Article

Supplementation of clomiphene citrate cycles with *Cimicifuga racemosa* or ethinyl oestradiol – a randomized trial

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Abstract

The anti-oestrogenic activity of clomiphene citrate (CC) on the cervical mucous and endometrium may be the reason for the relatively low pregnancy rates in CC induction cycles. Various follicular-phase supplements have been tried to improve cycle outcome in these patients. This study compared follicular-phase supplementation with either phytoestrogen (PE) or ethinyl oestradiol (EE) in CC induction cycles for the treatment of unexplained infertility. A total of 134 patients were randomly allocated to each treatment group (67 each). The PE group needed significantly fewer days for adequate follicular maturation, had a thicker endometrium and higher oestradiol concentration at the time of human chorionic gonadotrophin injection (all $P < 0.001$). The PE group had higher luteal-phase serum progesterone compared with the EE group. No significant difference was found regarding clinical pregnancy rates (14.0% versus 21.1%, respectively). In conclusion, the cycle characteristics in unexplained infertility women treated with clomiphene citrate induction and timed intercourse improved after follicular-phase supplementation with PE compared with EE supplementation. Further studies are needed to confirm the mechanism beyond these effects.

Keywords: *clomiphene citrate, ethinyl oestradiol, follicular-phase, phytoestrogens, unexplained infertility*

Introduction

After repeated failure of induction cycles, couples with unexplained infertility are on the road to IVF. They yield to either the psychological pressure to conceive or the advice of their clinician. Although limited evidence exists that the routine use of IVF is more effective than expectant management or mild induction protocols in unexplained infertility (Horstein and Schust, 1996), cost-effectiveness data analysis supports the use of clomiphene citrate (CC) or gonadotrophins in combination with intrauterine insemination as a first line of treatment for unexplained infertility (Guzick *et al.*, 1994). Achieving better success rates through low-cost stimulation methods may spare these couples the dilemma of deciding whether to receive assisted reproduction treatments, which increase the risks of psychological stress (Verhaak *et al.*, 2007), operative risks (Kelada and Ghani, 2007), multiple gestations and ovarian hyperstimulation syndrome (Bhattacharya, 2003) as well as incurring a financial burden for IVF treatment, a major concern of infertility couples in developing countries (Shahin, 2007).

Clomiphene citrate, a nonsteroidal partial oestrogen agonist and antagonist (Biljan *et al.*, 1999), has an anti-oestrogenic effect on the hypothalamus, which induces ovulation, but its effect on other oestrogen-sensitive tissues may be primarily agonistic or antagonistic (Horstein and Schust, 1996). Although CC-induced ovulation rates are between 80% and 85%, conception rates are only around

40% (Clark and Markaverich, 1981; Massai *et al.*, 1993). This discrepancy may be secondary to either negative effects of CC on oocyte or granulosa cells, or prolonged anti-oestrogenic effects on the cervical mucosa and the endometrium, especially around implantation (Wu and Winkel, 1989). Ohno and Fujimoto (1998) found lower pre-ovulatory oestrogen receptor concentrations in the endometrium with CC cycles. In target tissues, the synthesis of progesterone receptors is regulated by oestrogen receptors (Jänne *et al.*, 1975).

Improving the pregnancy rate in CC induction cycles has been tried repeatedly through adjuvant treatment such as *N*-acetyl cysteine administration from cycle day 2 to day 6 (Badawy *et al.*, 2006), human chorionic gonadotrophin (HCG) injection around ovulation (Vlahos *et al.*, 2005); follicular-phase ethinyl oestradiol (EE) (Yagel *et al.*, 1992) and combination of conjugated equine oestrogens and micronized progesterone both in the pre-ovulatory and luteal phases (Biteker *et al.*, 2004). In addition, phytoestrogens have been successfully added to the follicular-phase of CC induction cycles managed by timed intercourse (Shahin *et al.*, 2008) or intrauterine insemination (Unfer *et al.*, 2004).

Phytoestrogens (PE) are natural products with oestrogenic activity, that occur in many plants and fungi (Cook and Samman, 1996) and include daidzein, genistein, and glycitein as well as black cohosh (*Actaea racemosa* and *Cimicifuga racemosa*). Black cohosh is a perennial plant member of the buttercup family that is native to North America. The constituents of the root rhizome extract include triterpene glycosides (acetin, cimicifugoside, 27-deoxyacetin), organic acids (isoferulic acid, cimicifugic acids) (A, B, E and F), fukinolic acid, caffeic acid, salicylic acid, cimicifugin, tannins and phytosterin (Brinker, 2000; Jellin *et al.*, 2004).

PE uterotrophic activity has been demonstrated in animal models (Diel *et al.*, 2000, 2001). The relative binding affinity of different PE for oestrogen receptors ranges from an oestrogen receptor β predominant affinity (Kuiper *et al.*, 1997; Salvatori *et al.*, 2003) to almost equivalent binding to both oestrogen receptor and oestrogen receptor β (Han *et al.*, 2002). The combination of *C. racemosa* with tamoxifene proved to be effective for a 12-month relief of post-menopausal hot flushes (Hernández and Pluchino, 2003), presumably due to its antidepressant activity (Winterhoff *et al.*, 2003). While in some studies, phytoestrogens were found to bind to oestrogen receptor *in vitro* or have an oestrogenic effect (Jarry *et al.*, 1985; Duker *et al.*, 1991; Kruse *et al.*, 1999), other studies reported a lack of binding to oestrogen receptors (Einer-Jensen *et al.*, 1996). An anti-oestrogenic effect, however, was suggested due to the ability of *C. racemosa* extracts to antagonize the proliferation of cells induced by oestradiol *in vitro* (Zierau *et al.*, 2002).

The aim of this study was to compare follicular-phase supplementation with either PE or EE in couples with unexplained infertility induced by CC after repeated failures.

Materials and methods

Inclusion criteria and randomization

The study was approved by the institutional council of the Department of Obstetrics and Gynecology, Assiut University. Couples attending the infertility clinic at Assiut University Hospital were approached. The study was conducted from March 2007 to January 2008. Patients younger than 35 years, presenting with primary or secondary infertility following regular marital life for at least 1 year and diagnosed with unexplained infertility who had received five unsuccessful CC-timed intercourse stimulation cycles were included. Patients were required to have at least 2 months rest after the last induction cycle. The diagnosis was based on the taking of a complete history, physical examination and a documentation of a complete infertility work-up within the previous 6 months, either conducted within the setting of the hospital or at a licensed infertility management clinic. A written informed consent was obtained. Patients had the right to refuse to participate and/or withdraw from the study at any time without being denied their regular full clinical care. Personal information and medical data collected were subjected to confidentiality and were not made available to a third party. A serum FSH concentration exceeding 10 IU/ml on day 3 (basal) served as an exclusion criterion.

Figure 1 shows a flowchart of the study including patient enrolment, allocation, follow-up and analysis. A total of 126 patients were eligible and agreed to participate. Participants were randomly assigned using a computer-generated randomization table produced by the secretary of the Statistics Unit, Assiut University Hospital. Patients were blinded to their treatment using previously prepared serially numbered closed envelopes. Women were randomly assigned to either Group one (CC plus PE) or Group two (CC plus EE). Primary outcome was pregnancy as estimated by positive serum pregnancy test as described below.

Sample size estimation

Sample size calculation was based on the primary outcome (pregnancy rate). After CC induction, in a setting of timed intercourse in normally ovulating women, the pregnancy rate was 22.2% per cycle (Fujii *et al.*, 1997). Assuming a 25% difference between study and control groups, the sample size required for the study was estimated to be 63 patients in each arm. This was based on an 80% power of the study and a 95% confidence interval.

Experimental protocol

All subjects received 150 mg/day CC from day 3 until day 7 of the cycle. Group 1 also received additional follicular-phase supplementation from day 1 to day 12 in the form of 100 μg/day EE (Kahira Company for Pharmarmaceutical and Chemical Industries, Egypt) whereas Group 2 received 120 mg/day oral PE in the form of film-coated tablets of cimicifuga rhizome dry extract preparation (Klimadynon; Bionorica, Germany). Transvaginal follicular monitoring was performed on all patients on days 7 and

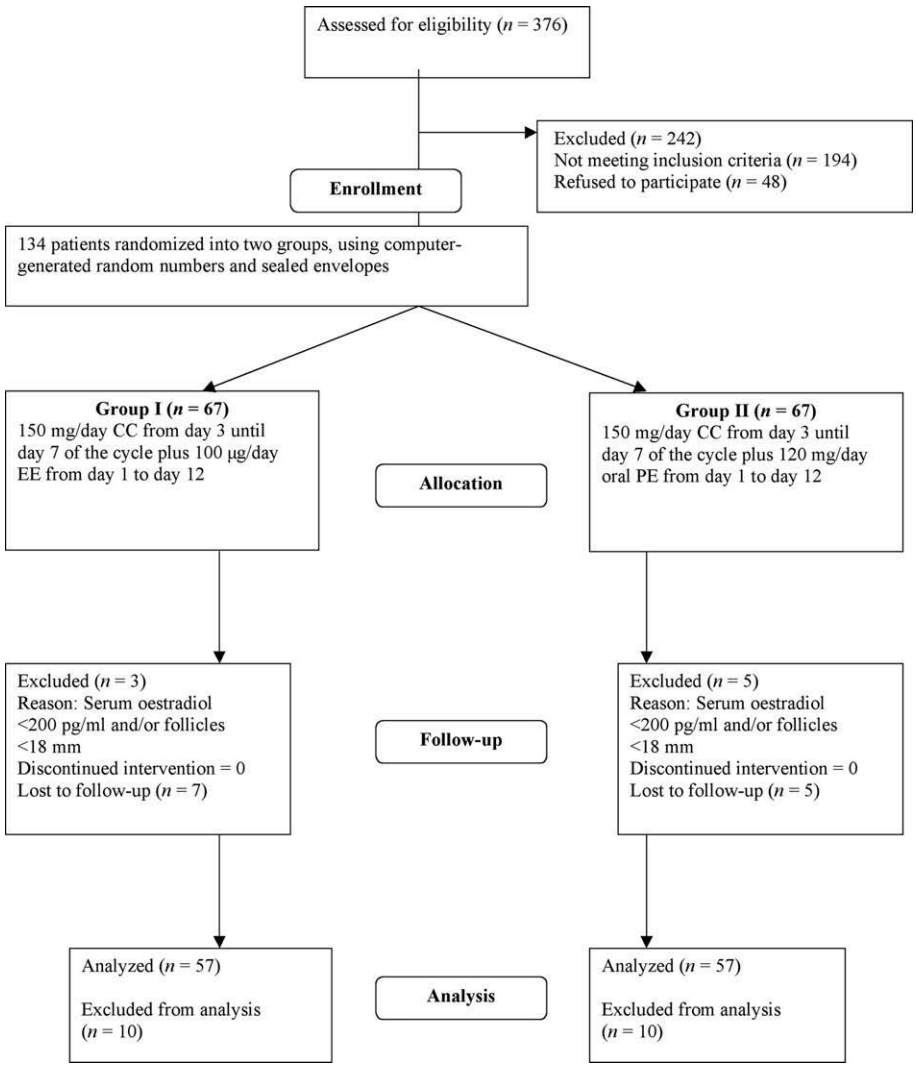

Figure 1. Flow diagram of patient enrolment, allocation, follow-up and analysis. CC = clomiphene citrate; EE = ethinyl oestradiol; PE = phytoestrogen.

9 and then individualized according to response. When one leading follicle attained a diameter of 18 mm or more, 10,000 IU HCG i.m. injection (Pregnyl; Organon, Holland) was given. Serum LH and oestradiol concentrations were estimated on the day of HCG injection. Timed intercourse was advised starting every other day for 1 week from the night of HCG administration. No luteal-phase support was given in either group.

Hormonal assay

Serum oestradiol was measured by radioimmunoassay using direct double-antibody kits (Pantex, Santa Monica, USA). Serum progesterone was measured on days 21–23 of the cycle by radioimmunoassay using an antibody-coated tube method (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, USA). A serum β-HCG concentration was determined 14 days after HCG injection if menses had not yet occurred. Pregnancy was diagnosed as an increase in the serum β-HCG concentration on serial determinations at least 2 days apart. Biochemical pregnancy was defined as a falling β-HCG concentration on serial determination. Clinical pregnancy was diagnosed by visualization of a gestational sac with fetal heart beat, using transvaginal ultrasound when the β-HCG concentration was more than 1500 IU/l. Miscarriages were defined as biochemical pregnancies and/or cases with positive β-HCG testing who aborted spontaneously before reaching the stage of clinical pregnancy and/or cases aborting before 12 weeks of pregnancy.

Statistical analysis

The analysis was carried out according to the intention-to–treat principle. The data were entered on Microsoft Access database and analysed using the Statistical Package for Social Science (SPSS version 13; SPSS Inc, Chicago). Comparisons between the groups were performed using Student's *t*-test to compare the mean values between groups in scale variables. However, chi-squared tests were used to compare the dichotomous and ordinal variables in the groups. For analysis, P-value < 0.05 was considered significant.

Results

Of the 376 patients assessed for participation in the study, 194 did not meet the inclusion criteria and 48 refused to participate. A total of 134 recruited patients were allocated to each treatment group (each group comprising 67 patients). Eight patients (three in Group 1 and five in Group 2) failed to reach a serum oestradiol value of >200 pgm/ml at time of follicular maturation and/or a follicular development of >17 mm. These patients were not given their HCG injection and the cycle was cancelled and they were excluded from the study. Twelve patients were lost to follow-up after HCG injection and were excluded from the analysis. Of these patients, seven were in Group 1 and five in Group 2. Analysis included 57 patients in Group 1 and 57 patients in Group 2. **Figure 1** shows a flow diagram of patients' enrolment, allocation, follow-up and analysis.

Both groups were comparable in regards to age (25.7 ± 3.2 versus 25.8 ± 2.6 years), body mass index (23.6 ± 1.3 versus 23.7 ± 1.5 kg/m^2), infertility duration in months (28.2 ± 2.6 versus 28.8 ± 3.1), number of patients presenting with primary or secondary infertility as well as day 3 serum FSH concentration (4.34 ± 0.39 versus 4.36 ± 0.41 IU/ml) (**Table 1**).

Although both groups produced a comparable number of pre-ovulatory follicles, with diameter of >17 mm, Group 1 patients needed significantly more days (13.55 ± 0.99 versus 11.65 ± 0.98, $P < 0.001$) to reach follicular maturation, compared with Group 2 (**Table 2**). EE users had significantly thinner endometrium (7.66 ± 0.68 versus 8.08 ± 0.59 mm, $P < 0.001$), lower serum oestradiol concentration at the time of HCG injection (245.10 ± 15.24 versus 277.0 ± 27.73 pg/ml, $P < 0.001$) and lower serum progesterone in the second half of the cycle (10.52 ± 0.89 versus 12.15 ± 1.99 ng/ml, $P < 0.001$), compared with PE users (**Table 2**). No significant differences were found between the groups in regard to the number of pregnancies (9/57, 15.8% versus 14/63, 24.6%) or clinical pregnancy rates (8/57, 14.0% versus 12/57, 21.1%), although both values were higher among PE users. Groups had comparable miscarriage and multiple pregnancy rates (**Table 2**).

Discussion

The problem of a relatively low pregnancy rate despite good ovulation in CC-induced cycles presents a challenge to reproductive specialists (Check, 2006). No evidence exists in the literature that PE affects human cervical mucous quality. It was thought that PE would, when used before CC in the follicular-phase of the induction cycle, act on the endometrium and oocyte quality by occupying oestrogen receptors and mostly inhibiting the anti-oestrogenic activity of CC (Casper, 2004; Unfer *et al.*, 2004; Shahin *et al.*, 2008). This is the likely mechanism of action for the favourable follicular maturation achieved in a shorter time and the endometrial response achieved both in this and similar studies (Unfer *et al.*, 2004; Shahin *et al.*, 2008). EE would improve the quality of cervical mucous around implantation time to enhance the results of timed intercourse (Check, 2006) by reverting the anti-oestrogenic activity of CC and improving both endometrial thickness and pregnancy rates (Yagel *et al.*, 1992; Gerli *et al.*, 2000). Based on these studies, the effects were compared of adding follicular-phase EE or PE (*C. racemosa*) to CC induction to revert its anti-oestrogenic effects and improve cycle characteristics in couples with unexplained infertility. This study found significantly better cycle characteristics in PE compared with EE users although there was no significant difference with regard to pregnancy rates in both groups.

Shahin *et al.* (2008) classified the effect of follicular-phase PE into direct and indirect effects. The direct effect operates through oestrogen receptor activation, displacing CC from its receptors (Unfer *et al.*, 2004). The indirect effect, however, may act through inhibiting the deleterious effects of CC on granulose cells and oocytes (Chung and Craig, 1989). This study's reported significant differences in cycle characteristics, although not in pregnancy rate, in favour of the PE group, could be attributed to the effect of PE on oestrogen receptors, a mechanism of action which is not shared by EE.

The higher progesterone concentration among PE users may be explained by its measurement at a more advanced time of the luteal-phase, secondary to earlier follicular maturation in this group (Shahin *et al.*, 2008). Moreover, this difference may be attributed to better follicular quality or the increase in the progesterone target gene expression secondary to oestrogen receptor activation by PE (Casper, 2004). The shorter induction interval among PE users was not reflected by a significant increase in the mean number of follicles (>17 mm) at the time of HCG injection (2.21 ± 0.77 for CC plus EE group compared with 2.16 ± 0.79 CC plus PE group). This negates an effect of

Table 1. Baseline characteristics of study participants.

Characteristic	*CC + EE (n = 57)*	*CC + PE (n = 57)*
Age (years)	25.7 ± 3.2	25.8 ± 2.6
Body mass index (kg/m^2)	23.6 ± 1.3	23.7 ± 1.5
Infertility duration (months)	28.2 ± 2.6	28.8 ± 3.1
Primary infertility *n* (%)	33 (57.9)	34 (59.6)
Secondary infertility *n* (%)	24 (42.1)	23 (40.4)
Day-3 basal serum FSH (IU/ml)	4.34 ± 0.39	4.36 ± 0.41

Values are mean ± SD unless otherwise stated; CC = clomiphene citrate; EE = ethinyl oestradiol; PE = phytoestrogen. There were no statistically significant differences between the two groups.

Table 2. Comparison of the clinical outcomes of the two treatment groups.

Item	*CC + EE (n = 57)*	*CC + PE (n = 57)*	P-*value*
Days until HCG injection	13.55 ± 0.99	11.65 ± 0.98	<0.001
Endometrial thickness (mm)	7.66 ± 0.68	8.08 ± 0.59	<0.001
Mean number of pre-ovulatory follicles >17 mm	2.21 ± 0.77	2.16 ± 0.79	NS
Serum oestradiol on day of HCG (pg/ml)	245.10 ± 15.24	277.0 ± 27.73	<0.001
Serum progesterone (ng/ml)	10.52 ± 0.89	12.15 ± 1.99	<0.001
No. of pregnancies (%)	9 (15.8)	14 (24.6)	NS
No. of clinical pregnancies (%)	8 (14.0)	12 (21.1)	NS
No. of miscarriages (%)[a]	1 (1.8)	2 (3.6)	NS
No. of multiple pregnancies (%)	1 (1.8)	1 (1.8)	NS

Values are mean ± SD unless otherwise stated; CC = clomiphene citrate; EE = ethinyl oestradiol; NS = not statistically significant; PE = phytoestrogen.

[a]Miscarriages were defined as biochemical pregnancies and/or cases with positive β-HCG testing who aborted spontaneously before reaching the stage of clinical pregnancy and/or cases aborting before 12 weeks of pregnancy.

more corpora lutea among the PE group, that could produce a higher luteal-phase serum progesterone and supports the theoretical effect of PE on oestrogen receptor activation, that consequently raises the progesterone target gene expression (Casper, 2004). The indirect effects of PE may antagonize the CC anti-oestrogenic activity on the oocyte and granulose cells, thus producing a mature follicle in shorter time and eventually attaining higher success rates (Shahin *et al.*, 2008).

In ovariectomized rats, PE exerts an oestrogenic-like effect on the uterine and vaginal morphology and uterine growth and can stimulate the growth of oestrogen receptor-positive cells. The uterine response to PE involves the activation of a large pattern of oestrogen-sensitive genes (Barkhem *et al.*, 1998; Pike *et al.*, 1999; Belcher and Zsarnovszky, 2001; Morito *et al.*, 2001, 2002). These results showed that PE at the used dose had a significantly greater effect on the endometrium and follicular growth, compared with EE. This study used PE at the prescribed dose to simulate the effect of successful PE adjuvant treatment with CC used in previous work (Shahin *et al.*, 2008). Such a high PE dose was considered as appropriate to make a proper comparison to the effects of a lower EE dose because the relative oestrogenic activity of PE is several orders of magnitudes less than EE (Casper, 2004; Unfer *et al.*, 2004). PE were found, nevertheless, to have a higher binding affinity to oestrogen receptor than EE and mestranol (Shutt and Cox, 1972).

This study aimed at inhibiting the anti-oestrogenic activity of CC in recurrent induction failure couples. This was targeted on two sites, which are complementary to each other: the first would be the endometrium and granulose cells of the ovary, the second would be the cervical mucous. It seems that the reverting of CC anti-oestrogenic activity on the endometrium and follicular growth requires blocking of the oestrogen receptor, which is mainly operated by PE and apparently to a lesser extent by EE, as studies proving its lower binding affinity to oestrogen receptor compared with PE, imply (Shutt and Cox, 1972). It is postulated that the higher oestrogen receptor-binding affinity of PE, compared with EE, may cause these differences in endometrial thickness and follicular growth. The higher PE doses were used to compensate for the lower oestrogenic activity of PE compared with EE (Casper, 2004). The lack of a significant difference in pregnancy rate between the two groups could be due to the sample size. A larger sample size would need a smaller difference in pregnancy rates to elicit significance. The sample size needed a 25% difference to show significance. Taking this difference as an acceptable measure, other explanations include a better action exerted on the cervical mucous by ethinyl oestradiol than PE, thus facilitating better-timed intercourse outcomes. This may have compensated for the relatively weaker EE effect on the endometrium and follicular maturation, thus, despite a less favourable effect on the endometrium and follicular growth, raising the pregnancy rate among EE users. This may be responsible for the non-significant difference in pregnancy rate between both groups. Short-term use of follicular-phase EE, specifically around the time of ovulation, has improved the results of post-coital test in a setting of infertility management (Check, 2006). On the other hand, the effect of PE on the human cervical mucous remains untested, both in this and in other studies (Casper, 2004; Unfer *et al.*, 2004; Shahin *et al.*, 2008). When clover-infertile ewes were exposed to PE, they had reduced spinnbarkeit of the cervical mucus, inability to store spermatozoa in the cervix and, consequently, a reduced fertilization rate (Adams, 1981). Further studies are needed to examine the effect of both PE and EE on the human cervical mucous.

Although treatments such as empirical CC and unstimulated intrauterine insemination showed higher patient acceptability, they were unlikely to offer superior live birth rates compared with expectant management (Bhattacharya *et al.*, 2008). Further studies should evaluate the potential value of adding follicular-phase phytoestrogens to CC-induced cycles in terms of pregnancy or live birth rates (Bhattacharya *et al.*, 2008). Other limitations of this study include lack of investigation of endometrial sonographic

or Doppler characteristics around mid-cycle and the inability to blind the investigators to the treatment arms due to limitations in preparing identical drugs. An experimental model that can study the direct effect of CC and PE on oestrogen receptor, progesterone receptor and cervical mucous should be proposed.

In conclusion, in unexplained infertility women with repeated failure of CC induction with timed intercourse, follicular-phase supplementation with PE significantly improved cycle characteristics compared with ethinyl oestradiol. It is postulated that both drugs act by reverting the peripheral anti-oestrogenic activity of CC. Further studies are needed to assess and compare the effects of each drug on the cervical mucous, endometrium and follicular growth.

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