensitivities of a combination of tests were assessed. It was found that a combination of smear and MGIT culture detected around 70% of the cases as positive. MGIT and LJ media provided no added advantage over MGIT alone since the only case where LJ culture was positive had been detected by a positive MGIT culture. A combination of smear and histopathological examination excluding all kinds of culture yielded a detection rate of 80%, whereas a combination

of smear examination for AFB, MGIT 960 culture and histopathological examination was able to detect all the positive cases. Thus, analyzing the diagnostic value of tests in the determination of positive cases in our study, it was found that LJ medium offered no help in diagnosis.

On the contrary, in as many as five cases out of 30 (16.7%), only MGIT culture was positive. Had MGIT 960 culture not been included in the diagnostic tests, these five cases would not have been detected by any routine method. Another important finding that is derived from the current study is no difference in MGIT positivity detected in smear-positive and smear-negative endometrial samplings. This is in contrast to other clinical samples where it has been determined that the culture failure was commoner in smear-negative samples than in smear-positive ones [12]. This also represents another probable difference between the results of endometrial samples and other materials analyzed. It can be easily concluded that addition of MGIT 960 culture media to the routine diagnostic battery in infertility patients for detection of genital tuberculosis will ensure accurate and an early diagnosis.

The absence of a gold standard test for diagnosis of tuberculosis as cause of infertility remains the biggest drawback of our study. The number of positive cases was taken to be those where any one test came out to be positive. We were not able to compute and evaluate differences between specificities of various methods since there were no false positives derived from our study. The option of using PCR as a gold standard was lucrative during the setting up of the trial protocol, but a deeper analysis revealed a large number of false positive cases by using that method. Although this may be perceived as a potential limitation, the literature reveals that no single test can be used as gold standard for detection of tuberculosis.

Authors' Contributions Study was conceptualized and designed by LKD and SS. The data were collected, analyzed and interpreted by NJ and SG. The drafting of the manuscript was done by NJ.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Assessing Receptivity of the Human Endometrium to Improve Outcomes of Fertility Treatment

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Abstract

Despite considerable improvements in assessment of embryo quality in infertility clinics, outcomes in terms of take-home baby rate have not improved substantially. Failure of the endometrium to achieve receptivity and the timing of the receptive period are now recognised as important issues in the success of IVF. Indeed, immunohistochemical and morphological studies show that the endometrium is highly disturbed in any cycle in which ovulation induction is performed,

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leading to recommendations that all embryos be frozen and replaced in a non-stimulation cycle. Assessment of any woman for endometrial receptivity either in cycles prior to treatment or testing for the potential for an embryo to implant in the cycle of transfer is urgently needed. However, careful consideration must be given to issues such as sampling, timing and rapid delivery of results as well as the best biomarkers, to enable in-clinic decision-making. Here these issues are considered, along with how endometrial receptivity testing might best be performed to optimise outcomes of infertility treatment.

Keywords: Endometrial receptivity, Biomarkers, IVF success

Endometrial Receptivity

An absolute need for synchrony of development between the maternal endometrium and a developing blastocyst was clearly demonstrated in embryo transfer experiments in animals some decades ago [1, 2]. In these studies using sheep and rabbits, pregnancy could not be established if endometrial-embryo asynchrony was >3 days. Subsequently, Psychoyos coined the phrase 'window of implantation' for the period of time when the endometrium is optimally prepared for implantation [3]: this is now generally referred to as the phase of endometrial receptivity.

In women, it was noted as early as the 1950s that in uteri removed at hysterectomy from young women, embryos were found attached to the uterine wall only if the endometrium was in the mid-secretory phase of the menstrual cycle [4]. This first established that the receptive phase in humans occurs between 6 and 10 days after ovulation [5] and was confirmed in a now classic large epidemiological study in which 189 human conceptions were confirmed by the detection of human chorionic gonadotrophin (hCG) in maternal urine only 6–12 days after ovulation [6]. This study also defined the limits of the optimal phase of endometrial receptivity, demonstrating that implantation was possible after day 10

post-ovulation but was suboptimal: up to 82 % of pregnancies which occurred 11 days after ovulation resulted in miscarriage.

Attainment of endometrial receptivity is driven by progesterone acting on an oestrogen-primed endometrium. Gene array studies [7] indicate that endometrial gene regulation alters with time of progesterone exposure with early and late response genes. Importantly, progesterone mediates both increased and decreased expression of specific genes in human endometrial cells with decreased genes being in the majority [7]. While a number of differential gene array analyses have compared times of the cycle (mostly mid-proliferative versus mid-secretory), very few changes in specific genes have been common to all studies. These observations indicate the difficulty of comparing gene array data between laboratories using (a) different arrays, (b) different patient cohorts, (c) different sampling techniques and (d) different methods of data analysis. A complication in comparing such studies likely lies in the staging of endometrium used for analysis; the classic 'Noyes' criteria of dating the endometrium are now well accepted as inadequate [8], and a better dating technique based on molecular markers is needed. This may be partially fulfilled by the use of the newly developed endometrial receptivity array (ERA) (see below) although other measures of endometrial dating, and particularly determination of endometrial receptivity, are needed.

Key features of receptive endometrium cannot be identified by morphology alone, except when the endometrium is clearly inadequately developed for the expected phase of the menstrual cycle (such as lack of secretory features of glands and spiral arteriole development). Furthermore, analysis of entire endometrial biopsies by gene array or multiplex protein assays is confounded by the considerable variability between cellular compositions of any sample. These differ considerably in the proportions of epithelial cells to stromal fibroblasts, cells of the vasculature and leukocytes. Laser capture and gene array analysis of individual cellular compartments [9] has clearly demonstrated this issue. Additionally, as demonstrated by histological assessment of stimulated (IVF) endometria, different cellular compartments (e.g., epithelium and stroma) may be 'out of phase' with each other adding to the complication of such whole tissue analyses [9].

Implantation-Specific Microenvironments

The peri-implantation microenvironment within the uterine cavity is represented in Fig. 1. The embryo first enters the uterine cavity as an unhatched blastocyst and undergoes its final development through hatching to attachment to the uterine luminal epithelium within the environment of uterine fluid. This fluid contains a plethora of proteins, lipids, ions, amino acids, nutrients and microvesicles/exosomes. The soluble factors can be derived from a number of sources including selective transudation from the blood, carriage from the Fallopian tubes and likely also the peritoneal cavity and, importantly, secretions from the endometrial glands. Thus, it is likely that factors released or accumulated during the mid-secretory phase, when implantation takes place, and also endometrial secretions from the early secretory phase will be important for the final stages of blastocyst development.

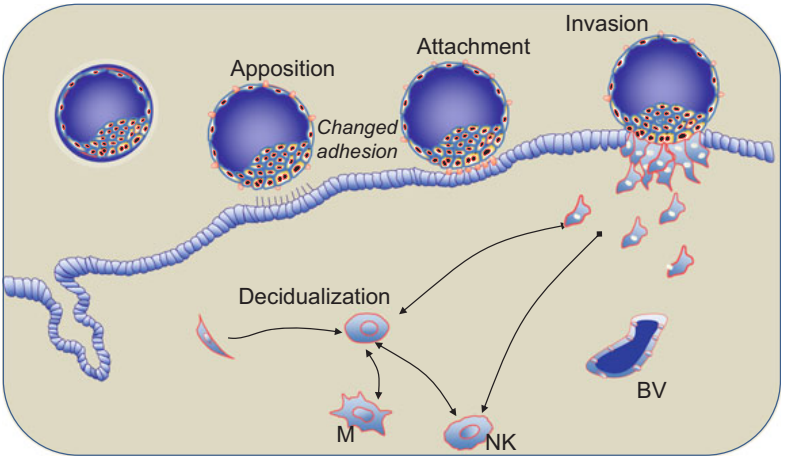

Fig. 1: Human embryo implantation. The embryo enters the uterine cavity as an unhatched blastocyst. After hatching it becomes apposed to the endometrial epithelium: the cell surfaces of both the trophectoderm and the endometrial luminal epithelium must change their adhesive properties to enable attachment and subsequent invasion of trophoblast cells into the decidualising stromal compartment, eventually to reach the blood vessels (BV). Macrophages (M) and uterine NK (uNK) cell numbers increase as decidualisation proceeds (Reproduced with permission from [10], CSIRO Publishing, http://www.publish.csiro.au/nid/44/paper/RD09145.htm)

The next important milieu for implantation is that of the developing decidua, which the blastocyst encounters once it has traversed the luminal epithelium. In women, differentiation of the stromal fibroblasts to decidual cells also occurs within each cycle, regardless of whether or not conception has occurred. Decidual development is dependent on progesterone acting through its receptors on the stromal cells: however, once initiated, a plethora of cytokines and other factors are released which, in turn, act on adjacent stromal cells in a cascade of intracellular events leading to a much wider decidualisation. These factors which include interleukin 11, activin A and relaxin act through separate pathways including but not restricted to cAMP [10, 11]. Decidualised stromal cells also secrete chemokines which act as chemoattractants for the macrophages and uterine natural killer (uNK) cells that are essential components of the decidua of pregnancy. The decidual milieu is overall favourable for trophoblast expansion and migration [12, 13]. Recently, early decidua has been identified as a 'sensor' of human embryo quality [14, 15]. It was found that the decidual cells could discriminate between 'good' and 'bad' quality embryos, except in the case of cells derived from women with recurrent pregnancy loss in whom the discriminatory capacity was absent [16]. Additionally, arrested 'bad-quality' embryos inhibited secretion of pro-implantation factors by decidualising stromal cells from normal women [15]. Genome-wide expression profiling of decidual responses to soluble factors released from competent embryos showed that only 15 genes were responsive, whereas some 449 genes were dysregulated by poor quality embryos [12]. Collectively, these data suggest the decidua is responsible for determining whether or not a pregnancy should proceed following successful pre and early implantation events.

Peri-implantation Embryo-Maternal Signalling

Blastocyst-Derived Human Chorionic Gonadotrophin Enhances Receptivity

Human chorionic gonadotrophin (hCG) is the most well-known product of the developing embryo. Its gene expression is detectable by the time the blastocyst is formed [17] and hCG levels in serum underpin early pregnancy detection kits. hCG is formed of an alpha- and a beta-subunit

and has considerable similarity to luteinising hormone (LH) with which it shares a common subunit. It binds to both the LH/hCG receptor and a mannose receptor [18] through which its signals are transduced: LH/hCG receptor mRNA [19] and protein [20] are present in the endometrium; immunohistochemistry shows them located to mid-secretory phase luminal epithelium. hCG exists naturally in minimally glycosylated and hyperglycosylated forms, a sulphated form and as a free beta-subunit which potentially have different bioactivities. The highly glycosylated form is produced in trophoblast-derived cells of the placenta [13]. Since acidic forms of hCG are secreted by early blastocysts, it is likely that these are likewise highly glycosylated [21]. An effect of hCG on the endometrium was clearly demonstrated in an elegant in vivo study in which hCG was infused into the uterine cavity of women in the mid-secretory phase and found to induce production of pro-implantation factors: leukaemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) [22], observations reinforced by studies in non-human primates [23]. In vitro, hCG stimulates secretion of selected cytokines by endometrial epithelial cells, confirming LIF and VEGF as hCG targets but also identifying IL-11, FGF2, GM-CSF and CXCL10 and prokineticin 1 as novel hCG-induced factors [24, 25]. Since all of these have known pro-implantation functions, it is clear that during a conception cycle, blastocyst-derived hCG acts to enhance endometrial receptivity. Given the progress in proteomic technologies, it is likely that other secreted human blastocyst proteins of lower abundance will soon be identified and their functions elucidated.

Endometrial-Secreted Products Support Pre-implantation Blastocyst Development

Endometrial products, particularly proteins, secreted into the uterine lumen from the early to mid-secretory phases of the cycle can enhance features of blastocyst development in vitro and most likely promote both survival and development of blastocysts. While some data has suggested that adding individual growth factors and cytokines (including HB-EGF, IGF-1, LIF and GM-CSF) to blastocyst culture prior to embryo implantation can improve blastocyst development in vitro [26, 27], the only one of these followed through to clinical trials is GM-CSF. This had a modest positive effect on ongoing pregnancy rate and live birth rate [28], but only

when the conventional level of HSA in the embryo culture medium was reduced. Given that uterine fluid contains multiple factors, it is important that their impact on blastocyst development is determined in combination. Exposure of human or mouse embryos to human uterine lavage in vitro has effects that differ depending upon the time of the cycle at which the lavage is harvested. Interestingly, mid-proliferative phase lavage is detrimental to embryo development: mouse blastocyst outgrowth on fibronectin is strongly and significantly inhibited. In contrast, pooled uterine lavage from the mid-secretory phase significantly enhances blastocyst outgrowth. In one study, this could be replicated by recombinant (r)VEGFA [29]. Furthermore, when mouse embryos were pretreated with either VEGF121, VEGF165 or rVEGFA, the time to cavitation and blastocyst number were also increased, and following transfer of these blastocysts to recipient mothers, both implantation rate and foetal limb development were enhanced [30]. Therefore, VEGF could be an important additive for embryo culture prior to IVF. However, in a study in mice, there was only a trend for VEFG165-treated and transferred embryos to improve viable pregnancies [30].

Lack of Synchrony in Controlled Ovarian Stimulation Cycles

Why Are IVF Success Rates So Low?

Nearly four decades after the birth of Louise Brown, the first baby to result from application of what is now commonly known as IVF treatment, the per-cycle success rates have not substantially increased worldwide, remaining at <30 % [31, 32]. This is in spite of technical advancement in embryo culture, selection and transfer. Indeed, implantation failure was identified as the most common outcome following embryo transfer [33]. The vital but often overlooked factor is the 'soil for the seed', the endometrium, which becomes receptive for embryo implantation only in the mid-secretory phase of the menstrual cycle in synchrony with blastocyst development. Inability to establish receptivity leads to infertility and is a major cause for implantation failure in IVF cycles. In many IVF cycles, the embryo is transferred without establishing whether the endometrium

is likely to be receptive (Fig. 2). This is despite the woman undergoing considerable hormonal treatment to induce oocyte development and ovulation, which may impact on endometrial development, and a wealth of recent knowledge regarding molecular changes essential for receptivity.

The Endometrium in Treatment Cycles

IVF cycles have severely abnormal hormone concentrations. This is due to the administration of gonadotrophins, as well as gonadotrophin-releasing hormone agonists and antagonists administered by differing protocols, resulting in very high oestrogen and progesterone concentrations, and compounded by the hCG administered for final oocyte maturation prior to oocyte harvest. Indeed, the precocious progesterone elevation often seen even as early as the day of hCG administration (0.08 ng/mL) is associated with a reduced probability of clinical pregnancy in fresh embryo transfer cycles, but not when the embryo is frozen and transferred in a normal cycle or in a donor-recipient cycle [34]. In addition, differential genomic analysis of endometrium from women with such elevated progesterone con-

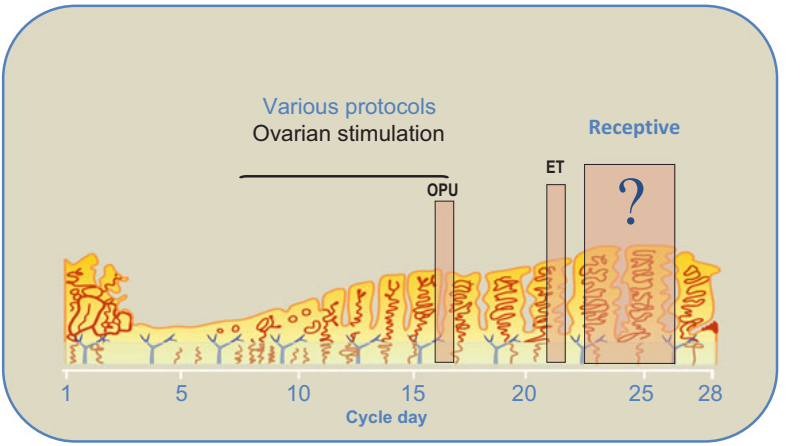

Fig. 2: Sequence of events in an IVF cycle. *OPU* day of ovum pickup, *E* day of embryo transfer.

centrations compared to women with normal progesterone concentrations revealed alterations in both miRNAs and mRNAs resulting from high progesterone exposure [35, 36]. However, while the endometrial receptivity profile (measured by DNA microarrays) in patients with premature progesterone elevation on the day of hCG administration showed an altered gene expression shift between the pre-receptive and receptive phases, it had no significant effect on a limited number of specific markers of endometrial receptivity [37] supporting earlier observations [38, 39]. These data, however, must be interpreted with caution as the gene arrays and subsequent validation studies were performed using a very small number of samples (n=4 different women per group), and it is therefore important that such observations be tested further before applicability to all progesterone-elevated cycles can be determined. It is also important to remember that endometrial progesterone receptor (PR) expression changes across the cycle in response to alterations in oestrogen and progesterone concentrations. Specifically, epithelial PRA and PRB are lost during the secretory phase of the menstrual cycle with these receptors maintained in the stromal compartment. Epithelial responses to progesterone in the secretory phase are therefore mediated by local stromal factors via an indirect rather than a direct mechanism. Haouzi and colleagues [37] propose that premature progesterone elevation may lead to a precocious downregulation of epithelial progesterone receptor, prematurely inhibiting the endometrial response to this hormone. Such cell compartment-specific responses may be masked in gene array analysis which examines whole tissue, and 'compartment-specific' analysis may be more appropriate for future studies [9]. Nevertheless, in accord with the historical animal studies, there is a complete failure to achieve implantation during IVF/ICSI cycles with stimulation by either gonadotrophin-releasing hormone (GnRH) agonist or antagonist, when histological dating (according to Noyes' criteria) [40] shows dys-synchrony of >3 days [41-44]. Further, microarray studies have indicated that ovarian stimulation may alter the receptive phase endometrium such that it is detrimental to implantation [45]. Probably the strongest proof to date comes from a recent comprehensive immunohistochemical and histological study of tissues taken on LH/hCG+2 (at the time of oocyte pickup) from normal women, and women stimulated

with either agonist or antagonist protocols and hCG, emphasising that the disturbance of the endometrium is much more than just developmental advancement [46]. The parameters investigated immunohistochemically included the progesterone receptor, leukocytes (CD45) and their subsets (uNK cells, CD56; macrophages, CD68; activated neutrophils, elastase), decidualised stromal cells (prolactin) and vasculature (CD34), in addition to morphological features (glandular development, oedema, blood vessel size). All parameters were scored and normalised against those for normal cycling women on LH +2. In the agonist stimulation group, outcomes of embryo transfer (pregnant or not pregnant) enabled stratification of data according to pregnancy outcome. Key data is summarised in Fig. 3 and clearly indicates that ovarian stimulation severely influences endometrial

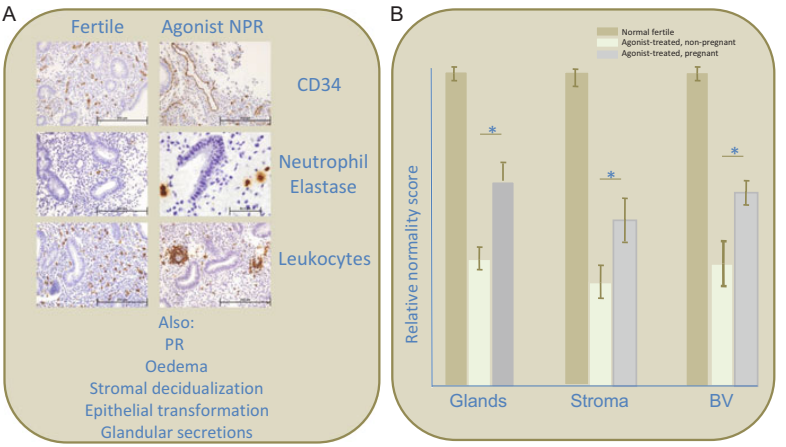

Fig. 3: Histological and immunohistochemical analysis was performed on endometrial biopsies taken 2 days after ovulation induction (OI+2) and on biopsies from normal cycling women on LH +2. Women in the OI group were stimulated by an agonist protocol and retrospectively separated into groups of women who did become pregnant or did not become pregnant (Pr) following fresh embryo transfer. Nine parameters as listed were examined. (**a**) Immunohistochemistry identifying blood vessels (CD34) and neutrophil activation (extracellular elastase). (**b**) Combined data from analysis of all parameters showing % of normal features (normal tissue expressed as 100 %) in glands, stroma and blood vessels. Note the high level of disturbance (<50 % normal) in all stimulated cycles and that this is significantly less in the women who became pregnant compared with those who did not become pregnant. *ER* oestrogen receptor, *PR* progesterone receptor (Derived from data in [46]).

development. Of prime importance is that the cohort of women who did become pregnant following embryo transfer showed significantly less endometrial disturbance than those who did not become pregnant. The endometrium of women who failed to become pregnant also contained highly activated neutrophils (Fig. 3a), a state which is normally only seen at menstruation where they contribute strongly to tissue breakdown and repair. No doubt these contribute to the 'menstrual-like features' in some of the tissues. In addition, this data, showing extreme disturbance to the endometrium as early as hCG+2 in a stimulation cycle, indicates that it may be possible to predict as early as the day of ovum pickup whether, based on the likelihood of not developing a receptive endometrium, the embryo should be frozen or whether it has a strong probability of implanting following fresh embryo transfer.

A detrimental effect on endometrial receptivity of the hCG administered for final oocyte maturation in ovulation induction as part of an IVF cycle has also been demonstrated. As detailed above, blastocyst hCG enhances endometrial receptivity. However, in women undergoing IVF cycles, the hCG receptor, by which the effects of hCG are transduced, is dramatically downregulated in the endometrial epithelium. Furthermore, functional studies in vitro show that the response to acute administration of hCG (mimicking blastocyst-secreted hCG), in terms of both endometrial adhesiveness and tight junction integrity, is lost if the acute hCG is preceded by a low chronic treatment of hCG (mimicking hCG administration during an IVF cycle) [20]. Thus, in an IVF stimulation cycle, the natural responsiveness of the endometrium to blastocyst hCG is lost, decreasing the likelihood of successful implantation.

These data, in concert with a number of clinical studies [47, 48], strongly support frozen embryo transfer rather than the fresh embryo transfer most commonly used. This would overcome the detrimental effects of ovarian stimulation and induced ovulation.

Testing for Receptivity

Given that endometrial receptivity is essential to establish pregnancy, testing for receptivity or, ideally, for prediction of receptivity later in the same cycle may provide a major boost for IVF success rates per cycle.

However, consideration must be given to which form of testing would be most useful.

What to Measure

Many potential individual biomarkers for receptivity have been identified within research programmes studying mechanisms of implantation (Table 1). In some studies the change between proliferative and mid-secretory phase of fertile women has been examined essentially to identify critical components of receptivity. Other studies have directly compared mid-secretory (based on Noyes criteria) samples from fertile and infertile women with idiopathic infertility. In most cases, comparison of their expression in infertile versus fertile women has not had the power for analysis of specificity and sensitivity. The newer technologies available for biomarker discovery utilise 'omics' technologies [49] that include genomics, transcriptomics, epigenomics, lipidomics, proteomics and metabolomics. The advantage of these methods is that they can differentially identify a vast number of molecular changes in matched samples: the disadvantage is they have generated large lists of 'potential' markers, for which validation has been limited or non-existent. Genomics, transcriptomics and epigenomics require tissue samples,

Table 1. Examples of individual proteins validated as differentiating between receptive and non-receptive endometrium.

Protein	Sample assayed	References
Proprotein convertase 5/6	Uterine fluid	[83]
Integrin β3	Tissue biopsy	[84]
Vascular endothelial growth factor A	Uterine fluid	[79]
Stathmin 1	Tissue biopsy	[85]
Annexin A2	Tissue biopsy	[85]
α-Dystroglycan-N fragment	Uterine fluid	[74]
Progesterone receptor membrane component 1	Tissue biopsy	[86]
Glycodelin A	Uterine fluid	[87, 88]
α2-Macroglobulin	Uterine fluid	[79]
Antithrombin III	Uterine fluid	[79]

whereas proteomics, lipidomics and metabolomics can be applied also to body fluids and are thus less invasive. Appropriate handling of samples prior to analysis is essential: RNA, proteins and many lipids are readily degraded. These limitations have been described in detail [49]. These powerful techniques themselves continue to evolve and gain increased power and are revolutionising the search for biomarkers.

Simultaneous measurement of more than one marker is becoming the standard for biomarker assessment in most fields, including cancers. Given the molecular complexity of disorders, it is unlikely that any one specific marker will discriminate or diagnose pathology. Particularly with regard to endometrial receptivity, defects probably represent a range of molecular changes, making it imperative that a large panel of biomarkers is used. Such multiple analysis or 'multiplexing' approach is well-served by the 'omics' techniques. Given that many individual biomarkers of receptivity have been proposed, there is now a critical need to test these in combination to determine their collective power to differentiate between receptive and non-receptive endometrium.

Type of Sample

A number of options are available for sampling. Tissue sampling, usually by pipelle biopsy, is the most invasive [50], particularly for nulliparous women, but has been used in most tests available to date. Uterine aspiration and lavage are much less invasive. Given the very small volume of uterine fluid (<10 μl is retrieved by aspiration) and that aspirates usually contain blood indicating damage to the tissue, our laboratory and others favour a uterine lavage with 2–3 mL saline, gently infused into the uterine cavity and retrieved so that it washes over the entire endometrial surface. Importantly, aspiration and lavage are not interchangeable for the purpose of analyte analysis [51], presumably because soluble analytes are released from the endometrial glycocalyx during lavage. Aspiration may be the better technique if sampling is to be performed in the same cycle as embryo transfer, as it does not appear to influence implantation rates [52]. However, as indicated above, collection of an aspirate does carry a risk of tissue damage within the uterus and may therefore compromise the endometrium at the time of implantation. Uterine lavage offers a greater range of proteins for assessment of multiple factors,

thereby increasing the sensitivity and specificity of the predictive test. Clearly a minimally invasive test based on blood, urine or saliva would be optimal, as testing need not be limited to days when the patient is in the clinic, and indeed, consecutive days of testing to identify the optimal transfer time then becomes possible. However, this presents a challenge since most of the factors identified as potential biomarkers are produced locally by the endometrium in very low concentrations and not secreted directly into any of these fluids. Quality of the sample is of utmost importance: both collection and storage require adherence to strict standard operating procedures.

Variability in Timing of Receptivity Within and Between Women

The highly dynamic nature of the endometrium makes obtaining clinical samples extremely difficult. Uniquely, the cellular and molecular composition of the endometrium alters on a daily basis making histological dating of the endometrium by Noyes' classification highly variable, with considerable observer error [8]. Molecular markers are required to provide objective dating and prediction of receptivity. The recently described endometrial array, the ERA [53], suggests this is now possible, though this is not yet available for routine pathology applications [54]. Other confounding factors not yet known are the variation of the timing of onset of receptivity within one woman between cycles, between women, and/or even whether a woman attains receptivity in every cycle – repeat samples from the same women would provide valuable information but would place a great burden on study participants making collection difficult.

Time of Sampling

The first imperative is that sampling for the test does not require additional visits to the clinical specialist. In most instances, attendance is for: (1) early assessment of efficacy of gonadotrophin stimulation, (2) ovum pickup and (3) embryo transfer. Testing could be performed on any of these occasions.

If sampling is performed at early workup, an appointment could be arranged to coincide with the mid-secretory phase of the preceding

natural cycle. At this time, testing could establish whether or not endometrial receptivity is achieved during the woman's normal menstrual cycle. If normal, the woman could proceed to IVF. However, it must be taken into account that receptivity may be significantly altered in the stimulated versus the natural cycle [46]. If receptivity is not normally achieved, luteal phase supplementation may be favoured, and the test would enable assessment of its effectiveness.

A test performed following sampling at ovum pickup, the pre-receptive phase, would need to be able to predict development of receptivity and therefore likely pregnancy outcome, if fresh embryo transfer followed. It would enable decision-making as to whether to transfer in that cycle (if that was the woman's preference) or to freeze all embryos and transfer in a 'natural' cycle in which receptivity could be predetermined. The time between sampling at pickup and embryo transfer (usually at 5 days) should enable assay and reporting of the data – such assessments therefore need to be relatively rapid and robust, not requiring complex or lengthy methods and be suitable for use in any IVF/pathology clinic worldwide. However, should current data supporting 'freeze-all' strategies [48] be fully validated in internationally organised trials, this may become the most common protocol in IVF clinics.

It is much less likely that testing at the time of transfer would be useful, unless the test could be performed immediately on site in the clinic. In this situation, a very rapid test yielding a 'yes' or 'no' answer only is required as the time for decision-making and consultation with the patient is limited. Sampling from the uterus, particularly tissue biopsies, would not be appropriate at this time due to possible interference with the implantation environment.

Options for an endometrial receptivity test are summarised in Fig. 4.

Transcriptomic Studies

As summarised above, early transcriptomic studies on human endometrium failed to achieve consensus on a molecular signature of receptivity. The studies fall into different categories: (1) those that focussed entirely on normal tissue from women of known fertility, (2) those comparing natural and stimulated cycles [55, 56], (3) studies comparing mid-secretory tissue

Assessing receptivity. What is most useful?

What to sample?
- Blood, tissue, uterine fluid, urine

What to measure?
- mRNA, protein, lipids
- Single or multiple markers

When to sample?
- Mid-secretory in 'normal' cycle
- LH/hCG+2 in IVF cycle
- Day of embryo transfer

Fig. 4: Options for sampling for an endometrial receptivity test.

from fertile versus infertile women [57] and (4) tissue from women with recurrent implantation failure [58, 59]. In terms of biomarker discovery, those studies comparing non-receptive (proliferative or early secretory phase) with receptive (mid-secretory phase) endometrium [60-68] are the most relevant. The differentially expressed genes are both up and downregulated in the receptive phase. Many relate to known processes of cellular function at this time: cell adhesion, response to external stimuli, signalling, immune response, metabolism and cell-cell communication. Appropriately, there is decreased transcription of genes driving cellular proliferation and development, given that these processes are dominant in the proliferative phase. Details of these findings have been tabulated elsewhere [55, 56].

An important reason for the diversity of results in the studies detailed above is that the endometrial tissue biopsies used for analysis contain a diversity of cell types. In confirmation of this, microarray analysis following laser capture of cellular compartments showed distinct mRNA signatures for glands and stroma, each dependent on the day of the cycle [9]. Unfortunately, this approach is exceptionally time-consuming and not appropriate for routine use as a diagnostic tool for predicting receptivity. It does, however, highlight the redundancy of 'whole tissue' analysis.

An endometrial receptivity array (ERA) [69] is currently undergoing multicentre trials. For this test 238 genes that are differentially regulated within the endometrial cycle are customised on a single array. Of these,

134 genes represent a specific transcriptomic signature of the receptive phase. This test is of high specificity (0.8857) and sensitivity (0.9976) for endometrial dating and is being applied in clinical settings to establish accurately the timing of receptivity in individual women, enabling replacement of a blastocyst at an optimal time. The outcomes of the multicentre trial, particularly in terms of enhancing percentages of live births per cycle, are awaited. This test cannot be performed to predict endometrial receptivity in the same cycle as sampling.

Proteomic Studies

New developments in proteomic technologies provide the ability to detect relatively low-abundance proteins and have enabled considerable advances in the discovery of protein biomarkers. While early studies using gel-based proteomics provided changes in proteins of high abundance, predominantly structural proteins in tissue samples, the newer technologies are enabling identification of much lower-abundance proteins (particularly in biological fluids) that are part of important regulatory pathways. Proteins are the functional mediators of physiological changes, and there are a number of regulated steps between transcription and production of functional protein [55, 70, 71]. These include restriction of translation by miRNAs and post-translation modification of proteins by enzymatic processing, leading to activation or inactivation, glycosylation or phosphorylation. For example, the actions of proprotein convertase 5/6 are essential for receptivity and implantation [72, 73], at least in part by cleavage of a range of proteins including dystroglycan [74], caldesmon [75] and EPB50 [73]. The correlation between abundance of a transcript and its functional protein in the endometrium is often low, and thus examination of transcription by gene array can be misleading in terms of understanding function, although this is not necessarily relevant in provision of biomarkers. Application of proteomic techniques to endometrial biopsies and validation of proteins with relevance to receptivity has been undertaken by a number of research groups [76]: most of these proteins have not been further examined for their potential to assist in a clinical infertility setting.

Uterine fluid, the protein-rich histotroph within the uterine cavity, provides the microenvironment for implantation, including secretions

from uterine glands and endometrial luminal epithelium in addition to factors from the Fallopian tubes and blood transudates. Glandular secretions are essential for implantation in both sheep and mice [77, 78]: this has been functionally demonstrated in animals in which uterine gland development was inhibited during early postnatal life. Thus it is predicted that uterine fluid in women will contain important secreted proteins from uterine glands which may be actively involved in the blastocyst implantation process and that could be measured to assess uterine receptivity. This approach has proven very beneficial: proteomic and multiplex analyses of a cohort of factors have identified a number of proteins with considerable potential as markers of receptivity [55, 79]. It is implausible that all women currently defined as having idiopathic infertility have an endometrial-based infertility and, indeed, that those who do will have a single molecular change. Thus it is unlikely that any single biomarker will provide a definitive diagnosis of receptivity or infertility. Future studies will serve to validate and combine these markers using multivariate analysis and will incorporate physical measures (e.g., age, BMI, endometrial thickness) to provide a diagnostic fit of each woman to a spectrum of endometrial defects and produce predictive indices for IVF outcome. Such cohorts of markers will provide utility to monitor response to treatment regimens and provide a more personalised understanding of endometrial receptivity and therapy for infertile women.

The power of multiplex analysis of proteins to distinguish between fertile and infertile women during the mid-secretory phase is demonstrated in Fig. 5. Uterine lavage was performed during natural cycles in women <43 years of age who were of proven fertility (n =17) or infertility (<3 failed IVF cycles; n = 19). Male factor and tubal and ovarian pathologies were exclusion factors and dating was by Noyes criteria. Ten analytes secreted by endometrial epithelium and previously reported in the literature as potential biomarkers of receptivity were measured. Of the individual analytes, only four significantly discriminated between fertile and infertile women. Use of multivariate analysis combining eight of the markers distinguished between fertile and infertile women, with a sensitivity of 79 %, specificity of 82 % and significance of $P < 0.0001$. Of course, the likelihood of a 100 % specificity and sensitivity test is low given the acknowledged potential multiforms of endometrial failure and

that not all these women will have idiopathic infertility due to endometrial disturbance but rather some other undiagnosed condition. Indeed, as shown in Fig. 5, some primary infertile (PIF) women fall below the cut-off, thus apparently having normal receptive endometrium (based on this set of markers), while a small number of fertile women are predicted as non-receptive being positioned above the threshold. These women's endometrium may be incorrectly pathology dated, given the known inaccuracy of Noyes criteria. This could be determined more accurately with the ERA test discussed above. Further, it is still unproven whether previously fertile women (used as fertile controls) remain fertile in every cycle. Indeed, given the acknowledged relationships of environment, BMI, age and endometriosis with infertility, it is highly plausible that women who have been fertile may subsequently develop infertility. However, this is impossible to determine.

Lipidomics

In mice, double inactivation of cyclooxygenase (COX)-1 and COX-2 completely prevents pregnancy by blocking prostaglandin production and inhibiting implantation [80], providing a rationale for examination

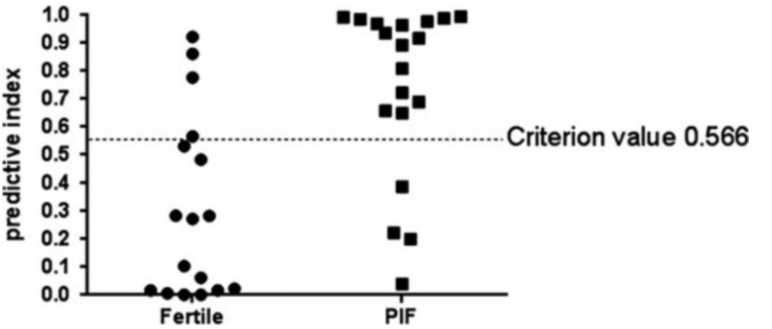

Fig. 5: The predictive index of receptivity in the mid-secretory phase of a natural cycle for a cohort of eight protein biomarkers in uterine fluid is shown for women of proven fertility (fertile) and those with primary infertility (PIF) who had >3 unsuccessful IVF cycles. The criterion value is 0.566.

of prostaglandins in the uterine cavity of fertile women. PGE_2 and $PGF_2\alpha$ assays have been performed on uterine fluid samples ($n = 39$) at different stages of natural menstrual cycles. In an initial experiment ($n - 13$), PGE_2 and $PGF_2\alpha$ were significantly increased between days 19 and 21 of the cycle – other lipids examined did not change. These data were replicated in a second sample set ($n = 26$). In the combined data set, PGE_2 showed a twofold increase and $PGF_2\alpha$, a 20-fold increase at their peak which coincided with receptivity [81]. A further larger study of endometrial fluid from 173 women [82] added to the evidence that lipid profiling may hold potential. Whether or not these lipids can be used as biomarkers for receptive endometrium remains to be established. Given that prostaglandins are highly unstable, careful sample preparation and storage guidelines are critical if their measurement is to prove clinically and biologically meaningful. Furthermore, the need for laboratory techniques/instrumentation beyond the norm (e.g., gas chromatography and mass spectrometry) may see lipidomics restricted at least in the short term, to a handful of specialist centres.

Conclusions

While many potential biomarkers for endometrial receptivity have been identified, international collaboration is now needed to adequately validate an optimal cohort of predictive biomarkers and provide a robust test. Standard operating procedures for sampling and storage need to be simplified for use in clinics worldwide. The impact of receptivity testing on clinical outcomes, including assessment of infertility and the impact of a predictive test on decision-making and thus the outcomes of IVF cycles, also remains to be established. Simple, rapid within-clinic testing will be an imperative for a major impact on pregnancy and live birth outcomes. Given the increasing numbers of couples presenting with infertility, such predictive testing to optimise pregnancy success is urgently required. This will reduce costs, both economic and emotional, and provide the best outcomes in terms of take-home healthy babies.

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Development of IVM Treatment: Combination of Natural Cycle IVF with IVM

Jin-Ho Lim, Ri-Cheng Chian

Introduction

Women with polycystic ovary syndrome (PCOS) have abnormal endocrine parameters, anovulation, numerous antral follicles within their ovaries, and frequently infertility. IVM treatment can be an option for infertile women with PCOS, but it seems quite difficult to offer IVM treatment for women with normal ovaries who develop a dominant follicle and ovulate its oocyte during menstrual cycle. In women, follicular development is characterized by the selection of a dominant follicle destined to ovulate from a cohort of growing follicles, and initiation of atresia in those remaining in the cohort. Interestingly, the traditional theory of a single cohort of antral follicles growing only during the follicular phase of the menstrual cycles was challenged that two or three waves of ovarian follicular development in the ovaries during menstrual cycle by daily transvaginal ultrasonography [1, 2]. Therefore, it seems clear that there are several follicles growing in each menstrual cycle as evidenced by baseline ultrasound scan for antral follicle counts (AFC) on day 3 of menstrual cycle, especially if women are healthy and under 35 years of age.

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After a dominant follicle has been selected in a spontaneous ovulatory cycle, secondary follicles are generally thought to be atretic with only the dominant follicle possessing the appropriate hormonal milieu necessary to achieve oocyte maturity and ovulation. It is a common belief that the development of a dominant follicle suppresses subordinate follicles and new growth of small follicles occurs only after the dominant follicle has ceased growing [3, 4]. Therefore, IVM treatment may be limited to those women undergoing infertility treatment who have polycystic ovarian syndrome with anovulatory cycles [5, 6].

Fertilization, embryo development, and live births have been achieved following the transfer of embryos produced from oocytes retrieved from secondary follicles in treatment cycles when the development of a dominant follicle in the ovaries [7] was observed. This indicates that oocyte viability is maintained in these secondary follicles in spite of the possibility that the dominant follicle may induce atresia and regression in them. Animal model study indicated that the developmental competence of oocytes from small antral follicles is not adversely affected by the presence of a dominant follicle [8] and phases of folliculogenesis [9]. These results indicate that the maturational and developmental competence of immature oocytes is not affected by the presence of the dominant follicle and the phase of folliculogenesis. This notion is not hard to understand from animal industry that oocyte collection is performed several times during one estrus cycle and immature oocytes are aspirated from small follicles from slaughterhouse materials regardless of follicular phases in cattle ovaries. Therefore, this is a very important point to understand how to further develop IVM treatment for infertile women with normal ovaries.

It has been reported that the maturational and developmental competencies of immature oocytes retrieved from women with PCOS are improved by human chorionic gonadotropin (HCG) priming before immature oocyte retrieval [10–12]. As an initial protocol, women with PCOS were excluded when development of a dominant follicle in ovaries occurred by day 8 of the cycle. Women with polycystic ovaries that have taken ultrasound scan but who have a normal menstrual cycle, or those with normal ovaries, usually produce a dominant follicle at the middle of cycle. If following the initial protocol of IVM treatment, most time, IVM treatment will be cancelled by women producing the dominant follicle in the ovaries. Modified IVM treatment has been applied to those

women, and natural cycle IVF combined with IVM treatment was proposed accordingly [13].

IVM Treatment for Poor Responders or Over Responders in the Stimulated Cycles

In conventional IVF treatment cycles, some women appear to respond to hormonal stimulation but have a low estrogen level or few or slow growing follicles, in which they have been considered as poor responders. This group of women requires a prolonged stimulation time and higher doses of gonadotropin. Following gonadotropin stimulation, the number of follicles may be normal, but the size of follicles may be smaller than in the usual treatment cycles [14]. In such case, IVM treatment may be an option for those poor responders instead of longer gonadotropin stimulation or treatment cancellation. It has been reported that pregnancies established following the immature oocyte retrieval and IVM with [15] and without [16] HCG administration before oocyte retrieval.

In addition, some women are extremely sensitive to the stimulation with exogenous gonadotropin and are at increased risk of developing OHSS that, sometimes, is a potentially life-threatening complication [17]. Several preventive strategies have been proposed to reduce the incidence and severity of OHSS, and one of them is to cancel the treatment cycle [18–22]. Normally, a woman is considered an over responder when there are more than 20 follicles with a mean diameter >10 mm in both ovaries and extremely high level of estradiol in serum following gonadotropin stimulation for several days. It has been proposed that IVM treatment may be offered as an alternative for these over responders when the leading follicle reached 12–14 mm in diameter, 10,000 IU of HCG was administered, and then oocyte retrieval was performed 36 h later before the treatment cycle cancellation [23].

Development of IVM Treatment

To further develop IVM treatment for women with normal ovaries, the natural cycle IVF combined with IVM was proposed [13]. If the mature oocyte from the dominant follicle together with immature oocytes from the small follicles were collected as well, the chances of a pregnancy are

greatly increased when we manage to mature these immature oocytes and produce several viable embryos. Interestingly, mature oocytes can be retrieved from the leading follicles and at the same time immature oocytes also be collected from the small follicles 36 h after HCG injection when the injection was performed at the size of leading follicle reached to 12–14 in diameter, suggesting that some small follicles can respond to an LH surge to trigger oocyte maturation, which might become fully mature in vivo [24]. In fact, in our previous study [10] of women with PCOS undergoing IVM treatment, when immature oocyte retrieval was performed 36 h after HCG administration with small follicles, it has been reported that approximately 46% of oocytes had initiated the process of oocyte maturation and undergone germinal vesicle breakdown (GVBD).

It may be an important point to mention that when HCG will be administered at the size of leading or dominant follicle reached for this group of women who are undergoing natural cycle IVF combined with IVM in order to prevent ovulation from the dominant follicle due to a spontaneous LH surge when the patients treated with natural cycle IVF combined with IVM. Our experience indicates that 10,000 IU HCG can be administered 36 h before oocyte retrieval when the size of the dominant follicle reached to 10–14 mm in diameter, and most oocytes collected from the dominant follicles were at M-II stage [25].

A selective group of ovulatory women can benefit from natural cycle IVF combined with IVM for treatment with acceptable pregnancy rates [26]. Also it is important to evaluate the efficacy of natural cycle IVF combined with IVM treatment as a clinical treatment for infertile women. We found that natural cycle IVF combined with IVM treatment might be offered to more than 50% of the total infertile women who were seeking infertility treatment, and with more than 40% pregnancy rate [27]. The detailed protocol for natural cycle IVF combined with IVM is shown in Fig. 1.

In addition, it has been reported that the clinical outcomes of different infertility causes (tubal factor, male factor, unexplained infertility, combination of tubal and male factors, and other/mixed factors) with natural cycle IVF combined with IVM treatment were evaluated [28]. They found that there were no significant differences in the rates of IVM, IVF, and cleavage as well as in the clinical pregnancy (30.4–46.9%) and live birth rates among the five subgroups, which suggests that natural cycle IVF and

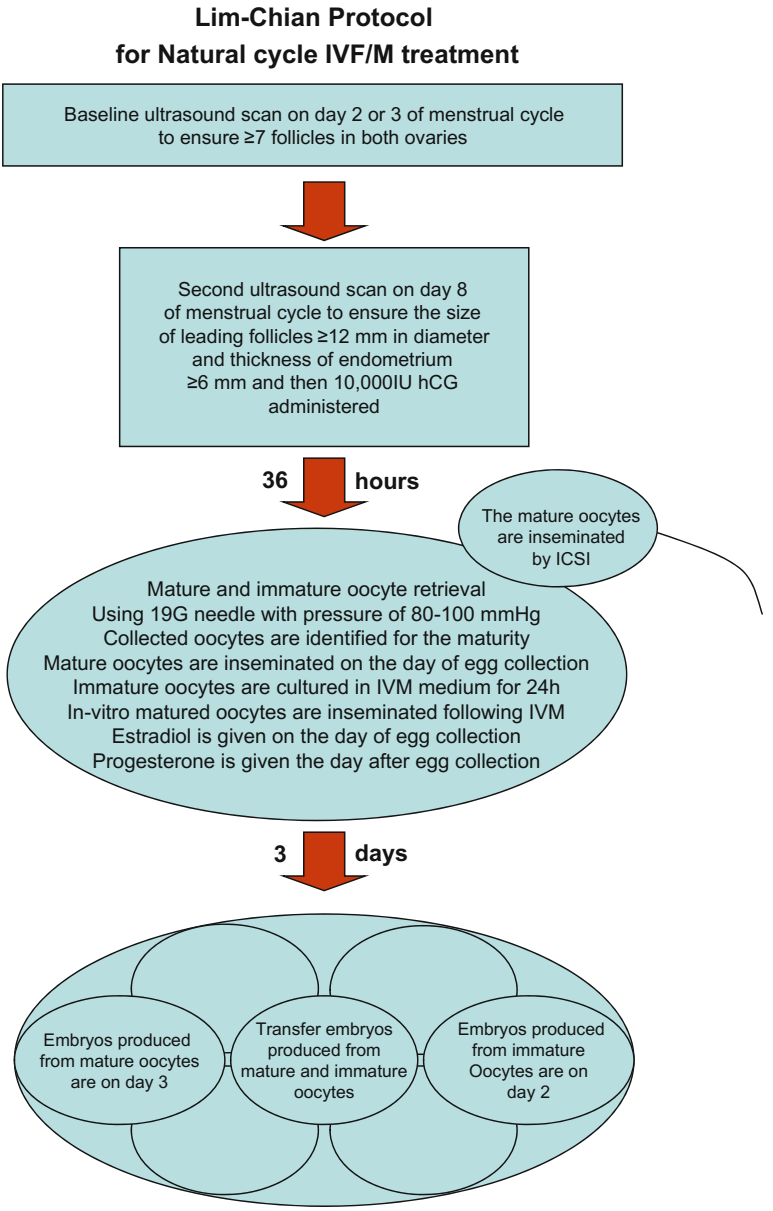

Fig. 1: Detailed protocol for natural cycle IVF combined with IVM treatment (Based on Lim et al. [27] with modification).

IVM treatment are the suitable treatments for infertility of various causes with acceptable pregnancy and live birth rates.

Interestingly, it also has been reported that the blastocysts produced from the immature oocytes derived from small follicles during natural cycle IVF combined with IVM treatment can be safely vitrified and give a healthy live birth, confirming that the presence of the leading follicle during natural cycle IVF combined with IVM treatment does not detrimentally affect the viability and health of the immature oocytes derived from small follicles [29]. Furthermore, it may be interesting to know that still whether or not during natural cycle IVF combined with IVM treatment there are differences in pregnancy and implantation rates between women with and without the presence of mature oocytes obtained at the time of the retrieval. It has been found that although the clinical pregnancy rates are not different regarding the retrieval of mature oocytes or the time of the egg retrieval, the live birth rate is higher ($P < 0.05$) when the mature oocytes are obtained at the time of the egg retrieval [30].

Table 1 shows the cycle outcome of Group A (women with mature oocytes) and Group B (women without mature oocytes). In Group A, a total of 739 mature oocytes were obtained at the time of retrieval from 314 cycles of natural IVF combined with IVM treatment with an average of 2.4 ± 1.6 per woman. Of these 739 mature oocytes, 644 (87.1%) were fertilized and 610 (94.7%) cleaved. There were also 2780 immature oocytes of which 1826 (65.7%) matured in vitro and 1457 (79.7%) were fertilized. In Group B, a total of 515 immature oocytes were retrieved from 55 cycles with an average of 9.4 ± 4.9 per patient. Of these 515 immature oocytes, 363 (70.5%) were matured in vitro and 294 (81.0%) were fertilized. There were no differences between the two groups in terms of total fertilization (81.9% vs. 81.0%) and embryo cleavage (92.1% vs. 89.1%) rates. Moreover, the quality of embryos was not different between the groups. Following embryo transfer, the clinical pregnancy rates and implantation rates between the groups were not different (40.1% vs. 34.5% and 16.2% vs. 15.0% in Group A and Group B, respectively). However, the live births per embryo transfer (29.6% vs. 16.4%) and miscarriage per clinical pregnancy (26.2% vs. 52.6%) rates were significantly different between Group A and Group B.

During natural cycle IVF/M, it is impossible to predict the stage of oocyte maturity according to the follicular size. In fact, as seen in the

Table 1. Comparison of pregnancy and live birth rates of natural cycle IVF/M treatment in women with or without mature oocytes collected at the time of egg retrieval (Reproduced from Yang et al. [30]).

Groups	A	B	*P* value
No. of patients (cycles)	283 (314)	53 (55)	–
Age (mean ± SD)	31.2 ± 3.6	30.4 ± 3.3	NS
No. of mature oocytes retrieved (mean ± SD)	739 (2.4 ± 1.6)	–	–
No. of immature oocytes retrieved (mean ± SD)	2780 (8.9 ± 5.0)	515 (9.4 ± 4.9)	NS
No. of oocytes matured in vitro (%)	1826 (65.7)	363 (70.5)	NS
Total numbers of oocytes matured (mean ± SD)	2565 (8.2 ± 3.5)	363 (6.6 ± 3.5)	NS
No. of oocytes fertilized (%)	2101 (81.9)	294 (81.0)	NS
No. of in vivo-matured oocytes fertilized (mean ± SD)	644 (2.1 ± 1.4)	–	–
No. of in vivo-matured oocytes cleaved (mean ± SD)	610 (1.9 ± 1.4)	–	–
No. of in vitro-matured oocytes fertilized (mean ± SD)	1457 (5.1 ± 1.4)	294 (4.9 ± 2.2)	NS
No. of in vitro-matured oocytes cleaved (mean ± SD)	1324 (4.2 ± 2.6)	262 (4.8 ± 2.7)	NS
No. of zygotes cleaved (%)	1934 (92.1)	262 (89.1)	NS
No. of embryos transferred (mean ± SD)	859 (2.7 ± 0.4)	147 (2.7 ± 0.5)	NS
No. of clinical pregnancies (%)	126 (40.1)	19 (34.5)	NS
No. of embryos implanted (%)	139 (16.2)	22 (15.0)	NS
Live births per cycle (%)*	93 (29.6)	9 (16.4)	0.043
Singleton	74	9	–
Twins	18		–
Triplets	1		–
Miscarriage rate per clinical pregnancies (%)*	33 (26.2)	10 (52.6)	0.019

Group A The patients with mature oocytes retrieved at egg retrieval
Group B The patients without mature oocytes retrieved at egg retrieval
*Significantly different between groups

patients of Group A, many in vivo mature oocytes were harvested from the small-sized follicles (<12 mm in diameter) than the leading follicles (≥12 mm in diameter). This observation should spur renewed interest in studying the process of folliculogenesis, and the resumption of meiosis during natural cycles and should caution in relying on a particular size of the follicle to predict oocyte maturity.

Lim et al. [27] have shown that the optimal sizes of follicles could be between 12 and 14 mm in diameter for natural cycle IVF combined with IVM treatment, which can prevent premature ovulation [25, 26]. Surprisingly, we found that some mature oocytes can be retrieved from relatively small follicles (between 8 and 10 mm in diameter). The embryo development from in vivo-matured oocytes had better quality than in vitro-matured oocytes, resulting in higher clinical pregnancy rate in the natural cycle IVF combined with IVM treatment with in vivo-matured oocytes compared to the cycles without in vivo-matured oocytes [25, 26]. However, the data show that there were no significant differences in terms of clinical pregnancy and implantation rates when the natural cycle IVF combined with IVM treatment with or without in vivo-matured oocytes was retrieved at the time of oocyte retrieval (Table 1).

Recently, it has been reported that non-dominant small follicles are a promising supplementary source of mature oocytes and that their use increase the live birth rate in natural cycle IVF treatment [31]. Also they demonstrate that the blastocysts from non-dominant follicles are as competent as those from dominant follicles in terms of pregnancy. This report confirms the notion that mature oocytes can be retrieved from non-dominant or leading follicles during natural cycle treatment.

Table 2 shows the pregnancy outcomes based on the mature oocytes retrieved from the different-sized follicles at the time of oocyte retrieval. Whether the mature oocytes were retrieved from leading follicles or small follicles, there were no differences in fertilization (81.8, 81.8, and 82.2%, respectively) and embryo cleavage (91.9, 93.9, and 90.5%, respectively) rates among those subgroups. Also the quality of embryos was not different among the groups assessed by morphology. In addition, there were no statistically significant differences in terms of clinical pregnancy (44.0, 34.9, and 38.5%), implantation (16.8, 14.7, and 16.6%), live birth per embryo transfers (30.7, 26.7, and 30.8%), and miscarriage per clinical pregnancy (30.3, 23.3, and 20.0%) rates among those subgroups. Interestingly, there are no significant differences in terms of clinical pregnancy rates between Group A (40.1%) and Group B (34.5%) independent of retrieving mature oocytes (Table 1). In fact, an average of 1.9 ± 1.4 embryos derived from in vivo-matured oocytes was transferred in Group A compared to Group B in which no embryo was produced from in vivo-matured oocytes transfer. In addition, the implantation rates were not different between these two

Table 2. Comparison of pregnancy and live birth rates based on the presence of mature oocytes retrieved from the leading or non-leading follicles at the time of egg retrieval (Reproduced from Yang et al. [30]).

Subgroups of A	1	2	3	*P* value
No. of patients (cycles)	137 (150)	82 (86)	76 (78)	–
Age (mean ± SD)	31.6 ± 3.8	31.2 ± 3.2	31.0 ± 3.5	NS
No. of mature oocytes retrieved (mean ± SD)	477 (3.2 ± 1.5)	89 (1.0 ± 0.2)	173 (2.2 ± 1.6)	–
No. of immature oocytes retrieved (mean ± SD)	1221 (8.1 ± 4.6)	808(9.4 ± 5.4)	751 (9.6 ± 5.2)	NS
No. oocytes matured in vitro (%)	811 (66.4)	532 (65.8)	483 (64.3)	NS
Total numbers of oocytes matured (mean ± SD)	1288 (8.6 ± 3.5)	621 (7.2 ± 3.4)	656 (8.4 ± 3.5)	NS
No. of oocytes fertilized (%)	1054 (81.8)	508 (81.8)	539 (82.2)	NS
No. of in vivo-matured oocytes fertilized (mean ± SD)	417 (2.8 ± 1.5)	81 (1.0 ± 2.3)	146 (2.0 ± 1.3)	–
No. of in vivo-matured oocytes cleaved (mean ± SD)*	395 (2.6 ± 1.4)	80 (0.9 ± 0.4)	135 (1.7 ± 1.3)	–
No. of in vitro-matured oocytes cleaved (mean ± SD)	574 (3.8 ± 2.6)	397 (4.6 ± 2.7)	353 (4.5 ± 2.5)	NS
No. of zygotes cleaved (%)	969 (91.9)	477 (93.9)	488 (90.5)	NS
No. of embryos transferred (mean ± SD)	410 (2.7 ± 0.4)	232 (2.7 ± 0.5)	217 (2.8 ± 0.4)	NS
No. of clinical pregnancies (%)	66 (44.0)	30 (34.9)	30 (38.5)	NS
No. of embryos implanted (%)	69 (16.8)	34 (14.7)	36 (16.6)	NS
Live births per cycle (%)*	46 (30.7)	23 (26.7)	24 (30.8)	NS
Singleton	41	15	18	–
Twins	5	8	5	–
Triplets	0	0	1	–
Miscarriage rate per clinical pregnancies (%)*	20 (30.3)	7 (23.3)	6 (20.0)	NS

Group 1 Mature oocytes were retrieved from both leading and small follicles
Group 2 Mature oocytes were retrieved from the leading follicles only
Group 3 Mature oocytes were retrieved from the small follicles only
*All embryos that produced from in vivo-matured oocytes were transferred

groups. Surprisingly, there was a higher miscarriage rate in Group B as compared to Group A, indicating that the embryos produced from in vitro-matured oocytes may be associated with a higher miscarriage rate. We cannot reconcile the higher miscarriage rate for in vitro-matured oocytes. Further study may be needed to answer this question.

Conclusion

With the development of IVM treatment for women with PCOS, the combination of natural cycle IVF with immature oocyte retrieval followed by IVM, namely natural cycle IVF/M, was proposed. Natural cycle IVF/M is an attractive treatment for women with all types of infertility without recourse to ovarian stimulation with acceptable pregnancy rate. Natural cycle IVF/M together with IVM-alone treatment can offer more than half of infertile women who are seeking for infertility treatment with an acceptable pregnancy and implantations rates. Although the clinical pregnancy rates are not different in terms of mature oocytes being retrieved or the time of egg retrieval with natural cycle IVF/M treatment, the live birth rate is higher ($P < 0.05$) when the transferred embryos were produced from the in vivo-matured oocytes. Natural cycle IVF/M may be the most suitable treatment for younger women who have regular menstrual cycles.

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Which Women Are Suitable for Natural and Modified Natural Cycle IVF?

A.K. Datta, B. Deval, S. Campbell, G. Nargund

What Is Natural Cycle IVF?

The term natural cycle in-vitro fertilisation (NC-IVF) applies 'when IVF is carried out with oocytes collected from a woman's ovary or ovaries in a spontaneous menstrual cycle without administration of any medication at any time during the cycle' [1]. The aim is to retrieve a single oocyte originating from a naturally selected follicle. Various modifications of the NC-IVF are possible to minimise the risk of premature ovulation and individualise the treatment protocol based on clinical needs and patient's choice. The first IVF baby was born from an oocyte collected in a completely natural cycle [2]. Subsequently, multi-follicular development to retrieve maximum number of oocytes, with concomitant suppression of premature luteinising hormone (LH) surge by gonadotropin-releasing hormone (GnRH) analogues, became the target of any IVF programme. Thus, pituitary 'downregulation' with GnRH agonists and ovarian

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stimulation with gonadotropins (the so-called long protocol) evolved as the standard protocol of today's conventional IVF (C-IVF). However, the side effects of intense ovarian stimulation and multiple embryo transfer (ET) were also being appreciated: ovarian hyper-stimulation syndrome (OHSS), twin or higher order births, or menopausal symptoms due to downregulation can rendered IVF a risky procedure. With the increasing complexity of administering multiple injections for prolonged period, along with significant hormonal changes often make the patients systemically unwell and emotionally stressed [3]. The use of medications for a longer period and at a higher intensity in C-IVF raises the overall treatment cost. In contrast, NC-IVF, being conducted on a spontaneous natural menstrual cycle with no or minimum medication(s), is usually well-tolerated by the patients and is less expensive and almost devoid of the above risks associated with C-IVF.

Why Natural IVF?

Presently, there is a drive in making IVF safer, more patient-centred and accessible worldwide [4]. Many IVF clinics around the world have appreciated the concept of NC-IVF and have reported their success stories [5–7]. It is increasingly being realised that quality, not quantity is the desirable goal of an IVF programme. By allowing a physiological approach to follicular recruitment, usually only the healthiest and most competent follicle(s) develop in NC-IVF and mild stimulation IVF (MS-IVF).

Indeed, a randomised controlled trial (RCT) showed same number of euploid embryos, whether created from a small oocyte cohort of MS-IVF cycles or from a larger pool of oocytes in C-IVF cycles [8]. Another RCT found significantly higher proportion of good quality embryo from MS-IVF cycles, compared to that following standard long protocol (61% vs. 29%, $p = 0.008$) [9]. A more recent RCT concluded that the number of available blastocyst did not correlate with the gonadotropin dose [10]. Rather, an inverse relationship has been depicted between increasing gonadotropin doses and blastocyst–oocytes ratio [10] or live birth rates (LBRs) [11]. To try to find an explanation of these findings at biochemical level, the follicular fluid hormonal milieu has been shown to be disturbed by high level of ovarian stimulation [12]. Follicular fluid Anti-Mullerian Hormone (AMH), which is believed to be a marker of

successful fertilisation and implantation, is maintained at a higher level in NC-IVF cycles compared to that of conventional stimulation [12].

In addition to possible direct influence of high ovarian stimulation on the oocytes or embryos [13], there is a number evidence of detrimental effect of very high oestrogen (and progesterone) levels on endometrial receptivity [13–17]. Supra-physiological hormone levels and high oocyte numbers have also shown to be associated with adverse perinatal outcomes including prematurity, low-birth weight [18, 19], intrauterine growth restriction [20] and cardiovascular disturbance in the neonates [21].

Despite obvious advantages mentioned above, NC-IVF remains under-utilised, mainly due to its alleged low success rates (average ongoing pregnancy rates: 7.2% per cycle and 15.8% per ET from a review of 20 studies) [22] and high risk of premature ovulation. Less flexibility in cycle scheduling resulting in 7-days-a-week service is not an acceptable option for most of the IVF clinics. However, the review by Pelinck et al. found only 3 small-scale RCTs, 2 of which compared pure NC-IVF with clomiphene citrate (CC)-stimulated IVF and the other with long GnRH agonist protocol; the bulk of evidence was derived from case series or retrospective studies [22]. The real effectiveness of NC-IVF is judged in its cumulative birth rates. Data since the early days of NC-IVF in unselected patients showed a 3–5 cycle cumulative pregnancy rates (PRs) of 41.7–46% [7, 23, 24] and a 32% cumulative LBRs [7]. A widely quoted non-inferiority RCT found no difference in cumulative 1-year LBRs, when a day 5 commencement of low-dose follicle stimulation hormone (FSH) regimen was assessed against C-IVF (43.4% vs. 44.7%) [25]. More recent studies comparing cumulative fresh and frozen single embryo transfer (SET) in C-IVF with multiple natural IVF cycles also demonstrated that NC-IVF could be a cost-effective alternative [26, 27]. The advent of GnRH antagonists made certain modification of NC-IVF possible that potentially has reduced the chance of premature LH surge [28, 29]. One of the largest series of modified natural cycle (MNC) (n = 1503 cycles) in recent time reported 14.5% PRs per cycle and 34.5% per ET for normal-responder women under 35 years of age, with 5.7% cycle cancellation rate due to premature ovulation [6]. Considering NC-IVF and MS-IVF safer, cheaper and more patient-friendly, a need for revival of this approach has long been voiced [29, 30].

What Are the Types of Natural IVF?

To streamline the use of various terminologies to describe different ways of ovarian stimulation, the International Society of Mild Approach Assisted Reproduction (ISMAAR), a consensus paper was published in *Human Reproduction* [1]. A brief protocol for each type of Natural IVF has been described in Table 1.

Other than the follicular size and serum estradiol (E2) levels, assessment of perifollicular blood flow by Doppler ultrasound also aids in managing a natural cycle IVF (Fig. 1). Good peak systolic blood flow in the perifollicular blood vessels in the pre-ovulatory period has been shown to be associated with probability of retrieving an oocyte and development of high-quality embryo [31]. Perifollicular blood flow velocity of 10 cm/second gave rise to 70% high-grade embryos, as opposed to only 14% when the flow was below 10 cm/s [].

To increase efficiency, NC-IVF is usually offered as a multiple-cycle package. While availability of oocytes or embryo per OC is less, accumulating oocytes from multiple cycles and subsequent transfer of fresh and frozen embryos have been shown to improve the treatment outcome, when compared with multiple individual cycles (PRs: 34.4% vs. 16%) [33]. The opportunity of selecting the best embryo(s) and double ET, as opposed to usually SET in repeated fresh cycles, may explain this observation.

What Are the Indications of Natural IVF?

NC-IVF can be considered for anybody with regular menstrual cycle, whether on medical ground or on patient's request. However, there are certain situations where it appears to be particularly useful. The following are the most common ones.

Women with Poor Ovarian Reserve (POR)

Women classified of having POR based on low antral follicle count (AFC) and/or low AMH, with or without elevated baseline FSH usually, have a poor prognosis in C-IVF, despite having high dose of ovarian stimulation. Traditionally, a day 3 FSH level is regarded as predictor of ovarian

Table 1. Brief description of various natural IVF protocols.

Types	Definitions	Conduction of cycles (protocol)
Natural cycle	When IVF is carried out with oocytes collected from a woman's ovary or ovaries in a spontaneous menstrual cycle without administration of any medication at any time during the cycle	The cycle is monitored by serial ultrasound scans ± serum LH and E2, usually starting from day 4–6 onwards. Urine LH test is commenced once the dominant follicle reaches > 12 mm in diameter. Optimal timing of OC is determined by hormone levels and follicular diameter. Occurrence of endogenous LH surge necessitates OC within 24 h of the surge to prevent ovulation. Indomethacin may be added if there is a risk of premature ovulation. Luteal support is not necessary
Modified natural with hCG	"The use of hCG to induce final oocyte maturation" in a natural cycle	Elective 'trigger' of final oocyte maturation by hCG, once the dominant follicle reaches > 15 mm average diameter with satisfactory serum E2 levels. OC scheduled 35–36 h later. Triggering before endogenous LH surge reduces the need for emergency OC and the incidence of premature ovulation. Luteal support is optional
Modified natural cycles with addition of GnRH antagonist	'The administration of GnRH antagonist to block the spontaneous LH surge with or without FSH or HMG as add-back therapy'	The cycle starts with natural selection of the dominant follicle. Low-dose FSH or HMG at 150 IU/day is started along with Cetrorelix (antagonist), once the leading follicle is 13–14 mm size and serum E2 is >500 pmol/l. The hCG trigger is planned when the follicle reaches >16 mm in average diameter with a satisfactory serum E2 level and OC follows 35–36 h later. The luteal phase support is administered

LH Luteinising hormone. *E2* Estradiol. *OC* Oocyte collection. *hCG* Human chorionic gonadotrophin. *GnRH* Gonadotrophin-releasing hormone. *FSH* Follicle-stimulating hormone. *hMG* Human menopausal gonadotrophin. *MNC* Modified natural cycle

response, oocyte quality and IVF outcome, irrespective of women's age [34]. In 2011, the European Society of Human Reproduction and Embryology (ESHRE) working group on 'poor ovarian response' organised a meeting at Bologna to form consensus on universally acceptable definitions of POR, which are now regarded as 'Bologna criteria' [35]. However, many IVF centres decline treatment to women over 40 years of age or with high day 3 FSH values. Recovery of fewer oocytes is recognised as an under-response for C-IVF, whereas it is normal and a very intended response in NC-IVF. Low oocyte yield following high gonadotropin stimulation is believed to be a result of follicular dysfunction or

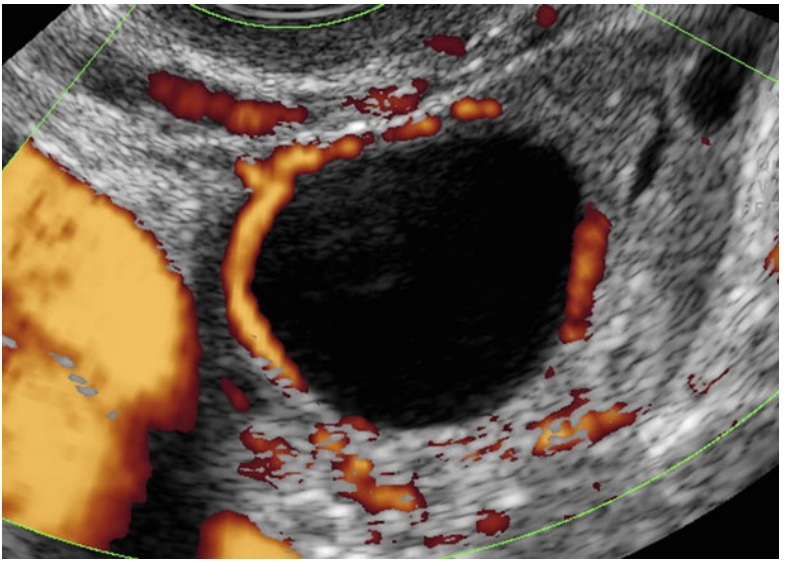

Fig. 1: Image by Doppler ultrasound for perifollicular blood flow.

ageing ovary. In contrast, NC-IVF encourages only the most competent follicle(s) to develop, and therefore, its outcome is not much dependent on oocyte yield. Being naturally selected, quality of oocytes and embryo in NC-IVF is expected to be better [36]. On this theoretical background, NC-IVF could be a cost-effective solution for those who had failed treatment with high-stimulation dose C-IVF.

Earlier, a small uncontrolled case series of 32 women with POR (defined as basal FSH >12 iu/l) and 1 previous failed IVF with <6 retrieved oocytes reported poor treatment outcomes with MNC [37]. A cohort study (n = 164) found application of MN protocol in women with POR, as defined by Bologna criteria, resulted in low LBRs (7.4% per patient); the outcome was assessed against that of normal responders which was not a like-to-like comparison [38]. In contrast, another larger cohort study found no such difference in women 35 years age group (LBRs-normal responders: 35.05% vs. poor responders: 29.63% per ET) [6]. However, in women older than 35 years who were poor responders as well, NC-IVF led to inferior outcome compared to normal responders of the same age group. This finding was similar to that by Kedem et al. who

found no benefit from NC-IVF among women aged between 35 and 43 years and also classified as 'genuine' poor responders (n = 111) according to Bologna criteria [39]. In contrast, a study of women aged 37–43 years and elevated serum FSH achieved 11.5% PR per cycle and 20.0% PR per ET by pure NC-intracytoplasmic sperm injection (ICSI) [40]. A more recent cohort control study that included women with POR according to Bologna criteria (n = 242) found significantly higher adjusted LBRs with MNC (7.5% vs. 3.1%; OR 4.01, 95% CI: 1.14–14.09) as compared to high-stimulation GnRH antagonist cycles [41]. Interestingly, more cycles were cancelled in the high-stimulation group mainly due to inadequate follicular growth (13.4% vs. 5.0%; p = 0.02) in this study, with no difference in cancellation rates due to premature ovulation. The reliability of these findings has been questioned (mainly on the principle of applying multivariate analysis), and re-emphasis was on the need for well-designed RCT [42].

Whether or not NC-IVF works better in women with POR in comparison with those with normal reserve, there is yet no evidence of superiority of C-IVF in this clinical setting, and rather, high stimulation is suggested to yield poorer outcomes [41]. Moreover, women often find intense ovarian stimulation regimen of C-IVF to be physically daunting, stressful and unrewardingly expensive [3]. NC-IVF being more patient-friendly and cost-effective, it could be a better option for these women.

Previous Poor Responders

There is no single strategy which is unquestionably beneficial after a suboptimal response with standard IVF treatment [43]. Earlier, several case reports, case series or small prospective trials on application of NC-IVF on previous poor responders revealed encouraging results [44–47]. As described above, the studies on women who had previous failed treatment and also had POR showed inconsistent results [37, 41]. The RCT (n = 215) that compared NC-IVF with 'micro-dose flare' protocol recruited women aged <43 years, with <4 dominant follicles in the previous treatment C-IVF cycle(s) [48]. It found similar PRs (per cycle: 6.1 vs. 6.9%; per ET: 14.9 vs. 10.1%) and a trend of higher implantation rates (14.9 vs. 5,5%; p = 0.05) with NC-IVF. A 3-cycle cumulative PRs/ET of 37.5% was achieved with negligible expense on medications [48].

Women dropped out from repeated C-IVF cycles more likely to find NC-IVF the way forward [3].

Advanced Women's Age

The role of NC-IVF in treating women of advanced age has not yet been fully evaluated. Theoretically, older women are more likely to produce poor quality oocytes or aneuploid embryos and therefore may benefit from 'natural selection'. As mentioned earlier, Shaulov et al. in their large uncontrolled study (n = 782 couples) found no significant decline in PR per ET (26.6% vs. 35.0%) in the older age group (>35 years) who had normal ovarian reserve and/or adequate response in the previous treatment cycle [6]. Cycle cancellation rates were also not significantly different. In this study, the PR in poor responder women of >35 years however was 6.25% per ET which was similar to those of Polyzos et al. (6.8% LBRs in women >40 years) [38]. Data are insufficient to compare relative effectiveness between NC-IVF and C-IVF in older age group. To date, the only RCT that compared NC-IVF (hCG only regimen) with one of GnRH agonist protocols (micro-dose flare) reported similar PRs per ET in 36-39-year age group (10% vs. 4%) and 40-43-year age group (8.0% vs. 9.7%) with a trend of higher implantation rates in favour of NC-IVF and minimal cost on medication [48]. Overall, the results are encouraging. Further work is needed to find the place of NC-IVF in women with advanced age.

Previous Conventional Stimulation Cycles with Poor Quality Embryos

High gonadotropin stimulation has been shown to generate higher proportion of poor quality of aneuploidy embryos [8, 9]. In the study by Arce et al., the number of blastocysts did not correlate with the FSH dose; however, the blastocyst–oocyte ratio and fertilisation rates declined significantly with the escalating gonadotropin dose [10]. By maintaining the follicular fluid hormone milieu (AMH, E2, androstenedione and LH) close to the physiological levels, NC-IVF improves fertilisation [12]. There was a paucity of comparative data between NC-IVF and failed C-IVF due to poor quality of embryos.

Contraindications to Ovarian Stimulation

Conventional ovarian stimulation possesses considerable risk in certain medical conditions including estrogen receptor-positive breast cancer, endometrial cancer or acute intermittent porphyria. Selective estrogen receptor modulators, particularly tamoxifen and aromatase inhibitors (e.g., letrozole), are being widely used in women with breast cancer requiring fertility preservation [49, 50]. In women with acute intermittent porphyria, even anti-estrogen may trigger disease flare up [51]. Pure NC-IVF could be an option for these patients undergoing fertility treatment. While multiple cycles of NC-IVF increase the number of oocytes or embryos to be cryo-preserved in cancer patients, very often the urgency of commencement of gonadotoxic chemotherapy or radiation does not allow the time for having repeated cycles: in vitro maturation (IVM) of oocytes from non-dominant follicles of a natural cycle could increase the number of available embryos [52]. Lim et al. reported a PR of 40.4% in a combined natural IVF+IVM cycles and 41.3% with IVM alone, as opposed to 37.8% with C-IVF among infertile couples undergoing treatment [53].

Women at Significant Risk of OHSS

Women with polycystic ovary are at risk of developing OHSS. Treatment of women who have already had severe OHSS is always a challenge. A number of very effective strategies to prevent OHSS have been described in recent years. GnRH agonist as an ovulation trigger followed by intense luteal phase support (by high dose of E2 and progesterone or low-dose luteal hCG) or freezing all embryos has made OHSS a rare event [54]. However, OHSS has recently been reported with agonist trigger and subsequent luteal-phase hCG [55] or even with freezing all embryos [56, 57]. NC-IVF with or without IVM may be an alternative option for high responder women who are at considerable risk of OHSS. Successful pregnancies can be achieved with this policy of NC-IVF and IVM [58]. However, a regular menstrual cycle is a prerequisite for NC-IVF. This option is not suitable for women with oligomenorrhoeic polycystic ovarian syndrome (PCOS). There is a large section of ovulatory PCO patients or high responders with regular periods who may benefit from NC-IVF-IVM treatment. Although theoretically reassuring, existing data

are too small to determine the risk of OHSS and the cycle outcome with this strategy. At present, most of the IVF centres do not practice IVM routinely. With further experience of NC-IVF-IVM, it may come up as a safe and effective treatment for high responders.

Patient's Choice

Finally, honouring patient's choice is a basic principle of any medical treatment. Mild/natural IVF has been regarded as more patient-centred and 'tailor-made' approach [4]. 'Natural' IVF appeals many women, particularly those who had multiple failed cycles with C-IVF [3].

Conclusion

NC-IVF provides a safe, low-cost and a patient-centred option for women wishing to avoid ovarian stimulation or where stimulation is medically contraindicated. NC-IVF should be regarded as a multiple-cycle approach, and cumulative success rates over 3 cycles (which can be done in successive cycles) are promising. The disadvantage of NC-IVF is that only one oocyte is obtained and that spontaneous ovulation can occur before the oocyte can be obtained. MNC-IVF overcomes this problem and with add-back FSH from the day of antagonist commencement can result in more than one oocytes being obtained. This strategy is particularly beneficial in poor prognosis patients with low ovarian reserve in whom standard IVF only adds cost to the treatment with no clear advantage in the final outcome. A simplified way of conducting the treatment cycles is physically and mentally less distressing to the patients and therefore appears to be more acceptable option to them. A 7-days-a-week dedicated service and expertise in advanced ultrasound assessment, e.g., follicular blood flow, are essential prerequisites to achieving an optimum outcome.

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G-CSF and Stem Cell Therapy for the Treatment of Refractory Thin Lining in Assisted Reproductive Technology

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Abstract

The study aims to describe two promising therapeutic options for resistant "thin" endometrium in fertility treatment: granulocyte colony-stimulating factor (G-CSF) and stem cell therapy. A review of the scientific literature related to patients with thin endometrium undergoing fertility treatment. Sufficient endometrial growth is fundamental for embryo implantation. Whether idiopathic or resulting from an underlying pathology, a thin endometrium of <7 mm is associated with lower probability of pregnancy; however, no specific thickness excludes the occurrence of pregnancy. We specifically reviewed two relatively new treatment options for resistant thin lining: intrauterine G-CSF and stem cell therapy. The majority of the reviewed trials showed a significant benefit for intrauterine G-CSF infusion in improving endometrial thickness and pregnancy rates. Early results of stem cell therapy trials seem promising. EMT <7 mm is linked to lower probability of pregnancy in assisted reproductive technology.

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Intrauterine G-CSF infusion appears to be a potentially successful treatment option for resistant cases, while stem cell therapy seems to be a promising new treatment modality in severely refractory cases.

Keywords Thin endometrium, Endometrial thickness, Pregnancy rates, G-CSF, Stem cell therapy

Background

Endometrial receptivity is a key step in the embryo implantation process [1–5]. During each menstrual cycle, and for a brief period of time, the endometrium represents the fertile "soil" for the implanting embryo [6]. Certain endometrial development appears to be crucial for adequate endometrial receptivity, and some reports proposed that a minimal endometrial thickness (EMT) of 6 mm is required to achieve implantation in assisted reproductive technologies (ARTs) [7]. However no cutoff appears to preclude implantation as successful pregnancies were documented at a much lower thickness (4 mm) [8–10]. In fact and up to this date, there is no consensus on a cutoff value of an EMT below which implantation rates decline in ART. Using receiver operating characteristic (ROC) area under the curve, a cutoff limit of endometrial thickness (on day of HCG trigger) above which implantation could be predicted was not detected by three reports, whereas two studies reported a threshold thickness of 8 mm [11–15]. A recent systematic review and meta-analysis of 10,724 cases showed that EMT as an independent variable is not predictive for the occurrence of pregnancy [16]. This meta-analysis, however, found that the most commonly reported cutoff of 7 mm occurred in only 2.4% of the cases (260/10,724), and it was associated with a significant drop in the probability of pregnancy [16]. In 2008, Senturk et al. reviewed thin endometrium and some of the available treatment modalities, and concluded that these were ineffective [17]. More recently, Lebovitz et al. concluded that the treatment of "thin" endometrium remains a challenge, with only minor improvements achieved with the currently available treatment

modalities [18]. We have recently published a detailed review that aimed at defining thin endometrium in infertility patients and concluded that of the available treatment modalities, only vaginal sildenafil and intrauterine granulocyte colony stimulating factor (G-CSF) were consistent in showing improvement in endometrial thickness and pregnancy rates of patients with thin endometrium, and that early stem cell results are promising [19]. Here, we provide a focused review on emerging and promising treatment options for refractory thin endometrium, namely, intrauterine G-CSF and stem cell therapy.

Materials and Methods

We conducted an electronic review of the literature pertaining to thin endometrium, its pathophysiology, and treatment through January 2017. Abstracts, case reports, original, and review articles were considered. The Cochrane database, Pubmed, Medline registries, and other online sources were searched using the broad terms: thin endometrium, thin endometrium and IVF, thin endometrium and ART, and treatment of thin endometrium. The titles and abstracts were screened, and relevant articles were analyzed in detail to determine which studies could be included in the review. Furthermore, the references cited in relevant studies and review articles were hand searched to identify further relevant studies. Studies were considered eligible if they were conducted to assess or compare different treatment modalities of thin endometrium. Changes in mean endometrial thickness and pregnancy rates were the two principle summary measures, and were independently extracted from individual studies.

Results (summarized in Table 1)

G-CSF

G-CSF, a hematopoietic growth factor, was reported to have positive effects in non-hematopoietic cells, including the endometrium [35]. It was hypothesized that intrauterine G-CSF might have a direct role promoting endometrial growth after successful treatment of four patients [20]. A pilot trial showed that intrauterine G-CSF (given 6–12 h prior to HCG trigger) significantly improved endometrial thickness in patients with

thin lining, resulting in an overall clinical pregnancy rate of 19.1% [21]. Lucena et al. successfully treated one patient, while Check and colleagues failed with another patient [22, 23]. Li et al. administered intrauterine G-CSF (100 μg) on the day of ovulation, or day of progesterone start, or day of HCG administration to 59 patients with thin endometrium (<7 mm) [24]. The cycle cancelation rate due to thin endometrium was significantly lower after treatment (69.39 vs. 48.75 vs. 17.5%; $P < 0.05$ self-controlled vs. control vs. treatment groups, respectively), with a trend towards better implantation and clinical pregnancy rates [24]. Similarly Kunicki et al. administered intrauterine G-CSF (300 μg) 6–12 h prior to HCG trigger to patients with an EMT of 7 mm, resulting in significant improvement in EMT within 72 h and the pregnancy rate of 18.9% [25]. Recently, Barad et al. administered intrauterine G-CSF to patients undergoing IVF or FET, regardless of endometrial thickness. The study group received 300 μg/cm^3 G-CSF on the day of HCG trigger without improvement in endometrial thickness [26]. In another prospective trial, Shah et al. administered intrauterine G-CSF to patients with thin lining (<8 mm) that were resistant to treatment with estradiol and vaginal sildenafil, and expanded the treatment to patients with repeated implantation failure who had an EMT >8 mm [27]. G-CSF (300 μg) was administered into the endometrial cavity after 10 days of oral estradiol and vaginal sildenafil to all 231 patients, resulting in a significant increase in EMT and clinical pregnancy rate of 38.07% [27]. In another trial, Eftekhar et al. compared intrauterine G-CSF therapy to direct embryo transfer in patients with thin lining (<7 mm) [28]. All patients received oral estradiol and vaginal sildenafil priming, and on either day 12 or 13, 34 patients accepted intrauterine G-CSF while another 34 refused. The cycle cancelation rate due to thin endometrium was similar between both groups (15.2%), and so was the increase in endometrial thickness and pregnancy rates. Interestingly, the EMT increased from 5.63 ± 0.78 to 7.91 ± 0.55 mm in the G-CSF group [28].

Xu et al. compared intrauterine G-CSF and G-CSF with endometrial scratch to no intervention [29]. The G-CSF was administered when the lead follicle measured 12 mm; the EMT increased significantly after treatment without a difference between G-CSF and G-CSF + scratch. The implantation and clinical pregnancy rates were significantly higher in both treatment subgroups [29]. Tehraninejad and colleagues treated 15

Table 1. Studies evaluating G-CSF and stem cell therapy in treatment of thin lining.

Author	Year	Study type	Diagnosis	Study vs. control group	Number of patients or cycles	Intervention	Mean EMT prior to intervention (mm)	Mean EMT post intervention (mm)	*P* value	Pregnancy rate	*P* value
Gleicher [20]	2011	Case series	POI, repeated IVF failure, AS		4 patients	Intrauterine G-CSF (300 µg) infusion 2–9 days before ET	5.0 ± 1.2	8.6 ± 1.1	N/A	100%	N/A
Gleicher [21]	2013	Prospective observational	DOR, male factor, uterine factors, PCOS		21 patients	Intrauterine G-CSF (300 µg) infusion 6–12 h before HCG trigger	6.4 ± 2.1	9.3 ± 2.1	<0.001	19%	N/A
Lucena [22]	2013	Case report	Thin endometrium in IVM cycles		1 patient	Intrauterine G-CSF (300 µg) infusion on day of oocyte retrieval	5.7	8.9	N/A	Full-term pregnancy	N/A
Check [23]	2014	Case report	Thin endometrium during IVF-ET		1 patient	Intrauterine G-CSF (30 million units) infusion 6–12 h before HCG trigger	5.7	5.0	N/A	No pregnancy	N/A
Li [24]	2014	Retrospective	Multiple	Study group	40 cycles	Intrauterine G-CSF (100 µg) infusion at varying times of the cycle	6.53 ± 0.65	6.75 ± 1.17	0.403	30.30%	N/S
				Self-controlled group	49 cycles	FET	N/A	N/A	N/A	20%	
				Control group	80 cycles	FET	N/A	N/A	N/A	29.27%	
Kunicki [25]	2014	Prospective observational	N/A		37 patients	Intrauterine G-CSF (100 µg) infusion 6–12 h before HCG trigger	6.74 ± 1.75	8.42 ± 1.73	<0.001	18.90%	N/A
Barad [26]	2014	RCT	All patients undergoing IVF or FET (regardless of EMT)	Study group first cycle	73 patients	Intrauterine G-CSF (300 µg) infusion on day of HCG trigger + IVF-ET	10.23 ± 2.01	11.68 ± 2.22	N/S	94.40%	N/S
			(regardless of EMT)	Control group first cycle	68 patients	IVF-ET	10.37 ± 2.14	11.62 ± 2.28		84.20%	
			Study group second cycle		19 patients	Intrauterine G-CSF (300 µg) infusion on day of HCG trigger + IVF-ET	9.96 ± 2.27	10.32 ± 2.13	N/S	70.43%	N/S

(continued)

(continued).

			Control group second cycle		16 patients	IVF-ET	11.1 ± 1.93	11.26 ± 2.01		60%	
Shah [27]	2014	Observational cohort	Thin endometrium <8 mm or repeated implantation failure		231 patients	Intrauterine G-CSF (300 µg) after 10 days of priming with oral estradiol and vaginal sildenafil	7.98 ± 1.3	10.97 ± 1.23	<0.0001	38.07%	N/A
Eftekhar [28]	2014	Non-randomized trial	Thin endometrium <7 mm	Control group	34 patients	Intrauterine G-CSF (300 µg) on cycle day 12 or 13	5.63 ± 0.78	7.91 ± 0.55	0.1	32.1%	0.1
				Study group	34 patients	FET	5.76 ± 0.86	8.23 ± 0.82		12%	
Xu [29]	2015	Prospective controlled	Unresponsive thin endometrium			Intrauterine G-CSF (300 µg) ± endometrial scratch when lead follicle = 12 mm	5.7 ± 0.7 l	8.4 ± 2.0 l	<0.001	48.10%	0.038
				Control group	52 patients	FET	6.5 ± 0.5	N/A		25%	
Tehraninejad [30]	2015	Non-randomized trial	Then endometrium <6 mm		15 patients	Intrauterine G-CSF (300 µg) on day of oocyte retrieval	3.6 ± 0.98	7.12 ± 0.84	<0.001	20%	N/A
Lee [31]	2016	Retrospective	Thin endometrium ≤8 mm		50 patients	Intrauterine G-CSF (300 µg) infusion on day of HCG trigger or egg retrieval	7.2 ± 0.6	8.5 ± 1.5	<0.001	22.0%	N/A
Nagori [32]	2011	Case report	AS		1 patient	Intrauterine endometrial angiogenic stem cells	3.6	7.1	N/A	Clinical IUP	N/A
Singh [33]	2014	Prospective case series	AS		6 patients	Mononuclear stem cell implantation in sub-endometrial zone	1.38 ± 0.39	5.48 ± 1.14	0.002	N/A	N/A
Santamaria [34]	2016	Prospective	AS or endometrial atrophy		16 patients	BMDSC injection into spiral arterioles	4.3 m and 4.2	6.7 m and 5.7	0.004 m and 0.03	10/16	N/A

POI premature ovarian insufficiency, *IVF* in vitro fertilization, *AS* Asherman's syndrome, *G-CSF* granulocyte-colony stimulating factor, *ET* embryo transfer, *DOR* diminished ovarian reserve, *PCOS* polycystic ovarian syndrome, *HCG* human chorionic gonadotropin, *IVM* in vitro maturation, *IVF-ET* in vitro fertilization-embryo transfer, *FET* frozen embryo transfer, *BMDSC* bone marrow-derived stem cells

patients who previously had an IVF cycle canceled due to resistant thin endometrium (<6 mm) with intrauterine G-CSF (300 μg) on the day of egg retrieval [30]. The patients suffered from various infertility diagnoses. After intrauterine G-CSF treatment, the endometrial lining significantly increased in the cohort, with 3/12 (20%) patients subsequently conceiving [30]. Most recently, Lee and colleagues also demonstrated that intrauterine G-CSF (300 μg) on either trigger day or retrieval day significantly increased the EMT in patients with thin lining, resulting in pregnancy rate of 22.0% [31]. Interestingly, there was a trend for higher implantation, clinical pregnancy, and ongoing pregnancy rates when the G-CSF was instilled on the day of HCG trigger vs. day of egg retrieval [31].

Stem cell Therapy

Emerging evidence supports that endometrial stem cells are present in both the basalis and functionalis layers of the human endometrium, and it is thought that these stem cells play a role in regenerating the endometrial lining during each estrous cycle [36–39]. Cervello recently demonstrated that human endometrial adult stem cells are able to generate human endometrium after transplantation in NOD-SCID mice renal capsules [40]. Non-endometrial stem cells seem to have a role in endometrial regeneration as well; in fact, hematopoietic and nonhematopoietic bone marrow-derived stem cells (BMDSCs) are recruited to the endometrium in response to injury as shown by Taylor and colleagues [41]. These findings were further affirmed when hematopoietic progenitor cells were shown to be recruited to the uterine epithelium to play an important role in epithelial regeneration and, even more interestingly, when male origin BMDSCs were shown to be present in endometria of female bone marrow transplant recipients [42, 43]. In their elegant study, Cervello et al. also demonstrated that BMDSCs exert their regenerative effects in a paracrine fashion by ultimately stimulating endometrial side cell population [44]. In fact, uterine ischemia/reperfusion injury results in a twofold increase in bone marrow-derived stem cell recruitment to the endometrium, which is independent of GCSF and only serves in uterine repair after injury rather than monthly cyclic regeneration of the endometrium [45]. The role of BMDSC in endometrial regeneration after injury was further validated in several murine models [46–48].

These findings triggered researchers to investigate the effects of bone marrow stem cells in the treatment of Asherman's syndrome and thin endometrium in a murine model. Female mice with AS were given BMDSCs via the tail veins and later bred after three estrous cycles [49]. Nine of 10 treated mice conceived compared to only 3/10 non-treated mice [49]. Similarly, Kilic et al. treated Asherman's syndrome in Wistar albino rats with either mesenchymal stem cells (MSCs) or oral estrogen or combined MSC and oral estrogen [50]. All treatment groups demonstrated improvement in the level of fibrosis when compared to control, which was mostly noted with combined MSC and estrogen treatment [50].

Zhao et al. further elucidated these regenerative capabilities of BMDSCs when rats with thin endometria were infused with BMDSCs and compared to endometria of normal rats [51, 52]. The treatment groups showed significant increase in EMT compared the control groups in both trials, respectively (325.35 ± 75.51 vs. 187.53 ± 34.38 μm; $P < 0.05$) and (359.13 ± 49.70 vs. 187.53 ± 34.38 μm; $P < 0.05$) [51, 52].

A recent comparative study delineated the role of various BM-derived cell subtypes in endometrial regeneration [53]. The investigators found that freshly isolated unfractionated BM cells, hematopoietic progenitor cells, endothelial progenitor cells (EPCs), mesenchymal stem cells, and in vitro cultured mouse $Oct4^+$ BM-derived hypoplast-like stem cells supported endometrial regeneration after injury [53].

The first human application was in 2011, when a patient with AS and refractory thin endometrium (3.6 mm) was treated with intrauterine autologous endometrial angiogenic stem cells [32]. The infusion was followed by high-dose estradiol valerate, aspirin (75 mg PO daily), and four cycles of cyclical estrogen and progesterone therapy until the EMT reached 7.1 mm. Three donor oocyte embryos were subsequently transferred resulting in a single viable intrauterine pregnancy [32]. Similar findings were noted in a case series of six patients with refractory AS who were treated with autologous mononuclear stem cells. The mean endometrial thickness improved from 1.38 ± 0.39 mm to 4.05 ± 1.4, 5.46 ± 1.36, and 5.48 ± 1.14 mm at 3, 6, and 9 months, respectively ($P < 0.05$) [33]. Cervello et al. investigated whether human bone marrow-derived stem cells would promote endometrial growth in rats with Asherman's syndrome [54]. The BMDSCs engulfed small endometrial vessels of damaged horns and resulting in proliferation of epithelial gland cells in a paracrine

manner by upregulation of thrombospondin 1 and downregulation of insulin-like growth factor 1 [53].

In the most recent human trial, Santamaria and colleagues treated patients who had Asherman's syndrome and endometrial atrophy with autologous CD 133+ BMDSCs [34]. The infusion, administered into the spiral arterioles, increased the EMT from 4.3 to 6.7 mm, and from 4.2 to 5.7 mm in patients with AS and endometrial atrophy, respectively. The improvement in endometrial lining lasted up to 6 months, and subsequent conception attempts resulted in three spontaneous pregnancies (2, 4, and 19 months after treatment), and seven other pregnancies following 14 embryo transfers [34].

Discussion

The diagnosis of "thin endometrium" is a frustrating condition that occurs not infrequently in clinical practice. It is not exactly clear how thin endometrium lowers the probability of pregnancy, but it is hypothesized that the proximity of the embryo to the reactive oxygen species-rich basal layer could be detrimental for embryo development and implantation, or possibly that with thin lining, there is not enough soil to sustain the "seed" [55].

The main challenge for clinicians is determining a certain thickness below which they consider an endometrium thin, and how to treat it when it is refractory to conventional therapy. The most widely accepted measurement is 7 mm; however, this cutoff is not absolute, as pregnancies can occur at lower cutoffs [8–10]. Another challenge is the reliability of endometrial thickness measurement as a predictor for the occurrence of pregnancies in IVF cycles, as it seems to be only a good factor for the assessment of conception probability, which significantly drops below an EMT of 7 mm [55]. Other key factors such as endometrial pattern play a vital role in successful implantation as well, and may be as important as endometrial thickness [56]. Newly emerging diagnostic tools such as endometrial receptivity assay (ERA) seem to be very accurate in determining the window of implantation. In one study, ERA determined that 75% of patients ($n = 13$) with an EMT ≤6 mm had a receptive endometrium according to the customized endometrial receptivity microarray test

[57]. This technique however is invasive and costly, and these early results must be further validated with larger studies.

Over the years, several treatment options have been suggested and assessed as a treatment for refractory thin endometrium with or without Asherman's syndrome [19]. Extended estrogen therapy, luteal GnRH-a supplementation, low-dose HCG during endometrial preparation, tamoxifen citrate as an ovulation induction agent, long courses of pentoxifylline and tocopherol or tocopherol only, aspirin, acupuncture, and neuromuscular electric stimulation with biofeedback were all inconsistent in improving endometrial thickness [19]. Vaginal sildenafil improved the endometrial thickness in a significant percentage of patients with thin lining due to various diagnoses that had prior IVF and ICSI cycles, indicating that it could be a reasonable first-line treatment option [19].

Treatment with intrauterine G-CSF gained significant interest despite its cost, and the majority of trials showed promising positive effects in patients with thin endometrium and possibly those with repeated implantation failure [27]. In fact, only one case report and one other retrospective study in FET cycles failed to demonstrate a positive effect of G-CSF in improving EMT [23, 24]. On the other hand, there is no evidence that G-CSF administration is beneficial to all patients undergoing IVF or FET [26]. While the use of G-CSF seems promising in increasing EMT and possibly pregnancy rates, most studies suffer from small sample sizes, and different studies used different doses and time points when G-CSF was administered, making interpretation rather difficult. In addition, GCSF has been given by intrauterine infusion but there is no data on whether other routes of administration, such as the subcutaneous route, are comparable. Larger prospective, randomized, placebo-controlled, trials are therefore sorely needed.

Stem cell therapy appears to be the most promising treatment option for cases with Asherman's syndrome with refractory thin lining (<5 mm). Initial reports on intrauterine angiogenic endometrial stem cells showed improvement in endometrial thickness in patients with Asherman's syndrome or refractory thin endometrium [32, 33]. Several murine models with induced thin endometrium showed significant improvement back to normal after bone marrow mesenchymal stem cell administration [51,

52]. In a promising early human trial, patients with AS and refractory thin endometrium had significant improvement in their endometrial thickness that lasted up to 6 months after autologous bone marrow-derived stem cells were infused via the uterine arterioles, with excellent pregnancy rates [34]. These results present a valuable viable option to patients who fail other treatment options; however, stem cell therapy remains a very invasive and expensive treatment option requiring bone marrow biopsy, cell sorting, and interventional radiology assistance for administration into the uterine arterioles.

In conclusion, a receptive endometrium plays a critical role in embryo implantation, and adequate endometrial growth is essential to this process. Poor endometrial development is linked to a lower probability of pregnancy, yet it is not the sole predictor of pregnancy occurrence. Of the several available treatment modalities, vaginal sildenafil appears to be a reasonable first-line option, whereas intrauterine G-CSF infusion before the HCG trigger could be a second-line treatment option, provided that large randomized studies evaluating outcomes, dosage, method, and timing of administration during the stimulation cycles are conducted. Stem cell therapy appears to be a very promising option for the most refractory cases; however, more research is warranted to evaluate the safety, effectiveness, and cost of this modality before it becomes integrated in the treatment of this frustrating condition.

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THANK YOU FOR **YOUR TRUST** THAT MADE US

† Schindler AE. Progestational effects of dydrogesterone *in vitro, in vivo* and on the human endometrium. *Maturitas*. 2009;65(1):S3-S11. * Data on file. ‡ Internal calculations based on Quintiles IMS database, IMS Health Analytics Link MAT03 2017.

Abbreviated Prescribing Information. Dydrogesterone Tablets IP. Duphaston® Composition: Each white film-coated tablet contains: Dydrogesterone IP 10 mg. Excipients q.s. Colour: Titanium dioxide IP. **Indications:** Progesterone deficiencies, Treatment of progesterone deficiencies such as: • Treatment of dysmenorrhoea • Treatment of endometriosis • Treatment of secondary amenorrhoea • Treatment of irregular cycles • Treatment of dysfunctional uterine bleeding • Treatment of pre-menstrual syndrome • Treatment of threatened and habitual abortion • Treatment of infertility due to luteal insufficiency. Hormone replacement therapy - To counteract the effects of unopposed oestrogen on the endometrium in hormone replacement therapy for women with disorders due to natural or surgical induced menopause with an intact uterus. **Dosage and Administration:** Dosages, treatment schedule and duration of treatment may be adapted to the severity of the dysfunction and the clinical response. Dysmenorrhoea: 10 or 20 mg dydrogesterone per day from day 5 to day 25 of the menstrual cycle. Endometriosis: 10 to 30 mg dydrogesterone per day from day 5 to day 25 of the cycle or continuously. Dysfunctional uterine bleeding: When treatment is started to arrest a bleeding episode, 20 or 30 mg dydrogesterone per day is to be given for up to 10 days. For continuous treatment, 10 or 20 mg dydrogesterone per day should be given during the Second half of the menstrual cycle. The starting day and the number of treatment days will depend on the individual cycle length. Withdrawal bleeding occurs if the endometrium has been adequately primed with either endogenous or exogenous estrogen. Secondary amenorrhoea: 10 or 20 mg dydrogesterone per day, to be given daily for 14 days during the second half of the theoretical menstrual cycle to produce an optimum secretory transformation of an endometrium that has been adequately primed with either endogenous or exogenous estrogen. Pre-menstrual syndrome: 10 mg dydrogesterone twice daily starting with the second half of the menstrual cycle until the first day of the next cycle. The starting day and the number of treatment days will depend on the individual cycle length. Irregular cycles: 10 or 20 mg dydrogesterone per day starting with the second half of the menstrual cycle until the first day of the next cycle. The starting day and the number of treatment days will depend on the individual cycle length. Threatened abortion: An initial dose of up to 40 mg dydrogesterone may be given followed by 20 or 30mg per day until symptoms remit. Habitual abortion: 10 mg dydrogesterone twice daily until the twentieth week of pregnancy. Infertility due to luteal insufficiency: 10 or 20 mg dydrogesterone daily starting with the Second half of the menstrual cycle until the first day of the next cycle. Treatment should be maintained for at least three consecutive cycles. Hormone replacement therapy: Continuous sequential therapy: An estrogen is dosed continuously and one tablet of 10 mg dydrogesterone is added for the last 14 days of every 28 day cycle, in a sequential manner. Cyclic therapy: When an estrogen is dosed cyclically with a treatment-free interval, usually 21 days on and 7 days off. One tablet of 10 mg dydrogesterone is added for the last 12-14 days of estrogen therapy depending on the clinical response, the dosage can subsequently be adjusted to 20 mg dydrogesterone per day There is no relevant use of dydrogesterone before menarche. The safety and efficacy of dydrogesterone in adolescents aged 12-18 years has not been established. Currently available data are described in section 4.8 and 5.1, but no recommendation on a posology can be made.

Contraindications: Known hypersensitivity to the active substance or to any of the excipients. Known or suspected progestogen dependent neoplasms. Undiagnosed vaginal bleeding. Contraindications for the use of estrogens when used in combination with dydrogesterone. **Warnings and Precautions:** Before initiating dydrogesterone treatment for abnormal bleeding, the etiology for the bleeding should be clarified. Breakthrough bleeding and spotting may occur during the first months of treatment. If breakthrough bleeding or spotting appears after some time on therapy, or continues after treatment has been discontinued, the reason should be investigated, which may include endometrial biopsy to exclude endometrial malignancy. **Pregnancy and Lactation:** Pregnancy: It is estimated that more than 10 million pregnancies have been exposed to dydrogesterone. So far there were no indications of a harmful effect of dydrogesterone use during pregnancy. Some progestogens have been reported in the literature to be associated with an increased risk of hypospadias. However due to confounding factors during pregnancy, no definitive conclusion can be drawn regarding the contribution of progestogens to hypospadias. Clinical studies, where a limited number of women were treated with dydrogesterone early in pregnancy, have not shown any increase in risk. No other epidemiological data are hitherto available. Effects in non-clinical embryo-fetal and post-natal development studies were in line with the pharmacological profile. Untoward effects occurred only at exposures which exceeded the maximum human exposure considerably, indicating little relevance to clinical use. Dydrogesterone can be used during pregnancy if clearly indicated. Breastfeeding: No data exist on excretion of dydrogesterone in mother's milk. Experience with other progestogens indicates that progestogens and the metabolites pass to mother's milk in small quantities. Whether there is a risk to the child is not known. Therefore, dydrogesterone should not be used during the lactation period. Fertility: There is no evidence that dydrogesterone decreases fertility at therapeutic dose. **Adverse Reactions:** The most commonly reported adverse drug reactions of patients treated with dydrogesterone in clinical trials of indications without estrogen treatment are migraines/headache, nausea, menstrual disorders and breast pain/tenderness. Undesirable effects that are associated with an estrogen-progestogen treatment : Breast cancer, endometrial hyperplasia, endometrial carcinoma, ovarian cancer • Venous thromboembolism • Myocardial infarction, coronary artery disease, ischemic stroke. Issued on: 3/4/14. Source: Prepared based on full prescribing information (version 03) dated 13/03/2015.

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