High Dose of Phytoestrogens Can Reverse the Antiestrogenic Effects of Clomiphene Citrate on the Endometrium in Patients Undergoing Intrauterine Insemination: A Randomized Trial

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OBJECTIVE: *To compare the effectiveness of clomiphene citrate (CC) alone or combined with phytoestrogens (PE) in ovulation induction in patients who had intrauterine insemination in a randomized, double-blind study.*

METHODS: *A total of 134 women aged 25–35 years, who were infertile for at least 2 years and who had oligomenorrhea or amenorrhea associated with a positive menstrual response to the intramuscular progesterone-challenge test were enrolled. They were randomly treated with CC (100 mg daily for 5 days) and CC (100 mg daily for 5 days) in combination with PE (1500 mg daily for 10 days). We estimated the difference in uterine artery pulsatily index, number of preovulatory follicles, endometrial thickness, and pregnancy rate.*

RESULTS: *Both treatments increased follicle-stimulating hormone, luteinizing hormone, and 17β-estradiol plasma concentrations, but the differences were not statistically significant. However, the differences in endometrial thickness of the two groups were statistically significant. No significant differences in the pulsatility index values and in the number of preovulatory follicles were noted.*

CONCLUSION: *A high dose of phytoestrogens can reverse the deleterious effects of clomiphene citrate on endometrial thickness and could contribute to higher pregnancy rates. (J Soc Gynecol Investig 2004;11: 323–8)*

KEY WORDS: Clomiphene citrate, phytoestrogens, isoflavone, intrauterine insemination.

Clomiphene citrate (CC), a nonsteroidal estrogen agonist and antagonist, was synthesized in 1956. It was reported to be effective in ovulation induction by Greenblatt et al[1] in 1961 and is now the most commonly used drug to treat infertility.[2] Clomiphene is most effective in inducing ovulation in women in the World Health Organization group II, which consists of women with anovulation or oligo-ovulation, a wide variety of menstrual disorders, relatively normal (or elevated) gonadotropin levels, and evidence of significant endogenous estrogen production.[3] The Food and Drug Administration–approved dosages for CC are 50 or 100 mg/day for a maximum of 5 days per cycle.[4] After spontaneous menses or the induction of menses by progesterone, CC is started on cycle day 3, 4, or 5 at 50 mg daily for 5 days. Obese women tend to require higher doses of CC to achieve ovulation.[5,6] CC is not stored in adipose tissue, and the increased dose used in obese women is more likely the result of a more intensive anovulatory state with higher androgen levels producing a more resistant hypothalamic-pituitary-ovarian axis.[7] The higher dosage of CC will eventually help to achieve the same therapeutic success in overweight women as in the lean ones.[8,9]

Over the years, evidence has accumulated that CC is successful in inducing ovulation in 50–75% of the cases,[10,11] but the number of pregnancies achieved after ovulation induction is much lower than expected.[12] This discrepancy has been attributed to a negative effect of CC on the endometrium, ie, to its prolonged antiestrogenic effects on endometrial receptivity[13] and cervical mucus.[14] Moreover, Hsu and colleagues[15] demonstrated that CC affects uterine blood flow, which was lower in the early luteal phase and in the perimplantation phase, compared with that of untreated women.

Many plants produce isoflavones that possess estrogenic activity in animals and are, thus, called phytoestrogens (PE). PE are nonsteroidal compounds present in a variety of dietary products.[16] Among the foods consumed by humans, soybeans contain the highest concentration of isoflavones, such as daidzin, genistin, and glycitin. Some of their metabolites, eg, daidzein, genistein, and glycitein, also show estrogenic activity.[17] These PE are of increasing interest for their possible

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Published by Elsevier Inc.

1071-5576/04/$30.00
doi:10.1016/j.jsgi.2003.12.007

influence on the physiology of the reproductive tract.[18] Epidemiologic studies have shown that the ingestion of food rich in PE might provide protection against certain estrogen-dependent cancers, such as breast and prostate cancer.[19,20] Several reports have suggested that some of the effects of PE are mediated by their ability to bind the estrogen receptor (ER).[21–24] This has been confirmed recently by numerous studies in which it was clearly shown that different PE such as genistein, coumestrol, diadzein, zearalenone, glycitein, and many others bind both the ERs (ERα and ERβ) and modulate a variety of estrogen-dependent processes.[17,25–28] PE exert an estrogenic-like effect on the uterine and vaginal morphology and uterine growth in ovariectomized rats and can stimulate the growth of ER-positive cells. Moreover, uterine response to estrogens also involves the activation of a large pattern of estrogen-sensitive genes. The analysis of this cluster of estrogen-sensitive endometrial genes is of help in identifying estrogenic substances, assessing their potency, and elucidating their mechanism of action.[29] The effects of many PE on estrogen-sensitive genes, as well as their uterotropic activity and their selective affinity to estrogenic-receptor subtypes, have been investigated and characterized in numerous studies.

The effects of genistein was investigated in normal and malignant experimental uterine models in vivo by Diel et al.[30] The effects of the 3-day oral administration of genistein (25, 50, or 100 mg/kg of body weight per day) on uterine and vaginal morphology, uterine growth, and uterine gene expression in the uterus and vagina of ovariectomized DA/Han rats were compared with those of ethinyl estradiol (0.1 mg/kg of body weight per day). A dose-dependent increase of the uterine wet weight and uterine and vaginal epithelial height, a dose-dependent up-regulation of complement C3, down-regulation of clusterin mRNA expression, and stimulation of the vaginal cornification were observed after administration of genistein. Uterine gene expression and vaginal epithelium responded to genistein at doses where no significant effects on uterine wet weight were detectable. In conclusion, four independent uterine and vaginal parameters indicated that genistein is a weak estrogen receptor agonist in the uterus and vagina of female DA/Han rats, and evidence was provided for a selective estrogen receptor modulator-like action of genistein in normal and malignant uterine tissues.

In another study by Diel et al,[29] daidzein also provoked a significant stimulation of the uterine wet weight and was able to modulate the expression of a large pattern of estrogen-sensitive genes. Although daidzein was a very weak stimulator of uterine growth in comparison to ethynyl estradiol, it was able to strongly alter the expression of the androgen receptor, ERs, and complement C3.[18]

Other studies have also confirmed that glycitein, diadzein, and genistein show clear estrogenic activity in in vitro and in vivo models and have high binding affinity for ERα and ERβ.[27,31–37]

Some clinical studies of the effects of PE on the endometrium have also been done recently, but their results are discordant.[38–41] Despite the evidence that PE act as an estrogen regarding uterotropic activity in animal models and the modulation of the expression of estrogen-sensitive genes, PE seem to exert an antagonistic effect clinically opposing the effect of estrogens on the endometrium. We have hypothesized that the dosage of PE administered in these studies may have been too low to cause any estrogen-like effects on the endometrium.

The aim of the present study was to compare pregnancy rates after intrauterine insemination (IUI) in two groups of women receiving CC, with or without high doses of phytoestrogens. We also investigated plasma hormonal levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2); follicular recruitment; and the differences in endometrial thickness and uterine artery blood flow, which are sensitive indicators of uterine receptivity.[42]

MATERIALS AND METHODS

Patients

A total of 134 women were enrolled in this study. Inclusion criteria were age 25–35 years, infertility for at least 2 years, and oligomenorrhea or amenorrhea associated with a positive menstrual response to progesterone challenge test (performed with 100 mg progesterone in oil). All patients had normal serum concentrations of thyroid-stimulating hormone, prolactin, and total testosterone. It was their first cycle of ovulation induction using CC, and no patient had received fertility medications in the past.

We excluded couples with male factor infertility (semen analysis according to World Health Organization criteria[43]), uterine or tubal abnormalities (hysterosalpingogram), and overweight women (body mass index >25).

Treatment Protocol

The protocol was approved by the Institutional Board of Research, and all patients gave written informed consent before being enrolled in the study. The day in which the menses started was designated day 1 of the treatment cycle. All patients were randomly distributed in a double-blind manner into the following two groups: in group A (65 patients), stimulation began on day 3 with the administration of 100 mg of CC daily for 5 days. From day 3 and for 10 days they received phytoestrogens (1500 mg daily). In group B (69 patients), stimulation began on day 3 with the administration of 100 mg of CC daily for 5 days. From day 3 and for 10 days placebo tablets identical to those containing phytoestrogens were administered to all patients of this group.

The soy product was formulated in tablets each containing 500 mg of soy isoflavones. The components of soy isoflavones were 40% to 45% genistein, 40% to 45% daidzein, and 10% to 20% glycitein.

Plasma 17β-estradiol concentrations and ultrasonographic analysis of follicular size and number were assessed on days 5, 7, and 12 of the stimulated cycles.

All patients were given 10,000 IU of intramuscular human chorionic gonadotropin (hCG) when serum 17β-estradiol

concentration exceeded 200 pg per mature follicle and when there was at least one follicle with a minimum diameter of 18 mm.

Intrauterine Insemination

A single intrauterine insemination was performed 24 to 36 hours after the administration of hCG.

Laboratory Determinations and Ultrasound Analysis

Plasma concentrations of FSH, LH, and 17β-estradiol were determined by radioimmunoassay on blood samples on days 1, 5, 9, and 12 of the menstrual cycle. Ultrasound scans were performed daily starting from day 1 (to rule out ovarian cysts) until the mean follicular diameter reached a length of 18 mm. The endometrial thickness was estimated on the day of hCG administration. Pulsatility index (PI) was recorded for both uterine arteries. A gynecologist experienced in transvaginal sonography performed all examinations with a 5-MHz broadband probe.

The examiner was blinded to the patient's group assignment. Color Doppler sonography was used for imaging the uterine arteries on cycle days 2, 8, and 12. The PI was calculated by subtracting the peak end-diastolic-shifted frequency from systolic-shifted frequency and dividing the result by the mean Doppler shift over the cardiac cycle. The intraobserver coefficient of variation for measurement of PI was 5.3%. All examinations were performed between 9:00 and 11:00 AM to reduce the effects of the circadian variation of PI.[42]

Determination of Pregnancy States

A biochemical pregnancy was defined as a small transitory increase in β-hCG levels followed by a decrease in β-hCG levels within 1 week. Clinical pregnancies were defined by visualization of a gestational sac at the first planned ultrasound examination obtained at 6–7 weeks of pregnancy or a serum β-hCG level over 1400 mIU in the absence of a scan. Ongoing pregnancies were defined as gestations that reached 20 weeks' gestation.

Statistical Analysis

Statistics were performed with the SPSS statistical package (SPSS Inc., Chicago, IL). Chi-square and Fisher exact test were used. Statistical significance was defined as a P value $< .05$.

RESULTS

On day 1, before the beginning of treatment, there were no statistically significant differences in the plasma levels of FSH, LH, and 17β-estradiol. Both treatments increased FSH, LH, and 17β-estradiol plasma concentrations, but the differences between the two groups were not statistically significant. However, the mean of FSH value in the group treated with CC in combination with PE was lower than that of the group treated with CC alone. There were no statistically significant differences in the plasma levels of FSH, LH, and 17β-estradiol between the groups during the entire treatment period (data not shown).

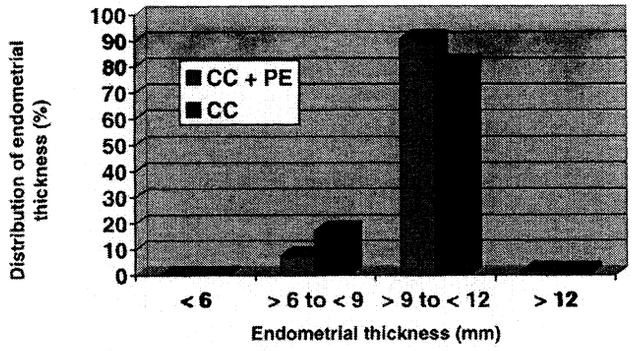

Figure 1. Percentage distribution of endometrial thickness on the day of hCG administration.

There were no statistically significant differences between the two groups in follicular development and in the number of preovulatory follicles (data not shown).

Endometrial Thickness and Uterine Blood Flow

The endometrial thickness was estimated on the day of hCG administration. In all cases it was more than 6 mm. It was between 6 and 9 mm in five cases in group A (7.7%) compared with 12 cases in group B (17.4%); between 9 and 12 mm in 59 cases (90.8%) in group A compared with 56 cases (81.2%) in group B; and greater than 15 mm in one case (1.5%) in group A compared with one case (1.5%) in group B (Figure 1). No significant differences in PI values were noted (Figure 2).

Pregnancy Rate

The miscarriage rate was 3.1% in group A compared with 8.7% in group B. The difference was statistically significant (Table 1). At the same time, the percentage of the ongoing pregnancies was higher in the group treated with CC in combination with PE (group A, 20.0%) than in the group treated with CC alone (group B, 4.4%; $P < .05$) (Table 1).

DISCUSSION

Clomiphene, which was introduced in 1967, is considered to increase the incidence of spontaneous abortion.[8,12] The in-

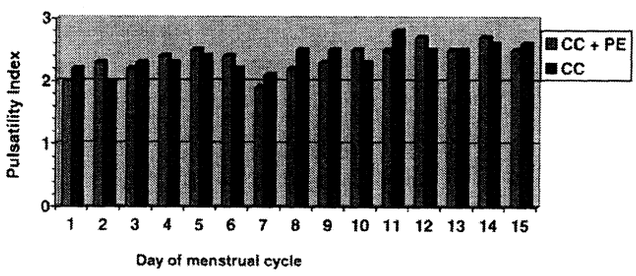

Figure 2. Pulsatility index (expressed as mean ± standard error) in the patients who had ovarian stimulation with clomiphene citrate alone (CC) or combined with phytoestrogens (CC + PE).

Table 1. Characteristics of Patients Who Received Clomiphene Citrate Plus Phytoestrogens or Clomiphene Citrate Alone

Characteristic	CC + PE Group A	CC Group B	*P*
No. of patients	65	69	—
Mean (± SD) age (y)	28 ± 5.6	26 ± 4.2	NS
Mean (+SD) duration of infertility (mo)	48.1 ± 18.5	36.7 ± 9.6	NS
No. (%) of pregnancies per cycle			
Biochemical	3 (4.6)	4 (5.8)	NS
Miscarriages	2 (3.1)	6 (8.7)	<.05
Ongoing pregnancies	13 (20.0)	3 (4.4)	<.05

CC = clomiphene citrate; PE = phytoestrogens; SD = standard deviation; NS = not significant.

crease in spontaneous abortion in clomiphene-treated pregnancies has been attributed to several factors, including impaired endometrial development.[9,44,45]

Goldstein et al[46] reported that estradiol concentrations that were either too low or too high and associated with normal progesterone concentrations caused desynchronized endometrial development. An antagonistic effect of CC on the endometrium has long been recognized in histologic studies.[44,47–49] Furthermore, numerous ultrasound studies have shown that CC may reduce endometrial thickness.[50–55]

It has been shown that the adverse effects of CC on the endometrium can be prevented by adding estrogens with clomiphene or after clomiphene.[56] However, in 1990, Bateman et al[57] demonstrated that exogenous estrogens did not improve the quality or quantity of cervical mucus in CC-treated women. Similarly, the use of CC for in vitro fertilization does not seem to reduce the implantation rate,[58] possibly because the contemporary use of human menopausal gonadotropin (HMG) with its more sustained estradiol production could have a positive effect on the endometrium.

Our study shows that adding PE to CC increased endometrial thickness and decreased the risk of abortion. A better pregnancy rate may have resulted from the improved endometrial characteristics caused by the administration of high-dosage PE (possibly having an estrogen-like action that balances the antiestrogenic effect of clomiphene), which facilitates embryo implantation. Results supporting this hypothesis were found in studies on IUI cycles.[56,58] These studies estimated that ovulation induction with sequential clomiphene-HMG resulted in fecundity that is double that of CC alone, which reaffirmed a possible positive role of estrogens on the endometrium. Furthermore, Dickey et al[58] noticed that the increased pregnancy rate achieved when HMG was administered after CC was related to the increased number of preovulatory follicles, and a significant doubling of the implantation rate per follicle was also calculated. In that study, the estradiol level per follicle nearly doubled for clomiphene-HMG treated women compared with women treated only with CC.

We determined the endometrial effects of high doses of PE. In vitro studies demonstrated that PE have estrogenic-like effects and promote the transcription of estrogen-sensitive genes. The in vivo studies on ovariectomized rats also confirmed the estrogenic effects of PE on the endometrium. Studies on the effects of PE on the endometrium in hormone replacement therapy has not only not confirmed the efficacy of these PE in hormone replacement therapy but also has often shown an antiestrogen-like effect of PE on the endometrium.[38–41] We hypothesized that this could be attributable to the insufficient length of the treatments, the insufficiency of the daily dosage administered, or both.

Phytoestrogens bind both estrogen receptors, ERα and ERβ, as shown in many studies,[17,25–28] with different affinity and efficacy, acting sometimes as a partial or a full agonist, depending of the ER.[26,27] For example, genistein acts as a partial agonist of ERβ, although it binds ERβ with an approximately 30-fold higher affinity than ERα in humans, and genistein binds ERα acting as a slight superagonist (range, 107–130% of the efficacy shown by the endogenous agonist estradiol).[27] Moreover, tissue distribution of ERs varies during the menstrual cycle and menopause[59–61] and is modulated by the same estrogens.[59] Finally, it is important to remember that isoflavones reduce the serum concentration of estradiol by feedback regulation.[17] The modulation of ERs is much more complex than thought before the discovery of ERβ. Thus, depending on tissue distribution and specific prevalence of ERα and ERβ on the target organ, PE could act in different ways resulting in different clinical effects. For example, a higher affinity combined with a lower efficacy in one of the ERs can result in an antiestrogen-like effect on the target organ, while the dosage could be insufficient to permit the displacement of the endogenous ligand on the other ER, where the PE could show a superagonist effect. The administration of a higher dosage of PE can help to displace the endogenous estrogen and bind to the receptor, or longer therapy could affect the tissular expression of ER subtypes. However, in two in vitro studies examining the effects of phytoestrogens on human endometrial cells, phytoestrogens were added to the cells at concentrations up to 10^{-5} M and, in the presence of estradiol, clear antiestrogenic effects were demonstrated.[62,63] An alternative hypothesis could be that phytoestrogens compete with the antiestrogenic isomer of clomiphene for estrogen receptors and have a less potent antiestrogenic effect than the cis-isomer of clomiphene citrate.

A previous study performed on postmenopausal women by our group[64] found that a longer therapy (up to 5 years) with PE had an estrogen-like effect on the endometrium, ie, led to an increased occurrence of endometrial hyperplasia in the PE-treated subjects compared with a placebo-treated group. This new study confirms our hypothesis.

In conclusion, in accordance with other studies, we noted that inadequate endometrial development might have a negative influence on the outcome of implantation. In fact, preovulatory endometrial thickness is predictive of a high risk of miscarriage. The addition of high doses of phytoestrogens to the treatment protocol of women treated with CC provoked a positive response of the endometrium. Based on our data, we conclude that a combined regimen of CC combined with high

doses of phytoestrogens could reverse the deleterious effect of CC on the endometrial development.

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