CLOMIPHENE CITRATE FOR INDUCTION OF OVULATION

By

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In the Name of

Allah

The Most Beneficent And Merciful

DEDICATED

TO

My Parents, **Who have always been a source of encouragement.**

My husband Tariq, **Who has always been a source of inspiration.**

My children

Amna,

Omer,

Kaneez Zahra.

Who have always been a source of joy.

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CERTIFICATE

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LIST OF ABBREVIATIONS

WHO	World Health Organization
CC	Clomiphene Citrate
FSH	Follicle stimulating hormone
LH	Leutinizing hormone
P	Progesterone
CRA	Clomiphene resistant anovulation
PCOS	Polycystic ovarian syndrome
PCO	polycystic ovaries
BMI	Body mass index
TVS	Transvaginal scan
CCR	Cumulative conception rate
IUI	Intra uterine insemination
ART	Artificial reproductive techniques
USG	Ultrasonography
Ag	Antigen
Ab	Antibody
EIA	Enzyme immunoassay
Yrs	Years
S.E	Standard error
MFD	Mean follicular diameter
RV	Reaction vessel
MUP	Methyllumbeliferyl phosphate
MU	Methyllumbeliferyl
P	Probability
%	Percentage
<	Lesser than
>	Greater than
mIU/ml	milli international units per milliliter
ng/ml	nanogram per milliliter
mg	milligram
mm	millimeter
ml	milliliter
Kg/m^2	kilogram per meter square
nmol/L	nanomol per liter
v/v	volume by volume

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Abstract

The present prospective and retrospective follow-up study on 102 nomogonadotropic oligoamenorrheic infertile female subjects (WHO group 2) registered at Federal Government Services Hospital, Islamabad and Islamabad Clinic Serving Infertile Couples in Islamabad Private Hospital Pvt. Ltd. Islamabad. The subjects selected were both with primary infertility (54.9%) and secondary infertility (45.1%) with polycystic and normal ovaries. The study was undertaken to determine whether clinical, endocrine and ultrasound characteristics could predict the chances of ovulation and conception in the subjects undergoing ovulation induction with Clomiphene Citrate (CC) medication. Additional inclusional criteria's were normal semen analysis and negative history for any tubal disease. All subjects underwent spontaneous or progestin induced withdrawal bleeding. Initial CC doses were 50mg daily for 5 days starting on day 2 of menstrual cycle. In case of an absent ovarian response doses were increased to 100, 150 and 200mg daily in subsequent menstrual cycles. The study was followed-up to four treatment cycle with daily CC doses of 200mg in 276 treatment cycles, 102 (36.9%) were ovulatory cycles. A cumulative conception rate of 33.3% was attained. The conception rate in case of subjects with polycystic ovaries was 40.6% while with normal ovaries was 30%. The subjects who conceived were young and with early menarche ($P<0.05$). The subjects who conceived had shorter duration of infertility ($P<0.04$). Those who conceived did so in the first 2-3 cycles. Patients with elevated day 3 serum LH concentration (>8mIU/ml) had a higher probability of conceiving. The LH: FSH ratio was <1.65 in the subjects who conceived. Serum FSH levels on day 3 were within normal limits (1-10mIU/ml). The mean follicular number and follicular size assessed by Transvaginal Ultrasound (TVS) were significant ($P<0.00002$) and ($P<0.0006$) in the subjects who conceived than in the CC failure. Subjects who did not conceive with higher doses of CC are Clomiphene resistant and do not ovulate (CRA). Follicular growth and follicular number improved during induction of ovulation with CC.

These observations suggest that there is more to be gained from CC ovulation induction in young subjects presenting with infertility (WHO group 2). It can be concluded that increased serum LH and LH : FSH ratio along with age, duration of infertility and cycle history predict chances of conception in WHO group 2. It is a step forward in decision making in the treatment of these subjects.

Introduction

Infertility or the inability to have children, affects both men and women of reproductive age in all parts of the world. For the individual infertility has profound social and personal implication. Besides the strain of personal failure, sometimes a tragedy, the infertile couple is often exposed to a variety of family and social pressures.

The problems of infertility have assumed an increased importance in health care system in recent years. Community awareness of newer reproductive technologies, together with the expectations of couples that they have the inherent right to determine their reproductive destiny, have led to an escalating demand for fertility–escalating services (Belsey, 1986).

The basic level of management of infertile couple involves comprehensive and expert investigations of both partners. This should achieve the composite aims of establishing diagnosis, formulating treatment, offering a prognosis and providing appropriate counseling (Dewhurts, 1995). Approximately one in six couples will seek medical help because they have been unable to conceive (Hull et al, 1985, Randall & Templton, 1991).

INFERTILITY

Infertility is defined as inability to conceive after 12 months of regular intercourse. The most common reasons for infertility are attributed to male factor, tubal factor and ovulatory dysfunction (Mitwally et al, 2003).

Infertility can be divided into primary and secondary infertility according to WHO scientific group on the epidemiology of infertility 1975.

Primary infertility

The woman has never conceived despite cohabitation and exposure to pregnancy for a period of two years.

Secondary infertility

The woman has previously conceived, but is subsequently unable to conceive, despite cohabitation and exposure to pregnancy for a period of two years.

Ovulation

Assessment of fertility deprivation requires the assessment of ovulation and hormone production. Ovulation is shedding of ovum from the ovarian follicle and not merely folliculation, whereas hormone assessment can be an indirect evidence of ovulation but cannot ensure ovulation (Aziz et al, 1998). Sonographic imaging can be used as a reliable method of detecting and predicting ovulation (O'Herliby et al, 1980), including fluid in the cul-de-sac and absence or collapse of previously described follicle, a decrease in follicular diameter, a blurring of the follicular diameter (Paulson et al, 1984, Cedars et al, 1995)

No women ovulate normally in every cycle. Hundred women were chosen randomly, 25-0% of them ovulated abnormally in any one menstrual cycle (Ahmed, 1998). If the women has a problem in one cycle but is completely normal in the next cycle, then that problem probably either does not exist or is of lesser significance in terms of her overall infertility situation (Birnbaum, 2000).

Normally ovulation is established within 1-2 years of menarche. Maximal reproduction efficacy is established by the age of 15 years and is maintained for approximately 20

years. Reproductive efficacy begins to wave in late 30's and perimenopause begins in the early to mid 40's (Jacobs et al. 1990).

Anovulation

Anovulation is the absence of ovulation and is the frequent cause of infertility. These women usually present with oligo or amenorrhea. In the great majority of infertile women presenting with cycle abnormalities, approximately 80% of females present with serum FSH and estradiol (E_2) levels within normal limits. These patients are referred to as world Health Organization Group 2 (WHO group 2) indicating a "pituitary–ovarian disbalance" (Imani et al, 1998). The WHO Group 2 females are normogonadotropic oligoamenorrheic infertile subjects. Women within this heterogeneous group may in addition to anovulation present with obesity and hairustism. Various endocrine abnormalities such as elevated serum LH hormone levels, hyperandrogenemia and insulin resistance, along with abnormalities in the insulin like growth factors have been observed (Pache et al, 1991). Moreover polycystic ovaries (PCO) may be found (Laven et al, 2001).Forty-70% of WHO Group 2 patients can be diagnosed as PCOS (Pache et al, 1992). This poorly defined syndrome is believed to be quite frequent among the female population. The incidence is 5% (Van Santbrink et al, 1995). They have a greater ovarian volume on Transvaginal scan (TVS). Excessive LH secretion may be responsible for the abnormal follicle dynamics encountered in women with PCOS (Fillicori, 1999, Eijkemans et al, 2003). The standard treatment for WHO Group 2 patients is ovulation induction. There are many options for ovulation induction, treatment should be individualized but Clomiphene Citrate (CC) in incremental doses is an excellent first line agent followed by exogenous FSH in cases of failure to ovulate or conceive (Imani et al, 1999, Wolf, 2000. Eijkemans et al, 2003).Thirty five years after the first clinical introduction CC still remains the first line treatment strategy in normogonadotropic oligoamenorrheic infertile female subjects (WHO Group 2). Rising serum FSH levels due to CC interference with estrogen negative feedback may be held responsible for

stimulating follicle growth (Greenblatt et al, 1961, Jacobson et al, 1968, Miyake et al, 1983, Kerin et al, 1985). A significant proportion of treated women do not respond (Imani et al, 1998).

Clomiphene resistant anovulation

Twenty to 25% of women, WHO Group 2 show Clomiphene resistant anovulation (CRA), that is, they remain anovulatory even after multiple attempts of ovulation induction with increased doses of CC. About 50% of ovulatory CC patients conceive within six CC induced treatment cycles. Of the women who fail to conceive with CC treatment, approximately 90% will ovulate and 50% will conceive subsequently following FSH ovulation induction (Fauser et al, 1997).

Steroidogenesis

The gonadotrophic hormones, FSH and LH, play a critical role in the growth of ovarian follicles. Gonadotrophic levels are low in the prepubertal period (Gulyas et al 1977). It is at the time of puberty that gonadotrophins begin to exert their full stimulatory action. The final development of ovarian follicle begins approximately 85 days before ovulation (Gougeon 1986, Gougeon et al, 1987). The follicles start to grow more rapidly in the luteal phase of the cycle preceding ovulation. The selective rise in FSH levels that occurs during the luteal–follicular transition is a potent stimulus for early antral follicles to enlarge in this phase of the cycle. FSH simulates promotion of GC growth and proliferation and stimulation of estrogen secretion (Ryan et al, 1966, Ryan et al, 1968). Theca cells contain LH receptors which is capable of stimulating androgen substrate production to be transformed into estrogen (Winter et al, 1975). FSH is required to induce the aromatase system. Estrogen is also produced by small antral follicles, elevated amount of this steroid drive mostly from large preovulatory follicle (Fritz et al, 1982).

Estrogen appears to protect the growing follicle from androgen induced atresia (Botero et al. 1984).

Ovulation Induction

Ovulation induction is the most common medical intervention for the treatment of infertility (Collins et al. 1995). Drugs for the treatment of infertility are used chiefly to stimulate ovulation. Ovulatory disorders, oligoamenorrhea and irregular ovulation, are the primary problem in 25-30% of couples (Kliger et al, 1984, Harrison, 1980, West et al, 1982). Fewer than 5% of infertile female subjects have amenorrhea, defined as the absence of menses for 6 months or more. Approximately one fifth of women with anovulatory disorders have a hyperandrogenic state, WHO Group 2 associated frequently with polycystic ovarian syndrome (PCOS), menstrual cycle disturbance, evidence of endogenous estrogen production and normal levels of prolactin and serum FSH. Many of these women have clinical or biochemical evidence of increased androgenicity (Insler.v, 1988). These patients respond less readily to ovulation induction treatment (Fluker et al, 1994). In women with anovulation and PCOS there are many options for ovulation induction, treatment should be individualized (Wolf, 2000). There are other causes of disorders of ovulation which include hyperprolactinemia, primary hypothalamic failure and defects of follicle maturation (Aslam et al, 1992). Induction of ovulation is primarily indicated for women with amenorrhea or oligomenorrhea (Soliman et al, 1994).

There are four basic type of medication that is used to induce ovulation.

1 Clomiphene Citrate (CC).
2 Injectable Gonadotrophine.
3 GnRH pump.
4 Bromocreptine.

Success rates for induction of ovulation vary considerably and depend on the age of the women, the type of medication used, whether there are other infertility factors present in the couple (Collins et al. 1993, Calaldo, 1998).

Ovulation induction is also used in the ovulatory infertile women to generate multiple mature follicles with the intent of increasing the likelihood of multiple ovulation and fertilization. The goal in ovulation induction is to generate 1 or 2 mature follicles (Collins et al. 1995).

Clomiphene Citrate (CC) is the most widely easy to use convenient, inexpensive and safe first choice medication for ovulation induction in normogonadotropic oligoamenorrheic infertile (WHO Group 2) female subjects As in WHO manual for the standardized investigations and diagnosis of infertile couples 1993 and Cataldo, 1998. The food and drug administration approved CC for human use in 1967 (Adashi, 1996). CC has been used for more than 30 years for the induction of ovulation and remains the most commonly used drug in the treatment of infertility (Fritz et al, 1991). CC therapy can be initiated on days 2-5 of the menstrual cycle without causing a significant difference in therapeutic outcome. Clomiphene is traditionally administered for 5days beginning on cycle day 5 (Cataldo et al, 1998), on cycle day 3-7 (Imani et al, 1999), on cycle day 2-6 (Hughes, 2000) following a spontaneous or progestin induced withdrawal bleed.

The administration of CC is followed by an enhanced release of pituitary gonadotrophic resulting in follicular recruitment. After the drug is stopped, there is continuing secretion of estradiol, selection of the dominant follicle and in successful cases ovulation. CC is indicated as the initial treatment in the majority of women with amenorrhea and oligomenorrhea. In women with irregular ovulation it seems to re-establish typical frequency of ovulation (Hughes et al, 2000)

Increased body mass index (BMI) is the only factor which is consistently associated with a decreased response to CC. It follows therefore that weight reduction should be an important part of therapy in anovulatory women. Increased serum LH level is related with adverse pregnancy outcome in women who conceive (Paulson et al. 1989, Kausta et al. 1997). Several studies have linked LH with detrimental effects on reproductive function. such as irregular menstrual cycle, anovulation, infertility and miscarriage (Shoham et al, 1993). Serum LH levels increases progressively during the normal follicular phase of the menstrual cycle and play an important physiological role in follicle steroidogenesis and development as well as in the mechanism of selection of the dominant follicle. Excessive LH secretion frequently is encountered in patients with PCOS (Waldstreicher et al, 1988). This excessive LH secretion may be responsible for abnormal dynamics encountered in women with PCOS (Yoshimura et al, 1986). The ratio of LH:FSH has been used as a useful laboratory indicator of inappropriate gonadotrophin secretion for the diagnosis of PCO (Lobo et al, 1981).

The staring dose of CC is 50mg daily. CC produces a rise in circulating LH and FSH levels which is followed by a rise in estradiol level accompanying follicular maturation both in cycling women and those with PCOS. CC increases the pulse frequency of LH and FSH (Kerin et al, 1985, Kettel et al, 1993). If ovlation does not occur in the first treatment cycle. dose is increased by 50mg in successive cycles until ovulation is achieved or a maximal dose is reached usually 150 to 250mg/day. As many as 30% women may fail to ovulate. A higher failure rate is in obese women (Lobo et al. 1982). Non responders have greater ovarian volume on USG and greater number of small follicles (Ficiciogle et al, 1996).

A course of 3-6 ovulatory cycles is usually sufficient to know whether pregnancy will be achieved using CC. fifty-75% of pregnancy achieved with CC occur within first 3 cycles of treatment (Gorlithsky et al, 1978, Gysler et al, 1982). Ovulation is rstored in 80% but will result in pregnancy in only 35-40% (Mac Gregor et al, 1968, Imani et al.

2002). Additionally around 20-25% of anovulatory women with PCOS will not respond at all and considered as CRA (Franks et al, 1996, Imani et al, 1998).

Body mass index

Body mass index is a reliable and convenient indication of body fat. It is derived by dividing body weight in Kg with square of height in meters. Values above 25 are abnormal. Individuals with values of 25-30 are overweight and those with values greater than 30 are obese (Ganong, 1993). But Imani et al (1998) has considered the BMI greater than 18 to be a criteria for inclusion in studies. BMI forms an important indication for predicting the response to stimulation by FSH due to CC. Patients with a raised BMI are less likely to respond to stimulation by FSH due to CC. A positive correlation between body weight and dose of CC required to induce ovulation has been established. Recent study has indicated that increased BMI is the only initial parameter that is significantly different between responders and non responders (Kousta et al, 1997). The correlation between BMI and ovarian response after CC treatment suggests that much emphasis should be focused on weight reduction though scientific proof for this approach is lacking, and that weight reduction may not necessarily result in normal response (Pache et al, 1992)

CLOMOPHENE CITRATE

Clomiphine citrate used for inducing ovulation, occupies an important position in the therapeutic repotoire of infertility treatment. CC is a synthetic non-steroidal compound that binds to estrogen receptors in various receptors in various tissues such as the hypothalamus, hypophysis, ovaries and the uterus and cervix. However unlike E_2, CC is unable to induce the synthesis of new E_2 receptors a process essential for the continuous binding of E_2 to the target cells as well as the expression of estrogenic action. It binds to the E2 receptors in the hypothalamus to create a state of hypoestrogenicity and

decreasing the amount of available estrogen receptors and causing the pituitary to increase secretion of FSH and LH (Kausto et al, 1997). After the drug is stopped there is continuing secretion of oestradiol, selection of the dominant follicle, and in successful cases, ovulation. (Collins et al, 1995).The available pharmaceutical preparation of Clomiphene citrate is a active and inactive isomers. Clomiphene is chemically related to Chlorotrianisene (sex hormone) and has both estrogenic and anti-estrogenic properties, the latter residing principally in the E-isomers (Speroff et al, 1999).

Clomiphene Citrate is an easy to use, convenient, inexpensive and safe first choice medication in normogonadotrophic oligo/amenorrhic infertility (WHO Group 2) (Dickey et al, 1996, Dickey and Holtamp, 1996, Imani et al, 1999). In women with irregular ovulation it seems to re-establish typical frequency of ovulation. Ovulation usually occurs within 5-10 days after thelast CC tablet is taken (Tarlatzis and Grimbizis, 1998). Cumulative pregnancy rates after Clomiphene Citrate treatment between 37-97% have been reported. It is well recognized that pregnancy rates achieved with CC is significantly lower than the ovulation rates (Scialli et al, 1986). The discrepancy between ovulation and pregnancy rates may be due to the antiestrogenic effect of CC on the cervical mucus and endometriium. Limited information is available however concerning the predictive value of initial screening characteristics for treatment outcome (Imani et al, 1999).

Therapy with CC will not be successful unless the woman though anovulatory is capable of ovulation and her partner is fertile. It is ineffective in primary pituitary or primary ovarian failure. It supposedly competes with natural estrogen for sites on specific estrogen binding proteins and blocks their inhibitory action on the hypothalamic-pituitary system (Jeffcoat's, 1987).

CLINICAL PROTOCOLS / DOSAGE

The initial dosage of CC is 50mg/day for 5 days beginning on the 3-5 day after the start of menstruation. If this is not successful the dosage may be raised to100mg/day for 5 days and subsequently to 150mg/day.If ovulation is confirmed at a dosage of 50mg/day, however, an increase in dosage is not subsequently indicated and if patients do not respond to 150mg/day for 5 days further evaluation and a change of therapy may be indicated (Collins et al, 1995). Doses of up-to 200mg daily may be given, depending on response and body weight (Dewhurts, 1995). Only six courses are recommended in succession (Eijikmans et al, 2003).

CLINICAL EFFECTIVENESS

In well selected cases CC induces ovulation in 70% but conception does not always result. Pregnancy occurs in 40-50% of women following treatment and even though the dosage is carefully controlled, 5-10% of the conceptions are multiple (Jeffcoat's, 1987).

ADVERSE EFFECTS

CC can produce a number of adverse effects. Foremost amongst them is over stimulation of the ovary resulting in reversible ovarian enlargement and cyst formation. Multiple pregnancies occur in 8-10% of CC induction and also there is increased risk of ectopic pregnancy. Other effects include hot flushes in11% (Hughes et al, 2000, Kausta et al, 1997) of patients and visual symptoms including blurring of vision and diplopia in 1-3%

(Collins et al. 1995). Clomiphene use is also related with increase risk of ovarian cancer which is mainly attributed.

In the present study an attempt has been made to assess the response of normogonadotropic oligoamenorrheic infertile (WHO Group 2) female subjects after ovulation induction with Clomiphene Citrate.

Subject And Method

This prospective and retrospective study was carried out at Federal Government Services Hospital, Islamabad and Islamabad Clinic Serving Infertile Couples in Islamabad Private Hospital Pvt. Ltd. over a period of two years from January 2000 to December 2002. A total of 102 couples were included, out of which 42 couples were selected prospectively and 60 couples were taken out from the files of Islamabad Clinic Serving Infertile Couples. These couples presented with normal cycles, oligomenorrhea (bleeding interval between 35 days and 6 months) or amenorrhea (bleeding interval > 6 months) and infertility were included in the present study. Complete history of each subject was taken which included age, duration of childlessness, years of living together, primary or secondary infertility, cycle history, any history of contraception, gynecological complaint and previous treatment for infertility. General physical as well as systemic examination was performed along with pelvic examination and BMI.

Additional Inclusional criteria's were :

1: Serum FSH level on 3rd day of the cycle, within normal limits and normal serum prolactin.

2: Spontaneous menses or positive bleeding response to progestagens withdrawal.

3: Ovulatory cycles and anovulatory cycles after CC induction for ovulation.

4: Age between 19 and 42 years.

6: Sperm analysis of the male partner with in normal limits.

7: There was a negative history for any tube pathology.

8: There was no indication for intra uterine insemination (IUI).

Standardized, clinical, endocrine and sonographic screening was undertaken before initiation of ovulation with CC medication. Ovulation with CC treatment was assessed by transvaginal sonographic monitoring of follicular growth until visualization of a preovulatory follicle (mean diameter > 18mm) and subsequent disappearance (Imani et al 1999).

TREATMENT PROTOCOL

Clomephene citrate (Duinum 50mg Medochemie, Thailand and Clomid 50mg Aventis, Belgium) was administered at a daily dose of 50mg (increased to 100, 150 and 200mg in subsequent cycles in the case of absent ovarian response) from cycle days 2 – 6 after initiation of spontaneous or progestin induced withdrawal bleeding. Conception was used as the end point. Duration of follow-up for all subjects included in the study was at least 2 – 4 cycles. All subjects underwent regular follow-up during follicular, ovulatory and luteal phases of one reproductive cycle keeping in mind the length of cycle. Regular ultrasonographic (USG) scans were performed, in women with regular 5/28-30 days cycle length on or around 10^{th}, 14^{th} and 21^{st} days of the menstrual cycle. Those with irregular cycles were followed accordingly. Ovulation was assessed by Transvaginal sonographic (TVS) monitoring of follicular growth as described previously. Responders were defined as subjects who ovulated during CC therapy, independent of the dose administered. The number of treatment cycles and the CC dose in which first ovulation occurred were recorded. Clomiphene resistant anovulation (CRA) was defined as patients who do not ovulate despite receiving CC 50, 100, 150, up-to 200mg in four consecutives cycles.

DATA SHEET

Date :

Name :

Husband's name :

Husband's occupation :

Female's occupation :

Age at presentation :

Date of Marriage :

Duration of living together (yrs) :

Duration of infertility (yrs)

Age of menarche (yrs)

Type of infertility :

Primary infertility :

Secondary infertility :

Menstrual cycle :

Duration :

Flow :

Pain :

Obstetrics History :

Gravida :

Para :

Abortion :

Live birth :

Still birth :

General examination :

Height :

Body weight :

General condition :

Abdomen :

Respiratory system :

Cardiovascular system :

Gastrointestinal system :

Pelvic exanunation :

Vulva / vagina :

Discharge :

Cervix :

Adenexa

INVESTIGATIONS

Hormonal profile :

FSH :

LH :

Prolactin :

Progesteron :

Semen Analysis

Volume :

Count :

Motility :

Morphology

Hysterosalphingography / Laproscopy :

Ultrasound :

BLOOD SAMPLE :

Blood sampling for baseline hormonal profile was undertaken to asses the individual reproductive status. Sample of peripheral venous blood was collected with the help of a disposable syringe from the cubital vein of the arm. The sample was collected on the 3rd day of the menstrual cycle for serum FSH, LH and prolactin and on 21st day of the menstrual cycle for serum progesterone. Blood was centrifuged within 24 hours of

withdrawal and serum was separated at 590-600 g for five minutes and stored at -20^{0}C until hormonal determination.

HORMONAL ASSAY:

Serum FSH, LH, Prolactin and Progesterone were determined by using commercially available enzyme linked immunosorbent assay (ELISA) kits Abbot AXSYM System (Abbot.Laboratories Diagnostics Division Abbot Park, IL 60064. USA.)

PROTEIN ASSAY

Quantitative determination of the protein hormones (FSH, LH & prolactin) was done by AXSYM instrumentation, which is a microparticle enzyme immunoassay.
Antigen – Antibody reaction, detected through disintegration of 4–methylumbeliferyl phosphate (MUP) into fluorescent methylumbeliferone (MU). The rate of production of MU is measured which determines the quantity of the particular analyte.

STEROID ASSAY

In quantitative determination of steroid hormone Progesterone by ELISA, a high affinity monoclonal antibody in P serozyme assay, was used which incorporated magnetic resonance phase separation. Hormone (Ag) present in the sample binds to monoclonal antibody (Ab). This results in the formation of Ag-Ab + Ag-Ab. Anti-fluorescein coupled to a magnetic solid phase was added in excess. The intensity of the colour developed was inversely proportional to the concentration of the hormone present

PRINCIPLE Of ELISA PROCEDURE FOR SERUM FSH, LH, PROLACTIN AND PROGESTERONE.

The samples and all AXSYM reagents, FSH, LH, Prolactin and Progesterone reagents for one test are pipetted by the sampling probe into various wells, of a reaction vessel (RV). The RV is immediately transferred into the processing center. Further pippetting is done in the processing center with the processing probe. Sample, anti Beta FSH, LH and Prolactin coated micro-particles and TRIS Buffer are pippetted into one well of RV. FSH, LH and prolactin binds to the Anti beta coated micro-particle forming an antibody-antigen complex. This antibody - antigen complex micro-particle bind irreversibly to the glass fiber matrix. The matrix is washed to remove unbound materials. The anti a subunit specific Alkaline Phosphatase is dispensed onto the matrix cell and binds with the antibody – antigen complex. The matrix cell is washed to remove unbound materials. The substrate 4-methylumbeliferyl phosphate is added to the matrix cell and the fluorescent product is measured by the MEIA optical assembly.

ELISA PROCEDURE FOR THE SERUM FSH, LH, PROLACTIN AND PROGESTERONE

Serum samples (0.05-0.15ml) in disposable round bottom (12x75mm) polystyrene test tubes were incubated with 0.2ml of the enzyme conjugate (fluorescein and bovine alkaline phosphatase labelled mouse monoclonal antibody to the protein or steroid hormones in Tris buffer with horse and bovine serum protein at 37^0 in a clean water bath. The incubation time varied with the type of the hormone to be measured i.e 15 minutes for FSH, LH and Prolactin (protein hormone), and 15 minutes for Progesterone with 0.2ml derivative (fluorescein labeled steroid in Tris buffer). After incubation, 0.2ml of thoroughly mixed separation reagent (sheep antiserum to fluorescein, covalently bound to magnetizable particles in Tris buffer containing bovine serum albumin and sodium azide) was added to each tube and incubation for 5 minutes for each hormone except for

progesterone for which the incubation was 10 minutes at 37^0 in a water bath. These incubations were followed by washing. The tube rack was fixed on a magnetic separator and particles were allowed to sediment for two minutes magnetically. The supernatant was decanted and 0.5ml of dilute wash buffer solution (a surfactant and preservative in Tris buffer) was added to each tube. Thorough mixing was performed to assure good assay performance. The rack of tubes was again fixed on a separator and particles were allowed to settle down. This washing was repeated in case of FSH, LH and Prolactin. The tubes were removed from the magnetic base and 0.3ml of serozyme substrate solution (Phenolpthalein monophosphate, an enzyme co-factor was dispensed into each tube including the blanks. The tubes were shaken and incubated for 15 minutes. After the last incubation, 0.1ml of serozyme stop solution (sodium hydroxide and a chelating agent in buffer solution, pH>10) was added into each tube including the blanks. The rack containing the tubes was fixed to the magnetic separator and particles were allowed to settle for at least 10 minutes. The tubes were then measured by the MEIA assembly.

NORMAL SERUM FSH, LH, PROLACTIN, P LEVELS IN DIFFERENT PHASES OF MENSTURAL CYCLES

Phases of Menstrual Cycle	**Serum FSH (mIU/ml)**	**Serum LH (mIU/ml)**	**Serum Prolactin (ng/ml)**	**Serum Progesterone (ng/ml)**
Follicular	3 –20	2 –15	1.9 –25.9	<0.3 –1.1
Ovulatory	9 –26	22 –105	1.9 –25.9	0.4 -3
Luteal	1 -12	0.6 -19	1.9 –25.9	1.8 –21

SEMEN ANALYSIS

Semen analysis was done under WHO criteria (WHO laboratory manual for the examination of human semen and cervical mucus interaction, 4th edition, 1999, ISBN 0521645999, Cambridge University Press). When investigating infertility, the basic analysis of seminal fluid usually includes:

1: Measurement of volume.

2: Measurement of pH.

3: Examination of a wet preparation to estimate the percentage of motile spermatozoa and viable forms and to look for cells and bacteria.

4: Sperm count.

5: Examination of a stained preparation to estimate the percentage of spermatozoa with normal morphology.

1: Measurement of volume:

Normal semen is thick and viscous when ejaculated. It becomes liquefied usually within 60 minutes due to a fibrinolysin in the fluid. Normal volume is usually 2ml or more.

2: Measurement of pH:

Usually a narrow range pH paper e.g 6.4–8.0, spread a drop of liquefied semen on the paper and record after 30sec. should be 7.2 or more. If more than 7.8 then infection, if less than 7 then no sperm.

3: Estimation of the percentage of motile and viable spermatozoa:

Motility:

Place one drop of well mixed liquefied semen on a slide and see under low power and high power. See several fields to assess motility whether rapid or weak. Count a total of 100 spermatozoa and note out of the hundred how many are motile. Normally over 50% of spermatozoa are motile.

Viability:

Mix a drop of semen with one drop of 0.5% eosine solution after 2 minutes count the viable and non viable spermatozoa. Viables remain unstained and non viables stain red. Normally 75% or more should be viable.

Sperm count:

Using a graduated tube mix the semen, 1 in 20 sodium bicarbonate–formaline. Using a pasture pipette, fill a neubauer chamber and count in two large squares and multiply the number by 100,000. Normal is $20x10^6$ spermatozoa / ml.

Estimate the percentage of spermatozoa with normal morphology:

Make a thin smear fix it with 95% v/v ethonal for 5-10 minutes and allow to air dry. Wash it with sodium bicarbonate formalin solution and rinse it with changes of water. Cover the smear with carbon fuchsin (1 in 20) wash with water, counter stain with loeffler's methylene blue for 2 minutes, wash with water and air dry. Normally 50% of spermatozoa should be normal.

SEMEN ANALYSIS

Parameters	Normal range
Volume	2 – 6 ml
Colour	
Consistensy	
Time of liquefaction	15 – 20 minutes
pH	6.4 – 8
Total count	20 – 250 x 10^6 / ml
Motility Active, Sluggish, immotile	50%
Morphology	50% normal

ULTRASOUND

Ultrasonography schedule has been designed so as to provide max information about the individual's status. Ultrasonosgraphy was done by Toshiba ultrasound diagnostic system Capasee model SSA-220 A. Transabdominal ultrasonography was performed with convex abdominal probe of 7 MHZ frequency model PVG-366V, while for transvaginal sonography convex endovaginal probe of 6 MHZ frequency model PVG-601V was used. Transaabdominal ultrasonography was performed to note gross structural abnormalities of uterus and ovaries while serial transvaginal sonography was performed to measure follicular size and its growth. Ovulatory and post ovulatory follicle was seen. Follicular rupture and ovulation was observed after induction with CC. The growing follicle can be recognized as a small cystic structure with well defined borders 8-10mm in diameter. The daily mean growth rate of the follicle is 2-3mm and represents an indication of normal follicular growth. The ultrasound was done in the late follicular phase 8-10th day of the cycle, in the preovulatory phase i.e. on the 14th day of the cycle and in the leuteal phase i.e. on the 21st day of the cycle. The growth of the follicle was observed. The mean reported pre-ovulatory diameter of the dominant follicle varied, usually between 18-23mm. After ovulation had occurred a sudden change in the appearance of the follicle could be observed. There could be a complete collapse of the follicle or appearance of the cyst or there could be fluid in the pouch of Douglas. Because of the higher resolution the transvaginal transducer makes possible detection and measurement of the above mentioned cycles with greater precision.

HYSTEROSALPHINGOGRAPHY

This procedure was done to check the tubal patency. It is carried out best during 7-10 days following the conclusion of a menstrual period. It entails injecting a non irritant radio-opaque material through the cervix into the uterus and tubes. The whole procedure is conducted on x-ray table in the radiological department. With the cannula fitted to the cervix and the patient lying on her back in the lithotomy position, the radio paque material is injected slowly from a syringe, the amount required varies from 2-20ml. For efficient studies it is essential to watch the flow through the uterus and tubes by screening, films being exposed at suitable intervals.

LAPROSCOPY

In this procedure a special endoscope is inserted under local or general anesthesia through the abdominal wall. For this to be done safely CO_2 is injected first into the peritoneal cavity. The operator must be reasonably certain that the gut is not adherent to the anterior abdominal wall at the site of entry of the trocar. The CO_2 is introduced either through the abdominal wall by needle or through uterus and tubes. The patient is than tilted head downwards and a tiny transverse incision made usually on the lower rim of the umbilicus. The trocar with its sleeve is inserted through this and made to enter the tense peritoneal cavity about half way between the umbilicus and symphysis pubis. The trocar is than replaced by the endoscope. To bring the organs into better view the uterus can be manipulated by way of a volsellum placed on the cervix. At the end of the procedure gas is expressed by way of cannula and the skin lesion closed with a single clip or suture.

BODY MASS INDEX (BMI)

Body mass index is a reliable indicator of body fat. It is calculated by the following formulae, dividing body weight in Kg with square of height in meter.

$$BMI = \frac{\text{body weight in kg}}{\text{height meters square}}$$

BMI between 18 and 25 are considered normal. Patients with increased BMI do not show good results with CC induction.

DATA ANALYSIS

Distribution of characteristics in patients groups is presented as the mean ± SE. Students t-test was used for exploratory comparison of initial parameters between responders and non responders .Excel and statistica statistical packages (statistica version 5. Stat Soft. mc, 2325 East, 13th street, Tulsa. OK 74104, USA) were used for data analysis. Limit of significance was set at $P<0.05$.

$$\text{Cumulative pregnancy rate} = \frac{\text{no of pregnancy}}{\text{Total no of treatment cycles}} \times 100$$

$$\text{Pregnancy rate} = \frac{\text{no of pregnancy}}{\text{Total no of subjects}} \times 100$$

$$\text{Percentage of conceived} = \frac{\text{No of conceived}}{\text{Total no of subjects}} \times 100$$

$$\text{Percentage of CC failure} = \frac{\text{No of CC failure}}{} \times 100$$

Total no of subjects

Percentage of normal ovaries = No of normal ovaries x 100
Total no of subjects

Percentage of PCO = No of PCO x 100
Total no of subjects

Percentage of regular cycles = No of regular cycles x 100
Total no of subjects

Percentage of irregular cycles = No of irregular cycles x 100
Total no of subjects

Results

SUBJECT CHARACTERISTICS

In this study a total of 102 infertile female subjects were selected and treated with Clomephene Citrate (CC) from day 2-6 of the menstrual cycle to induce ovulation. These female subjects after ovulation induction with CC were divided into two groups.

Group i: Subjects who conceived after treatment with CC.

Group ii: Subjects who had CC failure. They failed to conceive after induction with CC. they may had ovulatory or anovulatory cycles respectively (table 1).

Table 1.

Number of female subjects treated with Clomephene Citrate, number and percentage of Subjects with polycystic and normal ovaries along with the type and duration of infertility.

Characteristics	All subjects	Conceived Number (%)	CC Failure Number (%)
Number of subjects	102	34 (33.3)	68 (66.7)
Number of PCOS	32 (31.4)	13 (40.6)	19 (59.4)
Number of normal ovaries	70 (68.6)	21 (30)	49 (70)
Type of infertility			
Primary infertility	56 (54.9)	20 (35.7)	36 (64.3)
Secondary infertility	46 (45.1)	14 (29.1)	34 (70.9)
Mean duration of infertility (yrs)	6.61±0.46	5.1±0.74	7.13±0.56*

All values are given as mean ± standard error (S.E).
Values in parentheses represent percentage.
*P<0.04

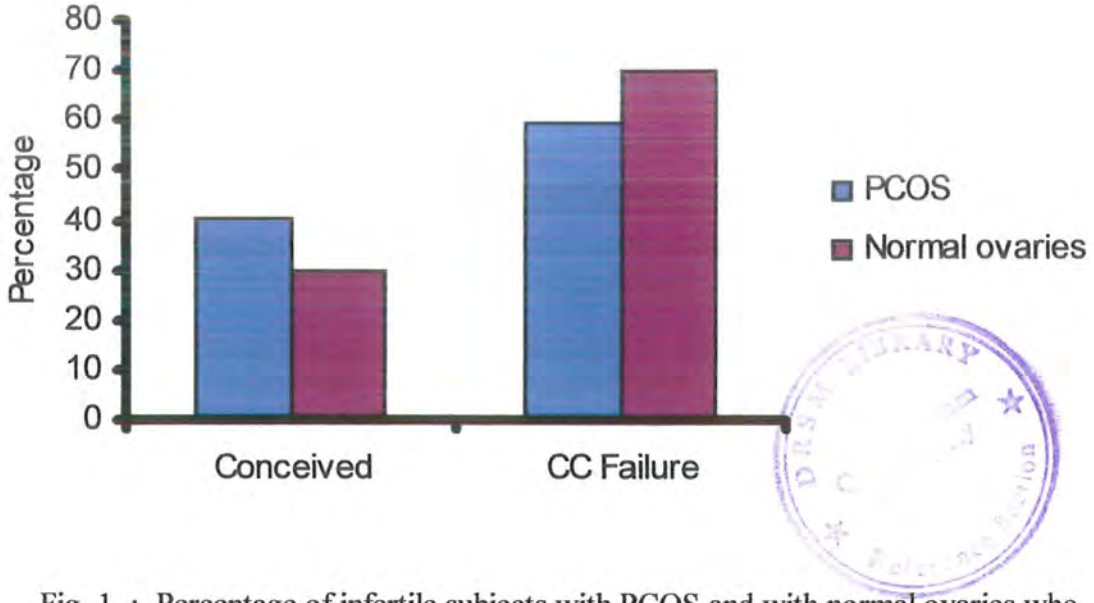

Fig 1 : Percentage of infertile subjects with PCOS and with normal ovaries who conceived with CC and with CC failure.

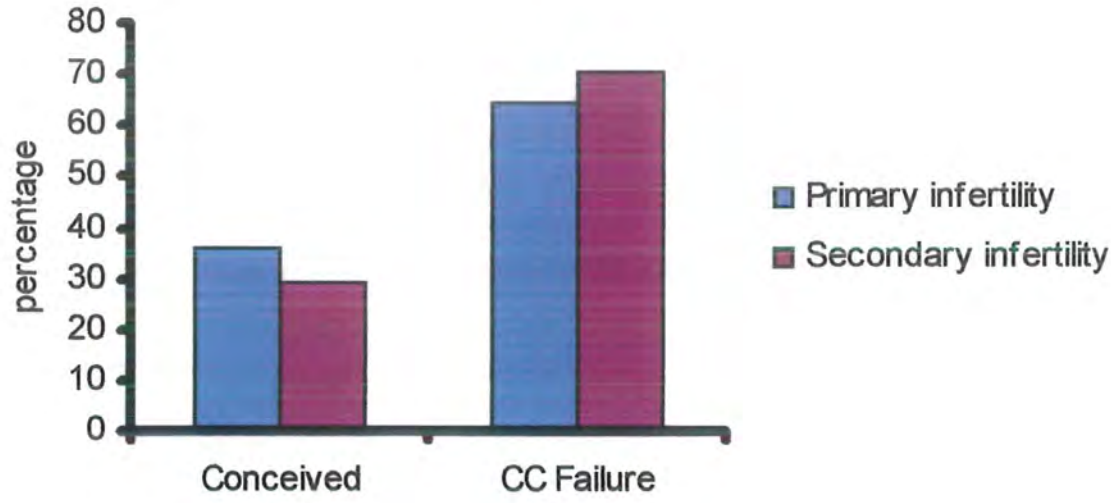

Fig 2 : Percentage of primary and secondary infertile female subjects who. Conceived with CC and with CC failure.

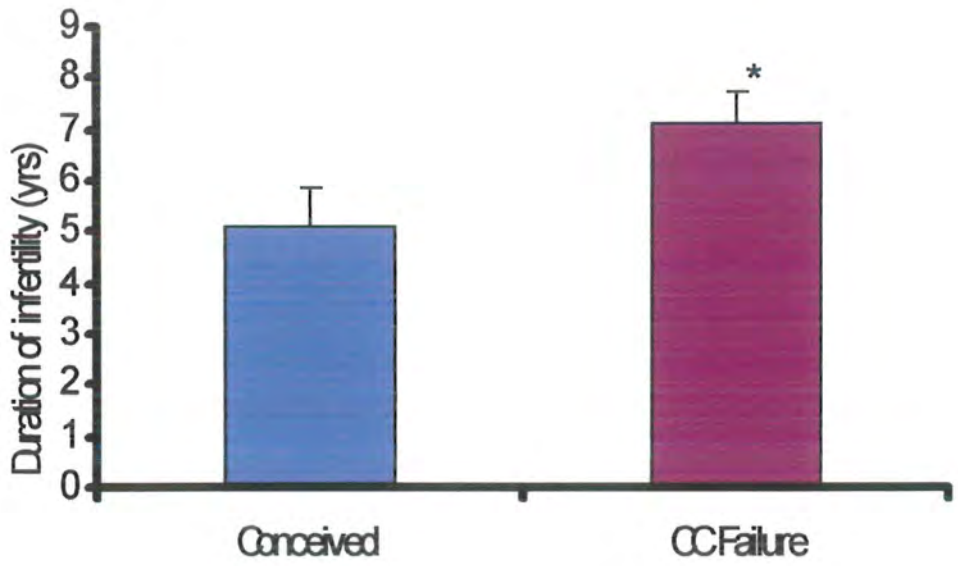

Fig 3 : Mean duration of infertility (yrs) of subjects who conceived with CC and with CC failure.
*P<0.04.

Total number of female subjects were 102, who underwent ovulation induction with CC. Conception was observed in 34 (33.3%) of female subjects while in 68 (66.7%) subjects was CC failure. The female subjects included in this study were both PCO and with normal ovaries as was observed on USG. The PCO subjects were 32 (31.4%) and with normal ovaries were 70 (68.6%) (table1). Ovulation induction with CC was performed in these female subjects. Out of 32 PCO subjects 13 (40.6%) conceived and 19 (59.4%) had CC failure. Out of 70 female subjects who presented with normal ovaries, 21 (30%) conceived after ovulation induction with CC and 49 (70%) ended up with CC failure (table1). In the present study both primary and secondary infertile female subjects were included. Ovulation induction was undertaken in 56 (54.9%) primary infertile subjects and 46 (45.1%) secondary infertile subjects. Out of 56 primary infertile subjects 20 (35.7%) conceived and 36 (64.3%) failed to conceive. Out of 46 secondary infertile subjects, 14 (29.1%) conceived and 34 (70.9%) ended up with CC failure (table1). Mean duration of infertility of female subjects at presentation was 6.61 ± 0.46 yrs. These subjects after ovulation induction with CC failure showed with mean duration of infertility of 7.13 ± 0.59 yrs. The female subjects who conceived have lesser duration of infertility of 5.1 ± 0.74 yrs. There was a significant ($P<0.04$) difference of the duration of infertility of the female subjects who conceived after ovulation induction with CC compared to the female subjects with CC failure (table 1).

TREATMENT CYCLES

Table 2

Mean number of treatment cycles per subject, number and percentage of ovulatory and anovulatory cycles after ovulation induction in female subjects with CC.

Characteristics	All subjects Number	(%)	Conceived Number	(%)	CC failure Number	(%)
Number of treatment cycles	276		63	(22.8)	213	(77.2)
Number of ovulatory cycles	102	(36.9)	63	(61.8)	39	(38.2)
Number of anovulatory cycles	174	(63.1)	-		174	(100)
Treatment cycle per subject	1.96±0.05		1.85±0.12		2.01±0.05*	

All values are given as mean ± standard error (S.E).
Values in parentheses represent the number.
Statistically significant.
*P<0.05

The total number of treatment cycles that were undertaken for ovulation induction with CC were 276, out of this total number only 63 (22.8%) helped in conception while 213 (77.2%) ended up with CC failure. In the CC failure both ovulatory and anovulatory cycles were included. Out of the total number of treatment cycles 102 (36.9) were ovulatory, out of these ovulatory cycles 63 (61.8%) helped to conceive and 39 (38.2) ended in CC failure (table 2). Out of the total CC failure cycles 174 were anovulatory. Mean number of treatment cycle per subject was 1.96+0.05. The female subjects who conceived had mean treatment cycles 1.85+0.12, while in the subjects who had CC

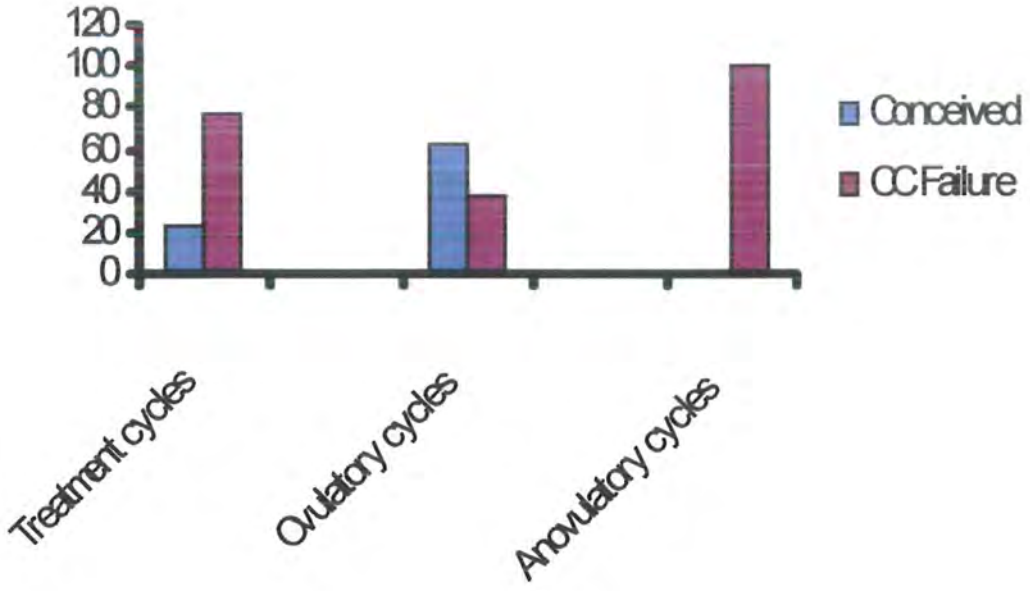

Fig 4 : Percentage of treatment cycles both ovulatory cycles and anovulatory cycles in subjects who conceived with CC and with CC failure..

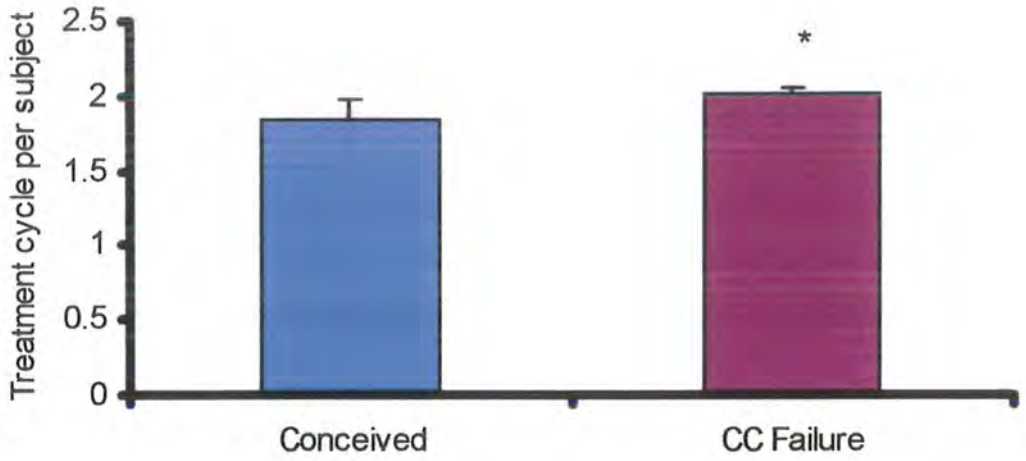

Fig 5 : Mean number of treatment cycles per subject, who conceived with CC and with CC failure.

failure had 2.01±0.05 mean treatment cycles. There was a significant (P<0.05) difference between the treatment cycles of female subjects who conceived and who had CC failure.

CLINICAL CHATACTERISTICS

Table 3.

Mean age (yrs) at presentation and menarche (yrs) in female subjects undergoing ovulation induction with CC.

Characteristics	All subjects	Conceived	CC Failure
Age at presentation (yrs)	28.4±0.5	27.09±1	29.04±0.66
Age at menarche (yrs)	13.2±0.11	12.9±0.17	13.38±0.14

All values are given as mean ± standard error (S.E).
Statistically significant.
[a]*P<0.05
[b]**P<0.04

Age at presentation, age at menarche and duration of infertility is shown in table 3. Mean age of female subjects at presentation was 28.4±0.5 yrs, the female subjects who conceived showed younger mean age of 27.09±1 yrs compared with CC failure subjects who had a mean age of 29.04±0.66 yrs. There was a non significant (P>0.05) difference in age at presentation of subjects who conceived compared to the subjects with CC failure (table 3). Age at menarche of the total number of female subjects undergoing ovulation induction with CC was 13.2±0.11 yrs. The female subjects who conceived after ovulation induction with CC showed an early onset of menarche at the mean age of 12.9±0.17 yrs, compared with the female subjects with CC failure at the mean age of 13.38±0.14 yrs. The mean age at menarche of female subjects who conceived compared with the female subjects with CC failure was statistically significant (P<0.05).

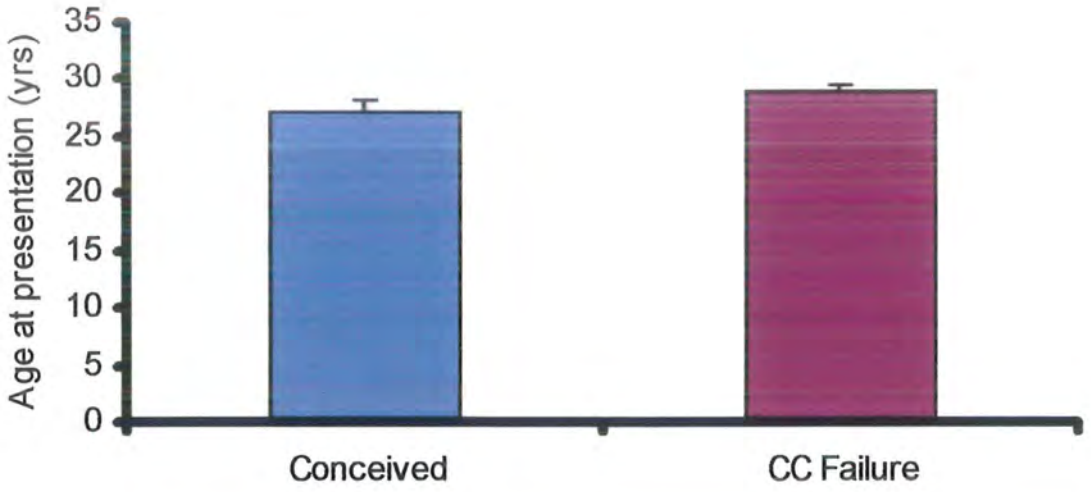

Fig 6 :Mean age at presentation in subjects who conceived with CC and with CC Failure.

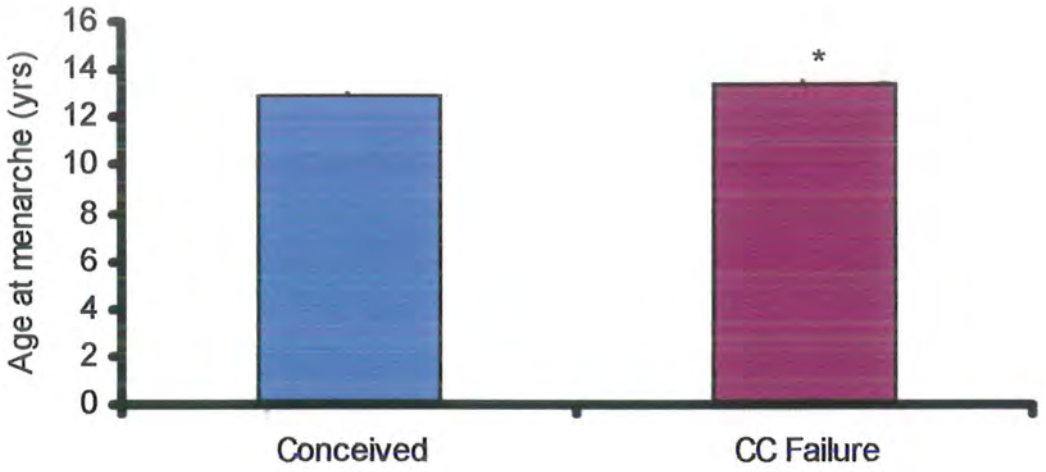

Fig 7 : Mean age at menarche (yrs) of female subjects who conceived after induction with CC and who failed to conceive.
*P<0.05

CHARACTERISTICS OF MENSTRUAL CYCLE

Table 4

Characteristics of the menstrual cycle. Duration of cycle, days of menstruation and regularity of cycles in females undergoing ovulation induction with CC.

Characteristics	All subjects		Conceived		CC failure	
Duration of cycle (days)	47.7±4.6		41.6±3.8		50.4±6.6	
Days of menstrual flow	4.7±0.23		4.9±3.8		4.6±0.25	
Regularity of cycle						
Percentage and number						
Regular cycles %	72.5	(74)	29.7	(21)	70.9	(53)
Irregular cycles %	27.6	(28)	39.2	(11)	60.8	(17)

All values are given as mean ± standard error (S.E).
Values in parenthesis represent the number.

Menstrual cycle of the female subjects undergoing ovulation induction with CC is shown in table 4. Mean duration of cycle of female subjects at presentation was 47.7±4.6 days, the subjects who conceived showed a lesser mean duration of cycle 41.6±3.8 days compared with CC failure subjects who had a mean duration of cycle 50.4±6.6 days. There was a non significant ($P>0.05$) difference in the duration of cycle of subjects who conceived compared with the subjects with CC failure. Mean days of menstruation of the total number of female subjects undergoing ovulation induction with CC was 4.7±0.23 days. The subjects who conceived after ovulation induction with CC showed a mean days of menstruation 4.9±0.52 days. While subjects with CC failure showed mean days of

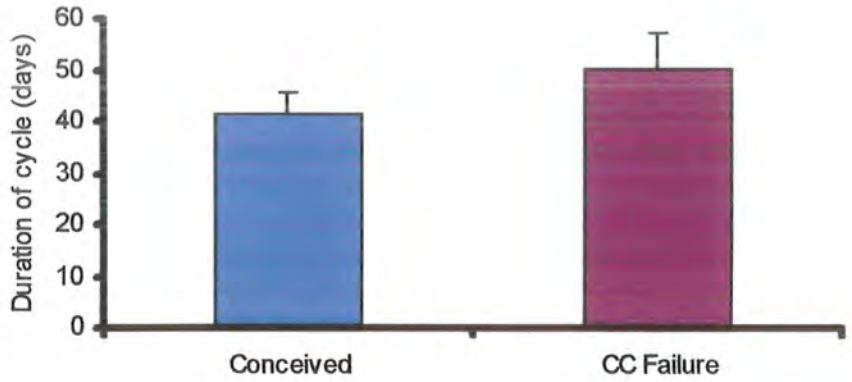

Fig 8 : Duration of menstrual cycle (days) in subjects who conceived with CC and with CC Failure.

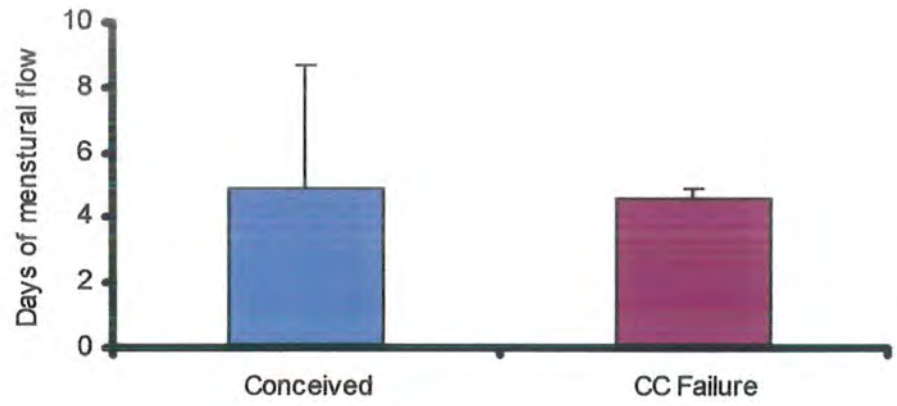

Fig 9 : Mean days of menstrual flow in subjects who conceived with CC and with CC Failure.

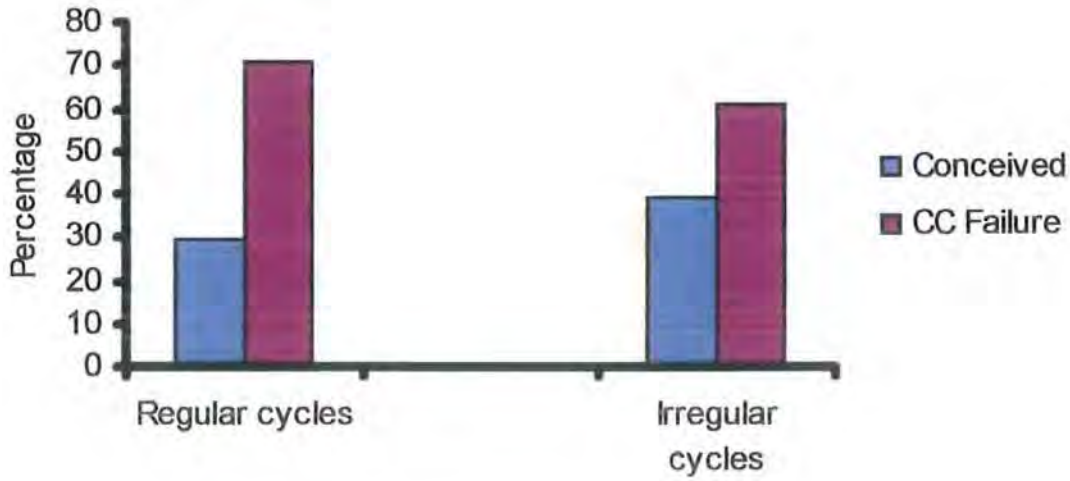

Fig 10 : Percentage of regular and irregular menstrual cycles in female subjects who conceived with CC and with CC failure..

conceived compared with CC failure subjects was statistically non significant (P>0.05). Regularity of the menstruation was observed in the subjects undergoing ovulation induction with CC. Number of subjects with regular cycles was 74 (72.50%). Out of the total number of subjects with regular cycles those who conceived was 21 (29.7%) and the female subjects who ended up with CC failure was 53 (70.9%). The total number of female subjects with irregular cycle was 28 (27.60%), the female subjects who conceived was 11 (39.2%) and the CC failure was observed in 17 (60.8%) of female subjects.

ENDOCRINAL PARAMETERS

Table 5

Endocrinal parameters mean serum FSH (mIU/ml), LH (mIU/ml), prolactin (ng/ml) levels of day 3 menstrual cycle of 102 infertile female subjects undergoing ovulation induction with CC.

Characteristics	All subjects	Conceived	CC Failure
Serum FSH mIU/ml	5.9±0.38 (46)	5.4±0.69 (13)	6.3±0.48 (33)
Serum LH mIU/ml	8.24±0.62 (46)	8.90±0.67 (13)	7.85±0.91 (33)
Serum prolactin ng/ml	11.4±0.71 (66)	11.4±1 (15)	11.4±0.89 (51)
LH : FSH	-	1.65	1.21
FSH ratio	-	1 : 0.91	1 : 1.07
LH ratio	-	1 : 1.08	1 : 0.95

All values are given as mean ± standard error (S.E).
Values in parentheses represent the number.
FSH follicle stimulating hormone, LH luteinizing hormone.

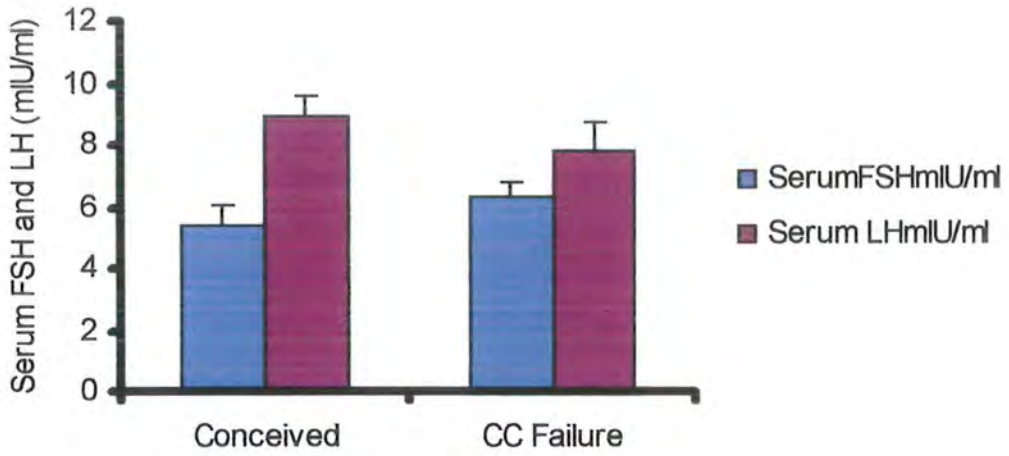

Fig 11 : Mean serum FSH and LH (mIU/ml) levels in subjects who conceived with CC and with CC Failure.

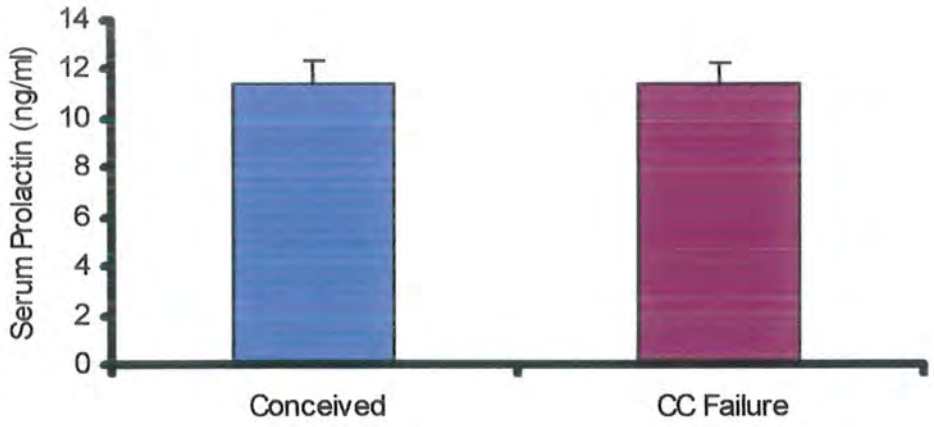

Fig 12 : Mean serum Prolactin (ng/ml) level in the subjects who conceived with CC and with CC Failure subjects.

Endocrinal parameters. mean serum FSH (mIU/ml), LH (mIU/ml) and prolactin (ng/ml) levels of day 3 menstrual cycle along with their ratio in the infertile female subjects undergoing ovulation induction with CC is shown in table 5. Mean serum FSH level at presentation of female subjects undergoing ovulation induction with CC was 5.9±0.38mIU/ml. It was observed in the subjects who conceived that the mean FSH level was less 5.4±0.69mIU/ml compared with the CC failure subjects who had a mean FSH level of 6.3±0.48mIU/ml.There was a non significant (P>0.05) difference in the FSH level of the female subjects who conceived compared to the subjects with CC failure. Mean serum LH level of the total number of female subjects was 8.24±0.62mIU/ml. The female subjects after ovulation induction with CC failure presented with mean serum LH level 7.85±0.91mIU/ml. The subjects who conceived had a higher LH levels which was 8.90±0.67mIU/ml. Statistically the mean serum LH level of subjects who conceived compared to the female subjects with CC failure was not significant (P>0.05). Serum level of serum Prolactin level of total number of subjects undergoing ovulation induction with CC was 11.4±0.71ng/ml. The female subjects who conceived after ovulation induction with CC was 11.4±1ng/ml and in subjects with CC failure was 11.4±0.89ng/ml. There was no significant (P>0.05) difference statistically in subjects who conceived compared to the subjects with CC failure after ovulation induction with CC. The LH : FSH ratio of 1.65 was observed in the female subjects who conceived after ovulation induction with CC while it was less than 1.21 in subjects with CC failure. The LH ratio was more than 1 in the subjects who conceived and less than 1 in the subjects with CC failure after ovulation induction with CC. The FSH ratio was less than 1 in the subjects who conceived compared to the subjects with CC failure which was more than 1 after ovulation induction with CC. Both the ratio's of LH and FSH in the subjects who conceived and with CC failure are the mirror images of each other.

Table 6

Mean serum Progesterone (ng/ml) level of day 21 of the menstrual cycle and BMI (kg/m^2) of female subjects undergoing ovulation induction with CC.

Characteristics	All subjects		Conceived		CC Failure	
Serum progesterone ng/ml	6.14±0.87	(41)	5.3±1.4	(12)	6.4±1.1	(29)
BMI kg/m^2	21.5±0.51	(42)	21.6±0.59	(12)	21.6±0.72	(28)

All values are given as mean ± standard error (S.E).
Values in parentheses represent the number.
BMI Body Mass Index.

Mean serum Progesterone (ng/ml) level of day 21 of the menstrual cycle and BMI (kg/m^2) of female subjects undergoing ovulation induction with CC was shown in table 6. Mean serum progesterone level of day 21 of the menstrual cycle of total number of female subjects undergoing ovulation induction with CC was 6.14±0.87ng/ml. The subjects who conceived showed mean serum progesterone level of 5.3±1.4ng/ml compared with CC failure subjects who had a mean progesterone level of 6.4±1.1ng/ml. There was a non significant ($P>0.05$) difference in the serum progesterone level of subjects who conceived compared with the subjects with CC failure. Mean BMI of female subjects at presentation was 21.5±0.51kg/m^2 , the subjects who conceived showed a BMI of 21.6±0.59kg/m^2 .The female subjects with CC failure presented with a BMI of 21.6±0.72kg/m^2 . There was a non significant ($P>0.05$) difference of BMI in the female subjects undergoing ovulation induction with CC who conceived compared to subjects with CC failure.

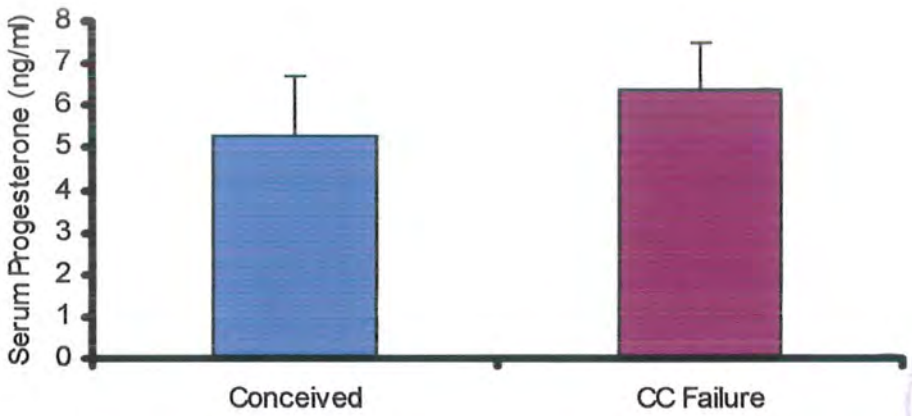

Fig 13 : Mean serum Progesterone (ng/ml) levels in the subjects who conceived with CC and with CC Failure.

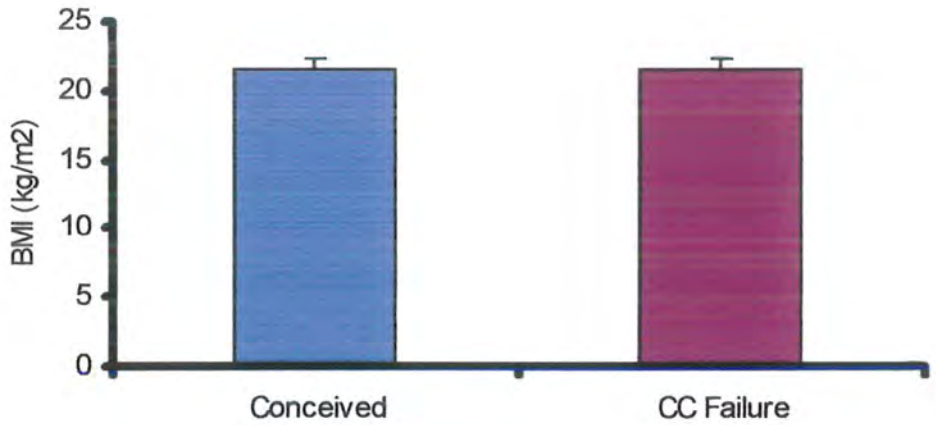

Fig 14 : Mean BMI (kg/m^2) of subjects who conceived with CC and with CC Failure.

CHARACTERISTICS OF SUBJECTS CONCEIVED

Table 7

Number of primary and secondary infertile female subjects along with relationship between conception and treatment cycles with increasing dose of CC in the same female subjects undergoing ovulation induction.

Characteristics	All subjects Number	%	Primary infertility Number	%	Secondary infertility Number	%
Number of subjects	102		56	(54.9)	46	(47.1)
Number of subjects conceived in treatment cycles						
I	12	(35.3)	6	(50)	6	(50)
II	15	(44.1)	11	(73)	4	(27)
III	7	(20.6)	3	(43)	4	(57)
IV	-		-	-	-	-

Values in parentheses represent percentage.
No of cycles represent:
I : induced with 50mg CC
II : induced with 100mg CC
III: induced with 150mg CC
IV: induced with 200mg CC

Total number of infertile female subjects with primary or secondary infertility that were included in this study were 102, out of which 56 (54.9%) were with primary infertility and 46 (47.1%) were with secondary infertility (table 7). The same number of female subjects underwent ovulation induction with the increasing dose of CC in four treatment cycles. In the first treatment cycle induced with 50mg of CC 12 (35.3%) female subjects conceived, out of which 6 (50%) were primary infertile and 6 (50%) were secondary infertile subjects. In the second treatment cycle induced with 100mg of CC 15 (44.1%)

female subjects conceived, out of which 11 (73%) were primary infertile and 4 (27%) were secondary infertile subjects. In the third treatment cycle induced with 150mg of CC, 7 (20.6%) female subjects conceived, out of which 3 (43%) were primary infertile and 4 (57%) were secondary infertile subjects. In the fourth treatment cycle induced with 200mg of CC none of the female subjects conceived (table 7).

Table 8

Number and percentage of regular and irregular cycles in female subjects undergoing ovulation induction with CC along with the relationship between conception and increasing dose of CC in the four treatment cycles.

Characteristics	All subjects	Regular Cycle Number	(%)	Irregular Cycle Number	(%)
Number of subjects	102	75	(73.5)	27	(26.4)
Number of treatment cycles.					
I	12	8	(67)	4	(33)
II	15	10	(67)	5	(33)
III	7	5	(71)	2	(29)
IV	-	-	-	-	-

Values in parentheses represent percentage.
No of cycles represent:
I : induced with 50mg CC
II : induced with 100mg CC
III: induced with 150mg CC
IV: induced with 200mg CC

Total number and percentage of regular and irregular cycles of female subjects undergoing ovulation induction CC along with the relationship between conception and

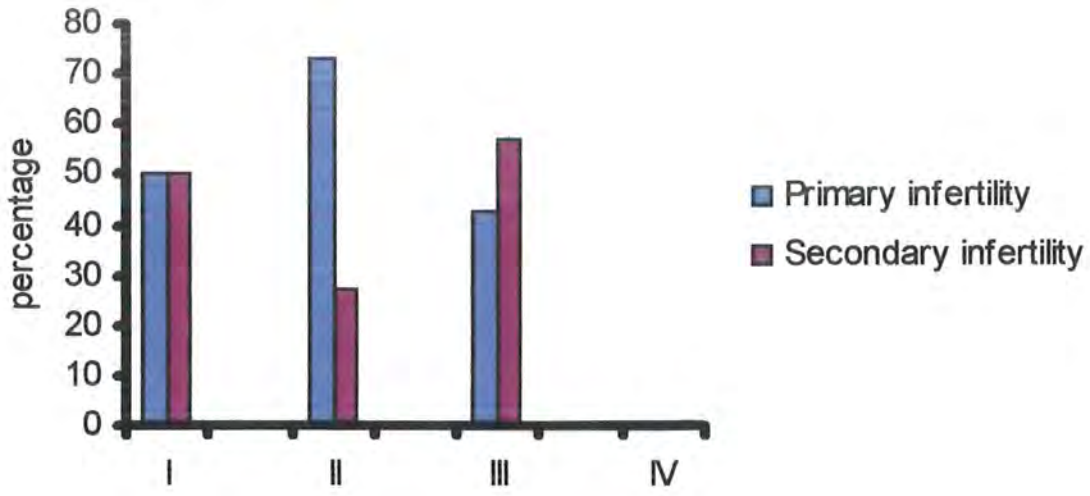

Fig 15 : Percentage of primary infertile and secondary infertile female subjects who conceived with CC in relation to the treatment cycles.

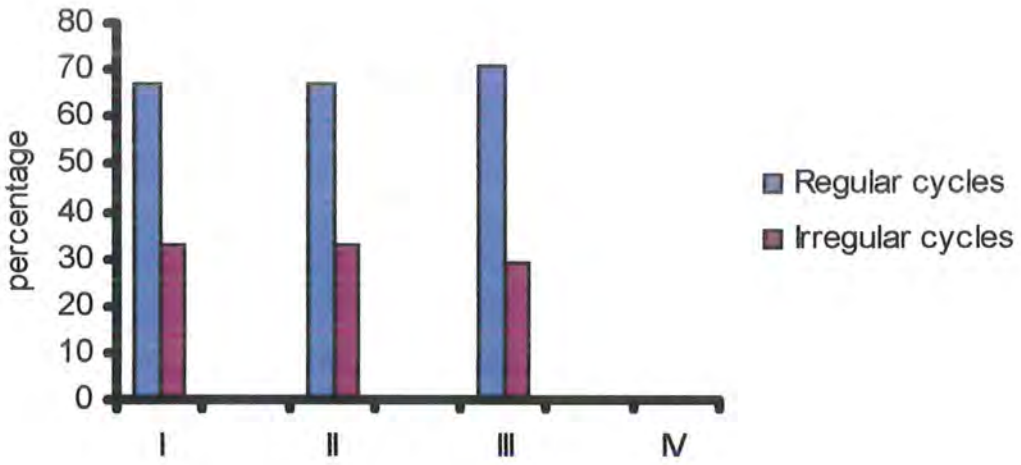

Fig 16 : Percentage of regular and irregular cycles in subjects who conceived with CC in relation to the treatment cycles

the increasing dose of CC in the four treatment cycles is shown in table 8. Out of the total number of female subjects who underwent ovulation induction with CC as described previously, 75 (73.5%) subjects had regular menstrual cycles and 27 (26.5%) female subjects had irregular menstrual cycles. The female subjects who conceived after ovulation induction with the increasing dose of CC in four treatment cycles is shown in table 8. In the first treatment cycle induced with 50mg of CC, 12 subjects conceived, 8 (67%) had regular cycles and 4 (33%) subjects had irregular menstrual cycles. In the second treatment cycle induced with 100mg of CC, 15 female subjects conceived, out of which 10 (67%) female subjects had regular cycles and 5 (33%) had irregular menstrual cycles. The female subjects who underwent the third treatment cycle with 150mg of CC 7 conceived, 5 (71%) had regular cycles and 2 (29%) had irregular menstrual cycles. The female subjects who underwent fourth treatment cycle with 200mg of CC ended up with complete CC failure.

Table 9

Number and percentage of female subjects with polycystic and normal ovaries undergoing ovulation induction with CC in relation to conception and the increasing dose of CC in the four treatment cycles.

Characteristics	All subjects	Polycystic ovaries Number	%	Normal ovaries Number	%
Number of subjects	102	32	(31.4)	70	(68.6)
Number of treatment cycles					
I	12	5	(42)	7	(58)
II	15	5	(33)	10	(67)
III	7	3	(43)	4	(57)
IV	-	-	-	-	-

Values in parentheses represent percentage.
No of cycles represent:
I : induced with 50mg CC
II : induced with 100mg CC
III: induced with 150mg CC
IV: induced with 200mg CC

Total number and percentage of female subjects with polycystic and normal ovaries undergoing ovulation induction with CC in relation to conception and the increasing dose of CC in the four treatment cycles is shown in the table 9. As mentioned previously out of the total number of 102 female subjects on USG 32 (31.4%) were PCO and 70 (68.6%) had normal ovaries. These female subjects underwent four treatment cycles with the increasing dose of CC. In the first treatment cycle induced with 50mg of CC 12 female subjects conceived, out of which 5 (42%) had PCO and 7 (58%) had normal ovaries. In the second treatment cycle induced with 100mg of CC 15 subjects conceived out of whom 5 (33%) had PCO and 10 (67%) had normal ovaries. The female subjects who

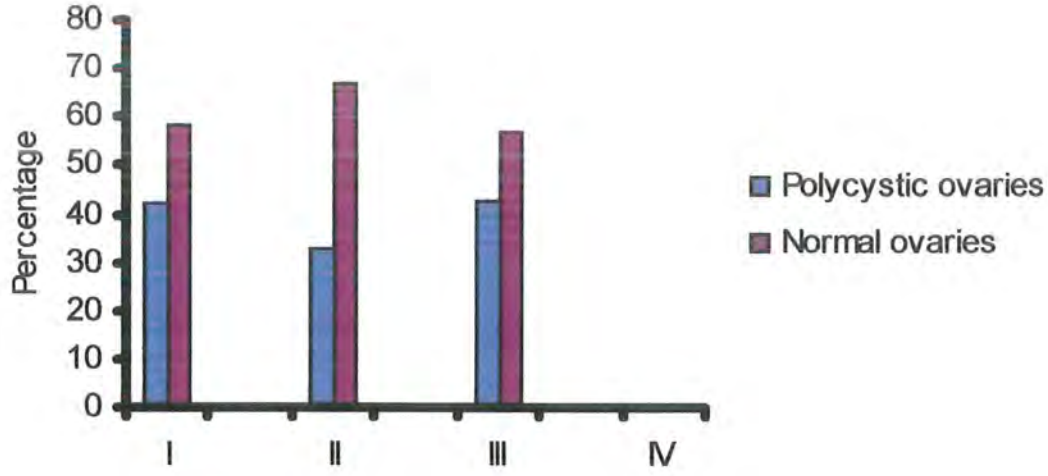

Fig 17 : Percentage of polycystic and normal ovaries in female subjects who conceived in relation to the treatment cycles.

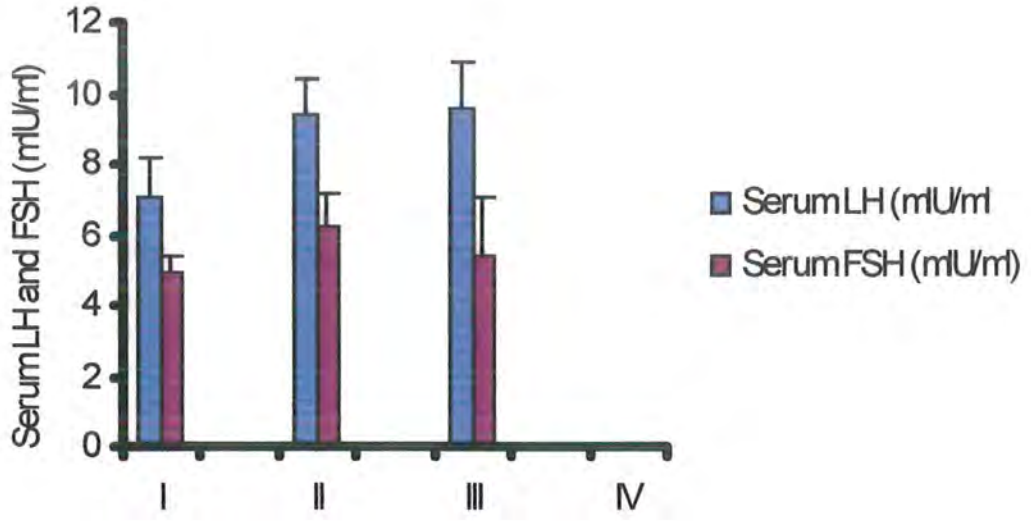

Fig 18 : Mean serum LH and FSH (mIU/ml)in subjects who conceived with CC in relation to the treatment cycles.

underwent third treatment cycle with 150mg of CC 7 conceived. Out of the one who conceived 3 (43%) had PCO and 4 (57%) had normal ovaries. In the fourth treatment cycle with 200mg of CC none of the female subjects conceived (table 9).

ENDOCRINAL PARAMETERS OF THE SUBJECTS CONCEIVED

Table 10

Mean hormonal profile, serum LH, FSH, Progesterone, and Prolactin levels along with period of infertility in relation to the increasing dose of CC in female subjects who conceived undergoing ovulation induction with CC.

Number of cycles	Serum LH (mIU/ml)	Serum FSH (mIU/ml)	Serum Progesterone (ng/ml)	Serum Prolactin (ng/ml)	Preiod of infertility (yrs)
I	7.07±1.13 (4)	4.89±0.53 (5)	5.88±2.5 (6)	11.4±1.52 (5)	4.04±1.11 (12)
II	9.41±0.99 (8)	6.27±0.92 (7)	7.1±1.38 (8)	11.2±1.12 (9)	5.28±1.27 (15)
III	9.54±1.30 (5)	5.38±1.7 (5)	7.93±3.75 (3)	14.21±3.3 (3)	7.35±1.42 (7)
IV	-	-	-	-	-

All values are given as mean ± standard error (S.E).
Values in parentheses represent the number.
No of cycles represent:
I : induced with 50mg CC
II : induced with 100mg CC
III: induced with 150mg CC
IV: induced with 200mg CC

Mean hormonal profile, serum LH, FSH, Progesterone, Prolactin and period of infertility in relation to the increasing dose of CC in four treatment cycles in female subjects who conceived undergoing ovulation induction with CC are shown in table 12. Female subjects who conceived in the first treatment cycle induced with 50mg of CC their mean

serum LH was 7.07±1.13mIU/ml in 4 female subjects. Mean serum FSH was 4.89±0.53mIU/ml in 5 female subjects while mean serum progesterone was 5.88±2.5ng/ml in 6 female subjects. Mean serum Prolactin was 11.4±1.52ng/ml in 5 female subjects. while mean period of infertility was 4.04±1.11yrs in 11 subjects. Female subjects who conceived during the second treatment cycle with 100mg of CC showed mean serum LH 9.41±0.99mIU/ml in 8 female subjects while mean serum FSH was 6.27mIU/ml in 7 female subjects. Mean serum Progesterone was 7.1±1.38ng/ml in 8 female subjects while mean serum Prolactin was 11.21±1.12ng/ml in 9 female subjects. Mean period of infertility was 5.28±1.27yrs in 14 female subjects. Female subjects who underwent third treatment cycle with 150mg of CC and conceived showed mean serum LH 9.54±1.30mIU/ml in 5 subjects. Mean serum FSH was 5.38±1.7mIU/ml in 5 female subjects. Mean serum Progesterone was 7.93±3.75ng/ml in 3 female subjects while mean serum Prolactin was 14.16±3.32ng/ml in 3 female subjects. Mean period of infertility in 7 female subjects was 7.35±1.42yrs. Female subjects undergoing fourth treatment cycle with 200mg of CC did not conceive.

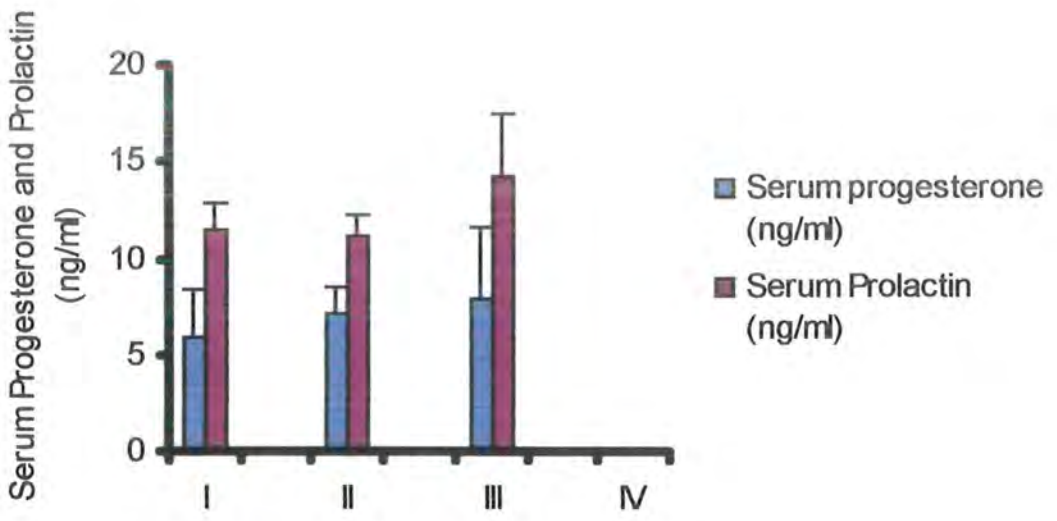

Fig 19 : Mean serum Progesterone (ng/ml) and Serum Prolactin (ng/ml) levels of females who conceived with CC in the four treatment cycles.

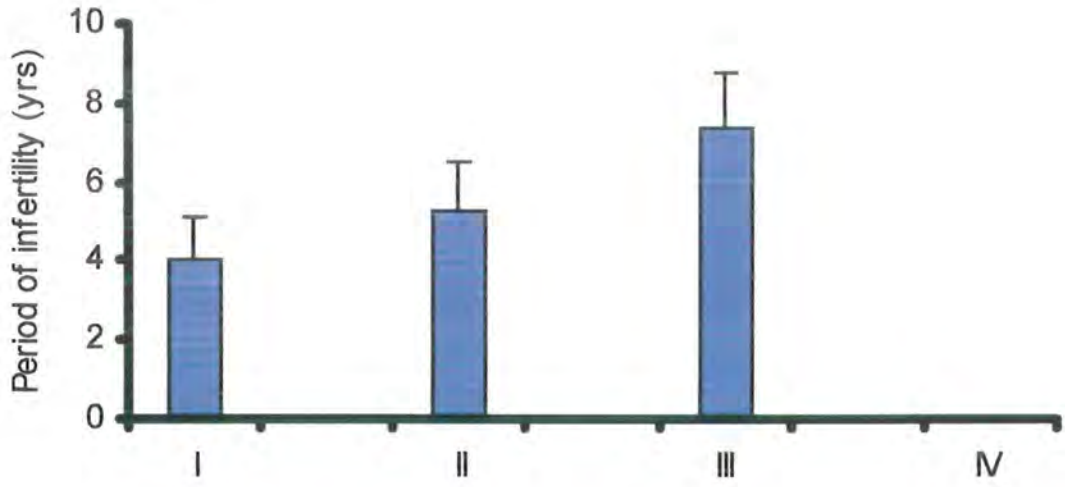

Fig 20 : Mean duration of infertility (yrs) in the female subjects who conceived with CC.in the four treatment cycles

TVS OF THE FOLLICLES

Table 11

Mean follicular diameter on TVS in follicular, ovulatory.and leuteal phase of menstrual cycle and the female subjects who conceived in relation to the increasing dose of CC in the female subjects undergoing ovulation induction with CC.

No of cycles	Follicular phase Diameter (mm) D10	Conceived Diameter (mm) D10	Ovulatory phase Diameter (mm) D14	Conceived Diameter (mm) D14	Anovulatory Leuteal phase Diameter (mm) D21
I	10.91±0.73 (102)	11.50±1.38 (12)	12.69±0.94 (102)	15.57±2.09 (12)	11.86±1.22 (82)
II	11.95±0.47[a] (90)	16.57±1.18[a*] (15)	14.02±0.65 (90)	17.80±2.55 (15)	14.31±0.75 (53)
III	12.47±0.41 (75)	12.52±1 (7)	14.87±0.55[b] (75)	17.33±1.39[b**] (7)	14.22±0.66 (34)
IV	12.68±0.41 (9)	-	14.86±0.56 (9)	-	15.33±0.66 (5)

All values are given as mean ± standard error (S.E).
Values in parentheses represent number of female subjects.
No of cycles represent:
I : induced with 50mg CC
II : induced with 100mg CC
III: induced with 150mg CC
IV: induced with 200mg CC
Statistically significant
[a*] $P<0.04$
[b**] $P<0.04$

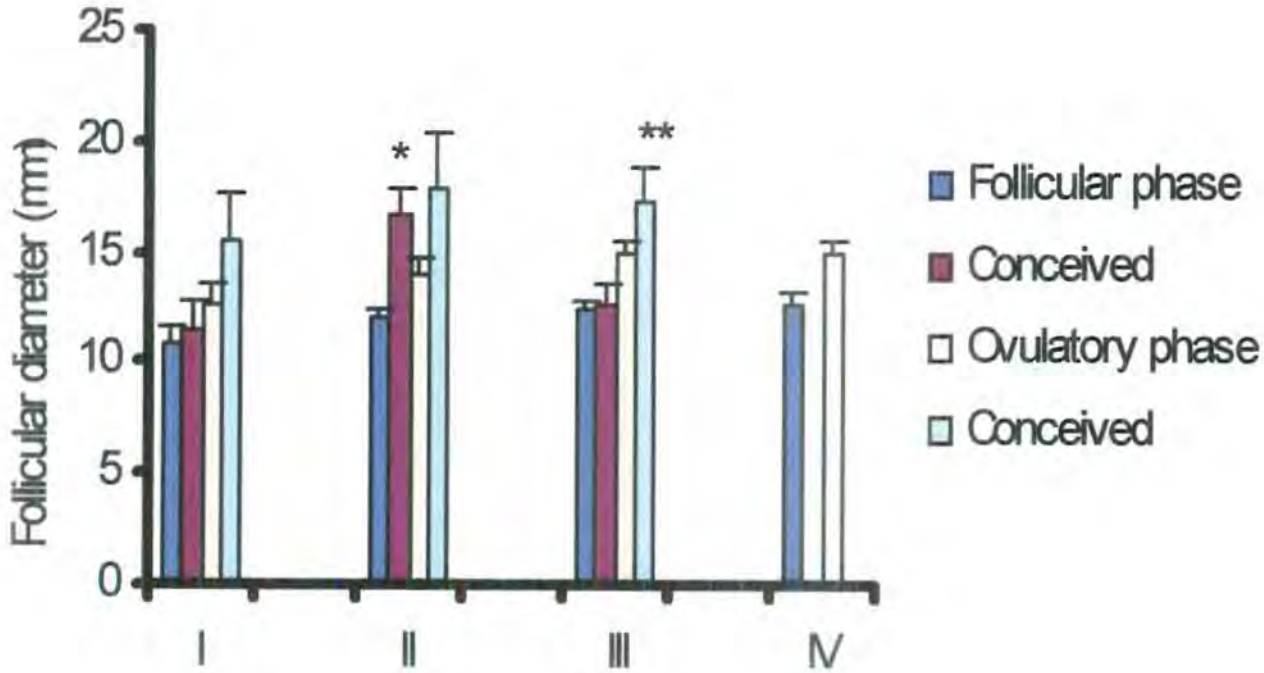

Fig 21 : Mean follicular diameter (mm) of female subjects in the follicular and ovulatory phase along with the follicular diameter of subjects who conceived with CC. in the four treatment cycles
*P<0.04.
**P<0.04.

Mean follicular diameter on TVS in follicular, ovulatory and leuteal phase of menstrual cycle and the female subjects who conceived in relation to the increasing dose of CC in the female subjects undergoing ovulation induction with CC is shown in table 11. Female subjects undergoing ovulation induction in the first cycle with 50mg of CC presented with mean follicular diameter (mm) in the follicular phase on day 10 of menstrual cycle 10.91±0.73mm (102), while the female subjects who conceived showed a mean follicular diameter 11.50±1.38mm with non significant (P>0.05) difference. The same female subjects underwent TVS on day 14 of the menstrual cycle in the ovulatory phase as shown in the table presented with mean follicular diameter (mm) 12.69±0.94mm compared to the female subjects who conceived 15.57±2.09mm with non significant (P>0.05) difference. The same subjects who failed to ovulate, in the leuteal phase on day 21 of the menstrual cycle on TVS presented with mean follicular diameter of 11.86±1.22mm with non significant difference (P>0.05) between the ovulatory and leuteal phase follicular diameters. The same female subjects (90) who failed to conceive underwent induction in the second cycle with 100mg of CC. Mean follicular diameter of all female subjects in the follicular phase was 11.95±0.47mm, which was markedly less compared to the subjects who conceived 16.57±1.18mm. There was a significant (P<0.04) as shown in the table. The same subjects on TVS in the ovulatory phase presented with 14.02±0.05mm, out of which the subjects who conceived presented with 17.80±2.55mm with a non significant difference (P>0.05). The same female subjects who failed to conceive and also failed to ovulate and ended up with anovulation presented with a mean follicular diameter of 14.31±0.75mm. The female subjects (75) who failed to conceive underwent induction with CC in the third cycle with 150mg of CC. In the follicular phase these female subjects presented with the mean follicular diameter of 12.47±0.41mm and the subjects who conceived presented with the mean follicular diameter of 12.52±1mm. There was a non significant (P>0.05) difference in the subjects who conceived and who failed to conceive. The same subjects in the ovulatory phase, day 14 of the menstrual cycle presented with the follicular diameter of 14.87±0.55mm and those female subjects who conceived among them presented with the mean follicular diameter of 17.33±1.39mm. There was a significant (P<0.04) difference in the mean follicular diameter of female subjects who conceived in the ovulatory phase of the

menstrual cycle than the subjects who failed to conceive. In the leuteal phase, on day 21 of the menstrual cycle, the subjects who failed to ovulate presented with the mean follicular diameter of 14.22±0.66mm.The female subjects who did not conceive should have followed up with the fourth cycle, but only nine female subjects followed the fourth cycle with 200mg of CC. Out of them none conceived as shown in the table 11. The female subjects who underwent four treatment cycles with CC, 82 subjects were anovulatory in the first cycle, 53 subjects were anovulatory in the second cycle, 34 subjects in the third cycle and only five out of nine in the fourth cycle as shown in the table 11.

Table 12

Mean follicular diameter on TVS in the female subjects who ovulated and who had anovulation in the four treatment cycles undergoing ovulation induction with CC

No of cycles	Ovulation Follicular diameter (mm)	Anovulation Follicular diameter (mm)
I	11.89±0.89	11.89±0.72
II	14.89±0.69	12.86±0.44*
III	14.77±0.60	13.44±0.39**
IV	-	14.02±0.38
Total diameter	14.77±0.60	14.02±0.38***

All values are given as mean ± standard error (S.E).
No of cycles represent:
I : induced with 50mg CC
II : induced with 100mg CC
III: induced with 150mg CC
IV: induced with 200mg CC
Statistically significant
* P<0.05
** P<0.01
*** P<0.0006

Mean follicular diameter on TVS in female subjects who ovulated and who ended up with anovulation after ovulation induction with CC in the four treatment cycles with increasing dose of CC is shown in table 12. Mean follicular diameter on TVS in the subjects undergoing ovulation induction with CC in the first cycle as shown in the table presented with 11.89±0.89mm who had ovulation and the subjects who ended up with anovulation presented with mean follicular diameter of 11.89±0.72mm. The difference in

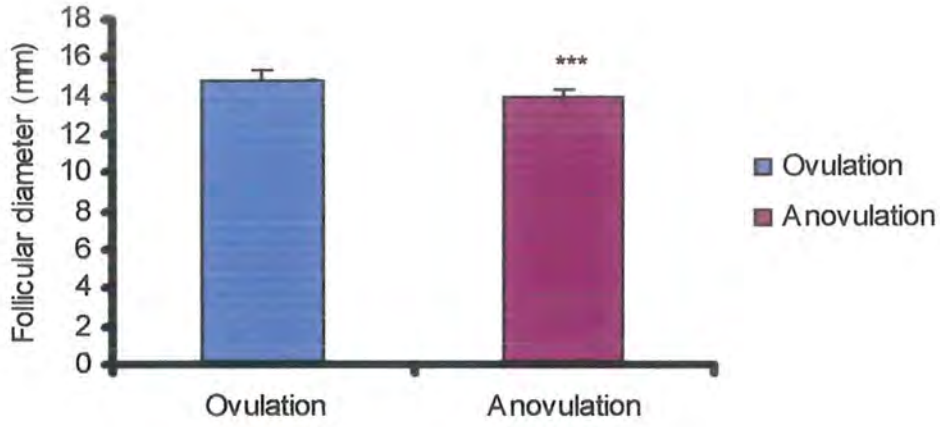

Fig 22 : Mean follicular diameter (mm) of female subjects with ovulation and anovulation after induction with CC.
***P<0.0006

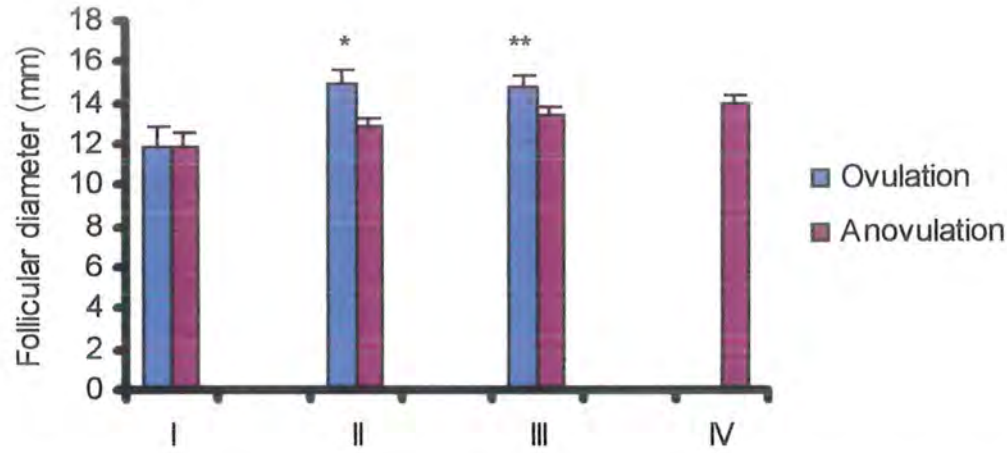

Fig 23 :Mean follicular diameter (mm) of female subjects with ovulation and anovulation in the four treatment cycles after ovulation induction with CC.
*P<0.05.
**P<0.01.

the ovulation and anovulation was non significant (P>0.05). In the second cycle of ovulation induction with 100mg of CC, the female subjects who ovulated presented with the mean follicular diameter of 14.89±0.69mm and who failed to ovulate presented with 12.86±0.44mm of mean follicular diameter. There was a significant (P<0.05) difference in the female subjects with ovulation and with anovulation. In the female subject who underwent ovulation induction with CC in the third cycle with150mg of CC presented with mean follicular diameter in the subjects with ovulation as 14.77±0.60mm and in female subjects with anovulation with 13.44±0.39mm. The difference in the mean follicular diameter in female subjects with ovulation and anovulation was significant (P<0.01). The female subjects presented with the mean follicular diameter in all the treatment cycles, those who ovulated showed mean follicular diameter 14.75±0.49mm and with anovulation with mean follicular diameter of 14.02±0.38mm. The difference in the mean follicular diameter of ovulation and anovulation was marked (P<0.0006).

Table 13

Total mean number of follicles, in the right and left ovary and in the female subjects who conceived after ovulation induction with CC.

CC Failure	Conceived	Left ovary	Right ovary
6.14±0.13	7.84±0.46*	6.29±0.19	6.0±0.18

All values are given as mean ± standard error (S.E).
Statistically significant
[a]*P<0.00002

Total number of follicles that were stimulated by induction with CC also in the right and left ovary and in the female subjects who conceived is shown in the table. Total mean follicular number after ovulation induction with CC was 6.14±0.13. Mean follicular number in the right ovary was 6.0±0.18 and in the left ovary was 6.29±0.19. The female

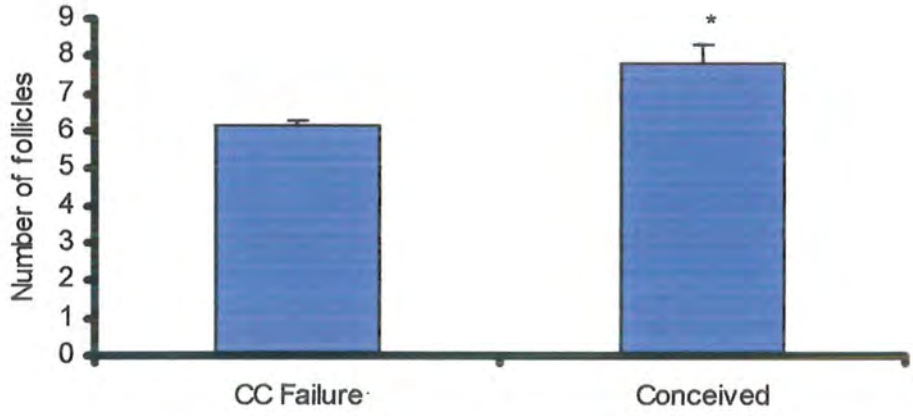

Fig 24 : Mean number of follicles in the subjects who conceived with CC and who failed to conceive.

subjects who conceived after ovulation induction with CC presented with the mean follicular number 7.84±0.46. There was a marked significant (P<0.00002) difference in the mean number of follicles after ovulation induction in the female subjects who conceived and the total number.

SEMEN ANALYSIS

Table 14

Semen analysis of the male partners of female subjects undergoing ovulation induction with CC.

Parameters	Values
Volume	3.19±0.19 ml
Count	86.60±8.19 x 10^6 / ml
MOTILITY	
Active	44.45±5.49 x 10^6 / ml
Sluggish	12.57±3.04 x 10^6 / ml
Immotile	17.13±3.64 x 10^6 / ml
MORPHOLOGY	
Normal (%)	82.09±3.87
Abnormal (%)	31.0±3.29
Colour	White
Consistency	Viscous

Semen analysis of the male partners of females undergoing ovulation induction was done. Females with their male partners having normal semen parameters were included in the present study. The semen which was included was white and viscous. The mean volume came out to be 3.19±0.19ml. Mean count of the sperms was 86.60±8.19 x 10^6/ml which was within normal range. Motility for active, sluggish and immotile sperms was done. Mean active sperms were 44.45±5.49 x 10^6/ml, mean sluggish were 12.57±3.04 x 10^6/ml and mean inactive were 17.13±3.64 x 10^6/ml. Mean morphology for normal sperms were 82.09±3.87 % and for abnormal was 31.0±3.29 %.

Table 15

Pregnancy rate and percentage of pregnancies in the female subjects undergoing ovulation induction with CC.

Characteristics	Pregnancy %
Pregnancy rate per treatment cycle.	12.3
Cumulative conception rate	33.3

The present study that was undertaken with 102 female subjects who were induced with CC showed the cumulative conception rate as 33.3 % and the pregnancy rate per treatment cycle as 12.3%.

Discussion

A retrospective and prospective follow-up study was carried out to determine whether initial screening could predict the chances of ovulation and conception in normogonadotrophic oligo/amenorrhic infertile women undergoing CC induction for ovulation. Late marriages are resulting in unprecedented number of couples who desire pregnancy relatively late in life. This has resulted in a decline in fertility and an increase in pregnancy wastage with advancing age and presents a new challenge for the clinician treating infertility (Speroff. 1994).

This study was carried out on 102 female subjects. The number of subjects who conceived was 34 (33.3%). while 68 (66.7%) showed CC failure after ovulation induction with CC. Imani et al (1998) carried out a study on 201 female subjects and found 77.5% to be responders whereas 22.5% failed to ovulate, was Clomiphene resistant anovulation (CRA). In a later study in 1999 he reported a conception rate of 51%. In 2002, Imani et al studied 256 (WHO group 2) normogonadotropic oligo / amenorrhic infertile female subjects, out of which 56% were ovulatory and 38% showed conception after induction with CC. All these reported figures were higher than those found in our study. Lidor et al (2000) observed 22 infertile subjects with PCOS and demonstrated a pregnancy rate of 33.3%. Although, lesser number of subjects was involved, the results are similar to the present study. Yolanda et al (1998) studied 45 infertile females undergoing ovulation induction in an increasing dose of CC from 50mg to 200mg, after four treatment cycles the pregnancy rate was 36%. These results are consistent with our study. Kausta et al (1997) observed the rate of pregnancy to be close to that expected in a normal fertile population. Hughes et al (2000) considered CC to be superior to no treatment or placebo.

Lower percentage of response has been shown by different researchers who evaluated the effectiveness of CC (Boostanfar et al, 2001, Fluker et al, 1996). Unsuccessful CC treatment was observed in twelve subjects with ovulatory infertility by Mitwally et al. 2001. Eijkeman's et al, 2003, prospective study of 201 subjects, 25% showed CRA, that is they remain anovulatory after multiple attempts with increased doses of CC. In the present study the percentage of CRA (63.1%) was more than the responders.

Infertility is defind as 1 year of unprotected intercourse without pregnancy. Epidemiologically it may be expected that 8 – 12% of couples experience some form of infertility, it may be primary infertility or secondary infertility (Belsey et al, 1986) affecting 50 – 80 million people world wide, according to a tabulation of available data on prevalence of primary and secondary infertility by WHO 1991. The percentage of primary infertility has increased to 70%. In the present study female subjects were taken randomly. The percentage distribution of primary infertility (54.9%) was more than secondary infertility (47.1%) in the present study which is a lesser percentage than the study done by WHO 1991. While in another study by WHO, which suggest that in most of the countries 60 – 84% of subjects needing infertility investigations have primary infertility (Ronald and Grey, 1990). Saeed and Rana, 1993, and Ghani, (2002), showed incidence of primary infertility to be 67.69% and 71.62% respectively. Primary infertile subjects showed a better response to CC, 35.7% conceived whereas only 29.1% showed conception in secondary infertile subjects. Imani et al, 1998 and 1999 carried out both studies in Netherland which showed primary infertility to be 72% in both studies with conception rate 38.75% after ovulation induction with CC in the former study which is close to our study and 71% in the later which is a higher percentage. Similarly Fuji et al (1997) in Japan had 72.2% primary infertile subjects, but the percentage of pregnancies was low 22.2%. Mustafa et al (1994) observed in Saudi Arabia that 41.2% suffered from primary infertility and 58.8% from secondary infertility. Similarly Idrees et al (2000) found that primary infertile subjects showed a pregnancy rate close to the present study 36% in the treatment group of CC protocol.

Duration of infertility in the present study was significantly less ($P<0.04$) in case of female subjects who conceived than in subjects with CC failure or CRA. Imani et al (1999) found shorter mean duration of infertility in both groups. This difference in study might be due to the fact that patient in the west more aware and therefore present early. Subjects with shorter duration of infertility, responds well to the CC protocol.

Ovulation induction in the present study was done in the increasing dose of 50, 100, 150 and 200mg from day 2–6 of the subsequent menstrual cycles. Each female subject

underwent 3 – 4 treatment cycles. Total of 276 cycles were induced with CC, 36.9% cycles were ovulatory and 63.1% were anovulatory or labeled as CRA. Results similar to the present analysis were observed by Fluker et al (1996) and Smith et al (1998). Imani et al (1998) observed 201 women, who underwent a total of 432 cycles in increasing dose of CC 50, 100, and 150mg from day 3 – 7 for 5 days. In the above mentioned study 22.5% subjects remained anovulatory (CRA). This percentage is less than our study, which might be due to early presentation and shorter duration of infertility. Mitwally et al (2001) studied subjects with CC treatment in PCOS and showed ovulation in 44.4% subjects which is close to the present study. Yolanda et al (1998) observed 76% and 75% ovulatory cycles which are higher percentages that are out of 134 cycles, 102 cycles were judged to be ovulatory. Mustafa et al (1994) carried out a study in which ovulation induction was done in 107 cycles and observed 22 ovulatory cycles (20.56%) this percentage is less than the present study. Different researchers have observed that 80% of subjects treated with CC in doses upto 250mg per day for 5 days will ovulate but only 40% conceive (Gysler et al. 1982. Hammond et al, 1983, Hammond et al, 1984). While in the present study 68.1% of the patients ovulated and only 33.3% showed conception. Other causes apart from anovulation might be the reason for this failure of conception.

The mean number of treatment cycles was significantly less ($P<0.05$) between the subjects who conceived and who showed CC failure after ovulation induction with CC. Fuji et al (1997) found that the mean number of treatment cycles were similar in the group who conceived and the group with CC failure after induction with CC in infertile subjects with <2 years duration of infertility. Imani et al (1999) found that the mean number of treatment cycle was 3.2 ± 2.6 in 159 subjects, when the duration of infertility was <2 yrs. In contrast to the above studies the present study showed shorter mean number of treatment cycles with >2 yrs duration of infertility. In couples with anovulation as the only infertility factor 75% of subjects will conceive within 3–6 months of therapy (Carolyn et al. 1997). Similarly Nasseri and Ledger (2001) found anovulatory women who are responsive to CC should be treated for at least 6 cycles and the treatment should be limited to 12 cycles.

The subjects included in the present study presented with mean age of 28.4±0.5 years. Conception was seen in the mean age of 27.09±1 years after ovulation induction, which was comparatively less than the subjects who showed CC failure with no significant difference. Young subjects have a higher probability to conceive during CC induced ovulatory cycles. The fecundability rate of the subjects decreases by approximately 10% / year. This is in agreement with reports that indicate that age is an important factor for the prediction of chances for spontaneous conception in untreated normoovulatory subfertile subjects (Mulders et al, 2003, Imani et al, 1999, Eimers et al, 1994, Collins et al, 1995, Scott et al 1995). Similar findings have been reported for the prediction of chances to conceive after exogenous gonadotrophin induction of ovulation (Dor et al, 1980) and in vitro fertilization treatment (Tempelton et al, 1996). Imani et al (1999) observed that those who conceived were younger 27±4 years as compared with CC failure 29±4. These results are consistent with the present study. All these studies revealed that predictive power of age was highest. While a study conducted by Fauji et al (1997) showed no effect of age on ovulation or the treatment cycles. There was no difference of age in the subjects who conceived after ovulation induction with CC and who conceived spontaneously. Yolanda et al (1998) observed two groups with average ages of 32 years and 29 years respectively. The results were almost similar with 96% ovulation in group 1 and 93% in group 2. Taylor and Braude (1994) state that if the female partner is young <30 years and there are no factors in either history suggesting an obvious problem, it is wise to wait and the response to CC is good. In older groups, therapies should be offered which are likely to be effective.

In this study mean age at menarche was 13.2±0.11 years. This figure is similar to other studies as the average age of menarche in UK is 13 years and 95% of female population reached it between 11 – 15 years (Edmonds, 1995). Ghani, (2002) observed mean age of menarche was 13.29±0.02 years with a range of 10 – 18 years in Pakistan. In the present study females who conceived showed a significantly less ($P<0.05$) mean age of menarche as compared with females who ended up with CC failure after ovulation induction. Although no relationship lies in early menarche, infertility and conception but in our study the responders had early menarche. The average age being 13.5 years in India, 13

years in Western Europe and 12.5 years in North America (Tindal, 1987) In China average age of menarche is 12.5 years (Ye, 1997). Bano, 1996 found age of menarche in Pakistani women ranged between 12 – 15 years. No significant difference was found among different socioeconomic groups.

Menstrual cycle length in determined by the rate and quality of follicular growth and development and is normal for the cycle to vary in individual women. In the present study mean duration of cycle was 47.7±4.6 days. The subjects who conceived showed a comparatively shorter duration of cycle 41.6±3.8 days. The response to CC ovulation induction was better with shorter duration of the cycle. (Imani et al, 1999) in a study divided the bleeding interval into 4 categories. Maximum number of subjects who conceived belonged to the first two categories with shorter duration of the cycle. The above study is consistent with the present study. Subjects with increased age and oligomenorrhea were predictive for delayed pregnancy after first ovulation with CC (Eijkemans et al, 2003). Mean days of menstrual flow showed no significant difference in the subjects who conceived and who failed to conceive. Ghani, (2002) found mean days of menstrual flow to be 5.88±0.72 days. in females with PCO, and 4.53±0.18 days in control subjects, which is consistent with our study.

The present study showed 72.5% regular cycles and 27.6% irregular cycles. The subjects who conceived after ovulation induction were more with irregular cycles 39.2% than with regular cycles 29.7%. Ghani, (2002) observed higher percentage of irregular cycles in PCO whereas the reverse was seen in subjects with normal ovaries. This is in accordance with our Study and the study conducted by Convey et al (1989) who reported irregular cycles to be a presenting complaint of females with PCO.

The subjects who conceived had non significant ($P>0.05$) higher concentration of serum LH on day 3 compared to those who did not conceive. Although, these levels were higher but were within normal limits. Hassan, (2002) found that serum LH levels on day 3, in subjects with PCOS group were within normal range (8.6±0.8 mIU/ml) but were significantly higher as compared to control (4.9±0.3 mIU/ml). Mean LH : FSH ratio was

also significantly high >2. In the present study LH : FSH ratio is 1:65 in subjects who conceived, is also slightly higher. Fasihunnisa (1994) demonstrated that PCOS might exhibit low, normal or high serum LH levels on day 3 in both regular and irregular menstruating females. It is suggested that abnormal LH secretion is not an obligatory cause of menstrual irregularities (Fasihunnisa, 1994, Balen et al, 1993). Raised LH had a significantly higher incidence of infertility than normal LH (37% verses 21%) and these subjects have a significantly higher probability to conceive once ovulatory cycles have been achieved by CC (Imani et al, 1999, Kousta et al, 1997, Balen et al, 1993). The present study is consistent with the above studies as serum LH on day 3 was higher (8.90±0.67mIU/ml) in the subjects who conceived as compared with CC failure (7.85±0.91mIU/ml). Although these levels were higher but were within normal limits. In contrast, a poor treatment outcome has been observed in subjects with high LH levels during follicular phase of CC induced cycles and higher LH : FSH ratio. The possible explanation for this may be that the serum LH level has been taken in the late follicular phase (Shoham, 1990). Lobo et al, (1982) found that compared to subjects who ovulated after induction with CC, the anovulatory subjects had higher LH levels, higher LH : FSH ratio and most had PCOS (Aziz et al, 1998). However, the assessment of LH levels in anovulatory patients is problematic due to effects of timing, the immunoassays used, and the pulsatile nature of LH release (Fauser et al, 1993). All these reports are on serum LH levels on day 3 before initiation of CC medication, rather than during CC induced cycles. Indeed, elevated LH levels may normalize only during CC induced ovulatory cycles (Eden et al, 1989). Clomiphene Citrate induces a discharge of LH as well as FSH and elevated LH concentrations are believed to impede conception. Those with high day 3 LH levels are less likely to respond to CC treatment (Homburg et al, 1988, Regan et al, 1990, Howles et al, 1986, Adams et al, 1985). Reduced fertilization rates have been reported when LH concentration has been elevated during follicular phase (Stanger and Yourch, 1985). Serum LH either normal or high with polycystic or normal ovaries does not seem to represent a different clinical entity. It seems justifiable to consider this as a subgroup of (WHO group 2) normogonadotrophic oligo / amenorrhic infertility (Laven et al, 2002). Also, serum LH concentrations do not predict ovarian response after CC medication (Kausta et al, 1997). Whereas Eijekmans et al (2003) reported LH to be a potential

predictor. Anovulation with an elevated LH in the presence of normal FSH is suggestive of PCOS. Low FSH and LH levels indicate dysfunction at hypothalamic or pituitary level which is amenable to ovulation therapy (Taylor et al. 1994).

The ratio of LH : FSH has been used as a useful laboratory indicator of in appropriate gonadotrophic secretion for the diagnosis of type of infertility (Lobo et al. 1996). Some researches have subdivided the subjects on the basis of whether or not LH levels are raised (Vejlsted et al. 1976 and Berger et al. 1975). Mean LH and LH : FSH ratio was increased in CRA and in females who failed to conceive and a diagnosis of PCO with infertility was made (Lobo et al. 1983). In the subjects who failed to conceive in the present study the Serum LH levels on day 3 were less with LH : FSH ratio less than in the subjects who conceived but within normal range.

Serum FSH concentration on day 3 are usually normal in women with (WHO group 2) normogonadotrophic oligo / ammenorrhic despite the fact that they have anovulatory infertility that responds to treatment which raise serum FSH concentration (Franks et al, 1985, Yen et al, 1980, Imani et al. 2002, Laven et al. 2002). In the present study the serum FSH on day 3 falls within normal limits. Actual low or absent FSH does not alter ovulation, the dominant follicle can survive in levels of FSH that are below the necessary threshold (Aziz et al, 1998).In the present study mean serum FSH levels (5.9±0.38 mIU/ml) were within normal limits.

Peak serum progesterone concentration on day 21did not vary with cycle number (Opsahl et al, 1996, Ficciolgn et al 1995) found no statistical difference of serum progesterone level in the luteal phase and ovulation was considered on the basis of TVS evidence. Paulson et al, (1984) observed a correlation between sonographic determination of ovulation and serum progesterone levels. A rise of serum progesterone level in the second half (luteal phase) of the cycle to be >30 nmol / L suggests ovulation has occurred. This is commonly measured on day 21 of 28 days cycle (Taylor and Braude, 1994, Paulson et al, 1984). A rise in serum progesterone occurring in midcycle has been reported by a number of authors (Dodson et al. 1975, Strott et al, 1969). It was originally suggested that

the level of this hormone might indicate follicular growth (Strott et al, 1969) but later publications (Dodson et al, 1975) indicated that serum progesterone was probably a product of early lutinization (Ghani, 2002) supported this by observing that no correlation lies between follicular growth (MFD) and serum progesterone levels. During the luteal phase i.e from 11 – 13 day of the cycle , progesterone concentration was 5 – 10.57ng/ml (Ghani, 2002). These findings are consistent with the present study, the subjects who conceived had mean progesterone concentration to be 5.3±1.4ng/ml as compared to 5–15 ng/ml reported by Aptu et al. (1978). In the present study mid luteal serum progesterone levels were 6.14±0.87 ng/ml which is in agreement with the limit 3 – 15ng/ml set for the adequate mid luteal serum progesterone levels in normal menstrual cycle by other researchers (Dorrison et al, 1980, Hull et al, 1982)

Serum prolactin level was found to be normal in (WHO group 2) normogonadotrophic oligo / ammenorrhic infertile subjects (Hull et al, 1987, Youlanda et al, 1998). Hyperprolactinemia is found to cause ammenohrrea or oligomenorrhea and once the correct diagnosis is made appropriate treatment results in the restoration of ovulatory cycles (Taylor and Braude, 1994, Kaplan et al, 1997, Adams et al, 1986). Subjects who had higher LH and LH : FSH ratio, serum prolactin levels were found normal.

Body mass index (BMI) of 19 – 25kg/m^2 is considered as normal. BMI (Ganong, 1993) with a low body weight can be associated with amenorrhea and an increased body weight exacerbates the hormonal derangements found in PCOS, both have adverse effects on reproduction (Taylor and Braude, 1994, Polson et al, 1994, Homburg 2003, Correa et al, 1978, Frisch, 1989). A loss of 5% of body mass in the obese PCO anovulatory patients results in spontaneous resumption of ovulatory cycles and pregnancy (Kiddy et al, 1992, Polson, 1994). In the present study the mean BMI was 21.5±0.59 within normal range with no difference between the subjects who conceived and who failed to conceive. These findings are consistent with that of Imani et al (1999). While other researchers found that BMI is one of the predictors of anovulation after CC medication (Eimers et al, 1994, Eijikmans et al, 2003, Snick et al 1997, Lobo et al, 1982). Our study on the contrary does not coincide with the above findings. Weight could not accurately predict

CC response. While Guzick (2004) found that increased BMI frequently complicates PCOS even though it is not a defining characteristic, behavioral weight management is a central component of the overall treatment strategy. Wang et al (2000) found that the prevalence of obesity in infertile women is high but there is no conclusive evidence that extremes of weight are associated with a low rate (24.1%) of pregnancy in ART and ovulation induction. Dickey et al (1997) studied large number of subjects and found a correlation between patient's weight and need for higher dose of CC. Similar were the findings of Lobo et al, 1982, Shepard et al, 1979). An increased BMI is the only factor which is consistently associated with a decreased response to CC. Weight reduction should be an important part of therapy in anovulatory women (Kousta et al, 1997, Clark et al, 1995, Kiddy et al, 1992.).

Cumulative conception rate (CCR) according to the dose and number of treatment cycles in our study was 35.3% with 50mg in the first cycle, 44.1% with 100mg in the second cycle and 20.6% in the third cycle with 150mg. While Imani et al, (1999) found CCR of 57%, 66% and 38% with in 5 ovulatory cycles with increasing dose of CC. CCR was 63% within six cycles and 73% within nine ovulatory CC induced cycles as compared to the present study where 33.3% was the CCR in four treatment cycles. At higher doses, chances of conception and ongoing pregnancy are not statistically significantly reduced, although absolute CCR were low in the 150mg CC group. Gortilhsky et al, (1978) noted 5.6% of total pregnancy occurred at doses of >100mg/day. Rust et al (1974) reported 22.5% of pregnancy occurred at doses of 105 – 250mg/day. Gysler et al (1982) found that 26.7% of pregnancies occurred at CC doses >150mg/day and Shephard et al (1979) found 28% of pregnancy at doses of >150mg, compared to the present study that shows higher percentage of pregnancies in the given doses. Two third of patients who conceive reach the end point within three ovulatory cycles CC induced treatment cycles (Gorlithsky et al, 1978). The present study and by other researchers are in agreement with previous reports regarding conception with CC treatment which is similar to spontaneous conception in normoovulatory women (Tietze et al, 1968, Gaicia et al, 1982, Gysler et al, 1982). Kousta et al, (1997) found CCR continues to rise after six treatment cycles with CC, reaches a peak by treatment cycle 12 and approaches that of the normal population. Imani et al,

(2000) showed CCR in three consecutive cycles to be 42%. Most of the pregnancies occur at a dose of 100mg/day. Hammond et al (1983) found that the most significant factor contributing to reduce overall pregnancy rate was patient's discontinuation of therapy and other factors like abnormal semen analysis, pelvic or tubal factors and poor cervical mucus. Along with these factors we found in our study lack of knowledge, awareness and social pressures as important factors. CCR corrected for discontinuation approach in 100% after ten cycle therapy with upto 150mg of CC. Gorlithsky et al (1978) administered high doses of 150mg and 200mg and found it effective in inducing ovulation and in three ovulatory cycles 50% of patients conceived. This does not represent discrepancy between ovulation rate and pregnancy results. The results of Gysler et al (1982) are similar to the present analysis that the great majority of subjects who conceive did so during the first three cycles.

In the present study subjects with regular menstrual cycles responded better as compared to subjects with irregular cycles. Hull et al (1987) on population studies revealed that irregular cycles accounted for 90% of patients with oligomenorrhea and 37% with amenorrhea or 73% with oligo or amenorrhea. Oligo or amenorrhea accounted for 21% of couples with infertility.

Subjects with normal ovaries in the present study were 68.6% as compared with PCOS (31.4%). With a standard therapy for anovulatory women a significant proportion with PCOS, fail to ovulate with standard dosage of CC and are called CRA (Amin et al, 2003). A comparative study was done (Mitwally et al, 2001) with PCOS and with infertile subjects with normal ovulation that showed 25% pregnancy rate in PCOS and 10% in the later. A similar workup was undertaken (Fauji et al, 1997) between PCOS and spontaneous normal cycles female subjects. The pregnancy rate in the PCOS was significantly lower than the other group. In the present study the conception rate of subjects with PCOS is higher (40.6%) as compared with the subjects with normal ovaries (30%). Takahashi et al (1994) induced PCOS patients with CC in dosage of 50mg to 200mg and observed the follicular number on TVS. It was found, 47% for the CC responders and 79% for CC non responders (PCOS). None of the non responders had

normal ovaries and 96% of PCOS with bilaterally abnormal ovaries were CC non responsive. Hull et al (1987) found that annual incidence of infertility due to PCOS was 180 per million. Of these, 140 appeared to respond well to CC (78%) but (40%) failed, requiring alternative therapy.

Relationship between ovarian morphological findings on TVS and CC responsiveness were studied in (WHO group 2 0 normogonadotrophic oligo / amenorrhic anovulatory subjects. The subjects who conceived presented with a significant ($P<0.04$) larger follicular diameter from the follicular diameter of CC failure. The number of follicles was significantly higher ($P<0.00002$). Takahashi et al (1994) made a comparative study between the CC responders and CC non responders of ovarian Ultrasonography (USG) features. He found that the number of small follicles in the CC non responders was large (96%) as compared to CC responders (16%). In the present study the subjects underwent 3 scan / cycle. Daly et al (1985) also observed subjects undergoing ovulation induction with CC, average of 3 scans / cycle. The growth and rupture of the follicle to confirm ovulation was observed on TVS. The mean follicular diameter observed was 22.1mm. Daly et al (1985) reported the incidence of anovulation to be 79%. None of the patients conceived, larger unruptured follicular diameters were seen upto 21mm, while in the present study anovlatory follicular size was significantly less ($P<0.0006$) than the ovulatory follicular size. Liukkonen et al (1984) observed un-ruptured follicles in 57% of cycles, out of those 29% were persistent. While Gibbons reported 10% incidence of unruptured follicles. Mustafa et al (1994) observed on TVS the follicular diameter around 17mm to 20mm.

In the present study the follicular growth patterns in cycles induced by CC was evaluated. It was observed that the leading follicular diameter was significantly larger but showed different follicular sizes. Adams et al, (1985). Haritha et al, (2003) scanned multifollicular ovaries by TVS after ovulation induction with CC and found that the ovarian morphology reverted to normal in ovulatory cycles, but despite the dominant follicle the PCO pattern persists. Different researchers have found that the leading follicle of CC induced cycle is larger (Randall et al, 1991, Nasseri et al, 2001, Adams et al,

1985). In the present study the subjects who conceived after ovulation induction with CC presented with larger follicular diameter as compared to those who failed to conceive and ovulation occurred on day 14 or 15 of the cycle as corroborated by Poulson et al, (1984). In both the studies follicles at the time just prior to ovulation ranged in size from 15mm to 21mm., which is consistent with the present study. Coulman et al (1982) showed ultrasonic evidence of unruptured follicle on day 21 of the cycle and were labeled as anovulatory cycles.Kousta et al (1997) observed that TVS of the ovaries during subsequent treatment cycles is important in order to choose the appropriate dose of CC.

Aziz et al (1998) found that the use of TVS is necessary to investigate the normal ovarian activity. Opsahl et al, (1996), studied the follicular development after 50mg to 150mg/day of CC from 5–9 day of the cycle. They found the peak follicular diameter of 21mm and that the increase in dose does not affect the size of the follicle, but the number of preovulatory follicles increases. These findings are not consistent with the present study, as the follicular size increases with the increasing dose. In our study the mean follicular diameter at 50mg dose was 15.57±2.09mm and at 150mg it was 17.33±1.39. Different researchers observed 3–25 number of follicles after ovulation induction with CC (Eijikmans et al, 2003, Imani et al, 1999 and 1998, Kousta et al, 1997, Pache et al, 1992). Our study is consistent with the findings of these researchers and showed 6–7 follicles. Adams et al, (1985) observed small follicles of >9mm in size in (WHO group 2) normogonadotrophic oligo / amenorrhic infertile subjects who have polycystic ovaries on TVS. While Agrawal et al. (1995) found that after ovulation induction with CC the follicular diameter increases to 18 – 24mm along with the number of follicles. These results are consistent to our study but the size of the follicles did not increase above 17mm.Dickey et al (1997) suggested that patients who tolerate lower doses should be moved up to higher doses. When dose of CC is increased above 100mg/day small additional numbers of preovulatory follicles appear. Follicles >12mm in diameter was observed with lower doses which increased to >15 - >18mm in diameter with the increase in dose (Dickey et al, 1992). According to Quigly et al (1984) one follicle per cycle is increased as the dose is increased. Shelen et al (1989) reported an increase in the number

of follicles and diameter >15mm in patients with regular cycles when CC is increased from 50 – 200mg.

The present study included only those infertile couples in which the male partner was normal. The semen analysis was within normal limits, the total sperm count was ($86.60\pm8.19 \times 10^6$/ml) motility and morphology of the spermatozoa within normal limits. Moreover it can be speculated that couple with better sperm parameters have a better chance of conceiving after treatment with Clomiphene citrate.

Conclusion

Clomiphene citrate still represents a highly effective means of fertility treatment in normogonadotrophic oligo / amenorrhic anovulatory women (WHO group 2) with low pregnancy chances without treatment.

The initial screening characteristics of normogonadotrophic oligo / amenorrhic anovulatory infertile women can aid in predicting response, ovulation or conception after induction of CC.

It is possible to predict the individual chances for conception which may be a step forward in optimizing the decision making process in the treatment of these women. Alternative first line of treatment options could be considered for some women who have limited chances for success.

Due to successful response to treatment by clomiphene citrate. It's use has increased manifold since it's initial trial in 1967. The drug has become one of the most popular modes of treatment for infertility. This has given rise to the injudicious use of the drug by unscuplous practitioners. Concern have arson that the drug is being prescribed in all scenarios of infertility whether required in those situations or not. Since it is possible to predict the individual chances for response to treatment by CC, therefore it should only be prescribed when the predicators points towards this drug.

More research is needed in this area, rather than referring these patients to assisted reproduction prematurely.

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