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Effect of Pyridostigmine on Ovulation Rate in Clomiphene-Resistant PCOS Women

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Abstract
Background: *Polycystic ovary syndrome is the commonest endocrine disorder in women of reproductive age. It affects round 4-9% of women in reproductive age.* ***Aim of the Work:*** *to investigate the effect of pyridostigmine on the ovulation rate in clomiphene resistance PCOS women.* ***Patients and Methods:*** *This clinical trial was conducted on 246 women with clomiphene resistant polycystic ovarian syndrome at Ain-Shams University Maternity Hospital on the period from November 2014 till August 2018. Each woman received clomiphene citrate 100 mg orally from day 3 until day 7. In addition to the clomiphene citrate, each patient received pyridostigmine, 60mg twice daily orally, from day 3 of menstrual cycle till the day of hCG injection.* ***Results:*** *pyridostigmine cotreatment during the follicular phase resulted in improvement of ovulation rate (51.2%) and pregnancy rate (15.4%). Although we did not have any control groups receiving placebo or CC alone, each patient can serve as her own historical control by her previous resistance to 150 mg of CC.* ***Conclusion:*** *Addition of pyridostigmine to CC is an effective, inexpensive and safe method for stimulating follicular development in CC-resistant PCOS and may be contemplated before gonadotrophins and laparoscopic ovarian drilling.*

Key words: *pyridostigmine, ovulation rate, clomiphene citrate, polycystic ovary syndrome*

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I. Introduction

Polycystic ovary syndrome (PCOS) is the commonest endocrine disorder in women of reproductive age. The syndrome affects around 4-9% of women of reproductive age [(1)].

The European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine (ESHRE/ ASRM) achieved a new consensus regarding the definition of PCOS. This is now defined as the presence of any two of the following three criteria: (1) polycystic ovaries; (2) oligo-and/or anovulation; (3) clinical or biochemical evidence of hyperandrogenism [(2)].

Clomiphene citrate (CC) is usually used as the first-line drug to induce ovulation in women with PCOS. Successful ovulation is achieved in ~70–85% of women and 40–50% will conceive[(3)].

Patients who do not ovulate on the dose of 150 mg for three cycles are considered to be clomiphene citrate resistant. This is common and occurs in approximately 15-40% in women with PCOS women. Complications of treatment are rare and usually mild [(4)].

Gonadotrophin treatment can be offered when these anovulatory women fail to respond to CC. The use of gonadotrophin is more expensive and associated with a much higher risk of multiple pregnancy and developing ovarian hyperstimulation syndrome [(5)].

For these patients who do not respond to CC, there are a few limited adjunctive therapies that can be tried before moving on to gonadotrophin therapy or laparoscopic ovarian drilling including bromocriptine (in the presence of hyper-polactinaemia or galactorrhea), insulin sensitizers (to treat hyperinsulinaemia), oral contraceptives (for pretreatment suppression of LH), pulsatile GnRH (to preserve physiological interactive feedback) and extended doses of CC [(6)].

However, their usefulness is limited to specific abnormalities, and many women with CC resistance do not present with any overt signs of a treatable disorder.

Pyridostigmine is an acetylcholinesterase inhibitor that may activate the cholinergic pathway, leading to inhibition of somatostatin release in the brain and thus, increasing GH secretion [(7)].

GH is a major regulator of IGF-1, which play a role in folliculogenesis, regulation of aromatase activity, and can augment FSH action on granulosa cells of the ovary [(8) (9) (10)].

These data prompted us to evaluate the use of pyridostigmine cotreatment to improve the ovarian response clomiphene citrate treatment.

Aim of the Work

To investigate the effect of pyridostigmine on the ovulation rate in CC-resistant PCOS women.

Patients and Methods

This is a clinical trial, conducted in Ain-Shams University Maternity Hospital on the period from November 2014 till August 2018. It included 246 women with clomiphene resistant polycystic ovarian syndrome.

Sample size justification:

The required sample size has been estimated using the Sample Size Calculator for Prevalence Studies v. 1.0.01 which is based on the formula described by **Daniel (1999).**

The expected ovulation rate for patients proved to be clomiphene-resistant may be assumed to equal 0%. Since there is currently no available information regarding the expected ovulation rate associated with combining pyridostigmine and clomiphene for induction of ovulation in PCO patients proved to be clomiphene-resistsant, the present study will seek an ovulation rate that may be regarded as clinically relevant.

Thus, it is estimated that a sample size of 246 patients would provide a precision of 5% for an assumed ovulation rate of 20%, which is estimated with a confidence of 95%. This would translate into a 95% confidence interval (95% CI) of 15% to 25% for the ovulation rate associated with the combination of pyridostigmine and clomiphene (i.e., an assumed ovulation rate of 20% with a 95% CI of 15% to 25%). This rate is chosen as it may be considered a clinically relevant effect size to seek in this type of research.

The formula used for calculation is as follows **(Daniel, 1999):**

$n = [Z^2 * P (1-P)]/d^2$, *where:*

n =sample size

Z = Z statistic for the targeted level of confidence (i.e., 1.96 for a 95% confidence level)

P = expected ovulation rate expressed as a proportion (i.e., 0.2)

d = precision (i.e., 0.05)

Inclusion criteria:

1. Age: 18 – 35 years old.
2. Women diagnosed as polycystic ovarian syndrome according to ***Rotterdam criteria (2004) (2):***

- Clinical hyperandrongenism (Ferriman-Gallwey Score >8) or Biochemical hyperandrogenism (elevated total/free testosterone).
- Oligomenorrhea (Less than 6-9 Menses per Year) or Oligo-Ovulation.
- Polycystic ovaries on ultrasound (≥ 12 antral follicles in one ovary or ovarian volume ≥ 10 cm^3).

3. Clomiphene citrate resistant PCOS women (defined as failure of ovulation to occur after reaching a dose of 150mg/day) ***(11).***Whether they were following up in the infertility clinic of Ain Shams Maternity hospital from the start or they came with the diagnosis settled and had enough data in their medical records.
4. Bilateral patent Fallopian tubes confirmed by HSG or laparoscope.
5. Normal semen analysis for all husbands.

Exclusion criteria:

1. Day 2 serum FSH ≥12 IU/L (to exclude poor responders).
2. Hyperprolactinemia.
3. Thyroid abnormality.
4. Renal impairment.
5. Abnormal liver function tests.
6. Any contraindication to pyridostigmine for example asthma, urinary tract blockage, stomach or intestinal blockage.

All patients were subjected to:

- Explanation of the study after which an informed consent was taken.
- Full history taking.
- Weight and height were measured then body mass index was calculated.
- Baseline hormonal profile was performed on the second day of spontaneous period or any time during the cycle if the patient was amenorrhea. This included serum FSH, LH, estradiol, free testosterone, prolactin, TSH.
- Baseline transvaginal ultrasonography was done to evaluate the endometrial thickness, uterus, ovaries (length, width, thickness and ovarian volume) and the number and diameters of the follicles on the third day of the cycle.

- Each woman received clomiphene citrate (clomid®) 100 mg orally from day 3 of menses or progesterone induced withdrawal bleeding, until day 7. In addition to the clomiphene citrate, each patient received pyridostigmine (pestinon® or mestinon®), 60mg twice daily orally, from day 3 of menstrual cycle till the day of hCG injection.
- If there was no follicle ≥ 12 mm by day 14, the cycle was presumed to be anovulatory and the pyridostigmine was discontinued.
- Follicular growth, ovulaion and endometrial thickness were followed by transvaginal ultrasonography. This was done every 2 days starting from the 10^{th} day of the cycle or as needed.
- Human chorionic gonadotropin (10,000 IU) was given IM to induce final follicular maturation if one or more follicles measure ≥18 mm in diameter.
- The patient was advised to have timed intercourse 36 hours after hCG injection.
- Two days after giving hCG, the patient was assessed for signs of ovulation (disappearance of pre-ovulatory follicle, fluid in the cul-de-sac and /or corpus luteum formation).
- Serum hCG was done in those who have ovulatory response after two weeks of hCG injection. Once positive, transvaginal ultrasonography was arranged to confirm intrauterine pregnancy.
- Each patient had one treatment cycle.

Outcome measures and Data collection:

Primary Outcome:

- Ovulatory response (number of follicles ≥ 18mm in diameter and ovulation rate in the treatment cycle.

Secondary Outcomes:

- Biochemical and clinical pregnancy rates.

Statistical methods

The collected data were coded, tabulated, and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 18.0, IBM Corp., Chicago, USA, 2009.

Descriptive statistics were done for quantitative data as minimum & amp; maximum of the range as well as mean ±SD (standard deviation) for quantitative normally distributed data, median and inter-quartile range for quantitative non-normally distributed data, while it was done for qualitative data as number and percentage.

Inferential analyses were done for quantitative variables using Shapiro-Wilk test for normality testing and independent t- test in cases of two independent groups with normally distributed data. In qualitative data, inferential analyses for independent variables were done using Chi square test for differences between proportions. The level of significance was taken at P value < 0.050 is significant, otherwise is non-significant.

II. Results

Table (1): Basal characteristics of the studied cases

		Mean±SD	Range
Age (years)		26.6±2.9	20.0–34.0
BMI (kg/m^2)		28.2±2.2	22.5–34.2
Duration of infertility (years)		2.7±1.0	1.0–5.6
LH (mIU/mL)		10.2±2.3	5.5–16.4
FSH (mIU/mL)		5.1±0.7	4.1–7.2
LH/FSH		2.0±0.4	1.0–3.1
Free testosterone (pg/mL)		3.8±0.9	1.4–5.8
E2 (pg/mL)		58.1±16.8	31-110.9
Endometrial thickness (mm) baseline		5.1±0.6	3.7–6.3
Endometrial thickness (mm) at trigger		9.1±0.7	6.9–10.2
		N	%
Infertility type	Primary	167	67.9
	Secondary	79	32.1
Presentation	Oligo or amenorrhea	195	79.3
	PCO	189	76.8
	hyperandrogenism	109	44.3

Total=246

Table (1) shows: **Basal characteristics** of the studied cases. More than two thirds of cases had **primary infertility**. The most frequent presenting symptom was oligomenorrhea/ amenorrhea (79%) of the patients, followed by PCO morphology in ultrasonography (76.8%) then hyperandrogenic symptoms (44.3%).

Table (2): Ovulation and pregnancy among the studied cases

Outcome		N	%
Ovulation		126	51.2
Chemical pregnancy		41	16.7
Clinical pregnancy		38	15.4
§Number of follicles ≥ 18mm	One	85	67.5
	Two	41	32.5
Multiple pregnancy		3/38	7.9
		Mean±SD	Range
§Number of follicles ≥ 18mm		1.3±0.5	1.0–2.0

Total=246, §In ovulatory cases only

Table (2) shows **Ovulatory response and pregnancy** among the studied cases. More than half of cases had **ovulation (51.2%). Chemical pregnancy** was detected in **(16.7%)** of the cases which decreased a little bit to **(15.4%)** when ultrasonography was done**.** About two thirds of ovulatory cases had monofollicular development ≥ 18mm, and the rest had two follicles.

Table (3): number needed to treat (NNT)

	CC alone	**CC + Pyridostigmine**	**NNT**
Ovulation rate	0%	51.2%	1.95
Chemical pregnancy rate	0%	16.7%	5.99
Clinical pregnancy rate	0%	15.4%	6.5

Table (3) shows that about two patients are needed to be treated with pyridostigmine for one of them to benefit from ovulation, and about 6 patients are needed to be treated, in order to detect biochemical pregnancy. From every thirteen patients, clinical pregnancy will occur in two patients

Table (4): Comparison according to ovulation

Variables		Positive (N=126)	Negative (N=120)	P
Age (years)		26.2±2.8	27.1±2.9	^0.020*
BMI (kg/m^2)		27.1±1.8	29.3±1.9	^<0.001*
Duration of infertility (years)		2.6±0.9	2.7±1.0	^0.285
LH (mIU/mL)		10.0±2.5	10.3±2.1	^0.464
FSH (mIU/mL)		5.1±0.7	5.1±0.7	^0.992
LH/FSH		2.0±0.4	2.0±0.4	^0.236
Free testosterone (ng/mL)		3.7±0.9	3.9±0.9	^0.129
Endometrial thickness (mm) Baseline		5.1±0.6	5.0±0.6	^0.620
Endometrial thickness (mm) At trigger		9.0±0.7	9.1±0.7	^0.217
Infertility type	Primary	88 (69.8%)	79 (65.8%)	#0.501
	Secondary	38 (30.2%)	41 (34.2%)	

^Independent t-test. #Chi square test. *Significant

Table (4) shows that patients who had responded to pyrdostigmine had significantly lower **age and BMI** compared to patients who did not respond to pyridostigmine. There were no difference between the two groups as regard duration of infertility, LH, FSH, LH/FSH, and free testosterone level.

Table (5): Comparison according to chemical pregnancy

Variables		Positive (N=41)	Negative (N=205)	P
Age (years)		25.5±3.1	26.9±2.8	^0.008*
BMI (kg/m^2)		27.4±1.6	28.3±2.2	^0.011*
Duration of infertility (years)		2.5±0.8	2.7±1.0	^0.136
LH (mIU/mL)		9.7±2.8	10.2±2.2	^0.178
FSH (mIU/mL)		5.0±0.7	5.1±0.7	^0.300
LH/FSH		1.9±0.4	2.0±0.4	^0.266
Free testosterone (ng/mL)		3.6±1.0	3.8±0.9	^0.109
Endometrial thickness (mm) Baseline		5.1±0.6	5.1±0.6	^^0.767
Endometrial thickness (mm) At trigger		9.2±0.7	9.1±0.7	^0.242
§Number of follicles≥ 18mm		1.4±0.5	1.3±0.5	^0.284
Infertility type	Primary	27 (65.9%)	140 (68.3%)	#0.760
	Secondary	14 (34.1%)	65 (31.7%)	

§In ovulatory cases only. ^Independent t-test. #Chi square test. *Significant

Table (5) shows that: **Chemical pregnancy** cases significantly had lower **age and BMI.**

Table (6): Comparison according to clinical pregnancy

Variables		Positive (N=38)	Negative (N=208)	P
Age (years)		25.3±3.1	26.9±2.8	^0.002*
BMI (kg/m^2)		27.2±1.5	28.4±2.2	^0.003*
Duration of infertility (years)		2.4±0.8	2.7±1.0	^0.078
LH (mIU/mL)		9.8±2.8	10.2±2.2	^0.332
FSH (mIU/mL)		5.0±0.7	5.1±0.7	^0.489
LH/FSH		1.9±0.5	2.0±0.4	^0.381
Free testosterone (ng/mL)		3.6±1.0	3.8±0.9	^0.188
Endometrial thickness (mm) Baseline		5.1±0.6	5.0±0.6	^0.613
Endometrial thickness (mm) At trigger		9.2±0.7	9.1±0.7	^0.163
§Number of follicles≥ 18mm		1.4±0.5	1.3±0.5	^0.279
Infertility type	Primary	25 (65.8%)	142 (68.3%)	#0.763
	Secondary	13 (34.2%)	66 (31.7%)	

§In ovulatory cases only. ^Independent t-test. #Chi square test. *Significant

Table (6) shows that: **Clinical pregnancy** cases significantly had lower **age and BMI.**

III. Discussion

Clomiphene citrate (CC) had been used as a first-line treatment for anovulatory polycystic ovary syndrome for decades, because it is a simple, cheap, and effective method to induce ovulation with minimal side-effects. However, some patients with PCOS are resistant to standard CC treatment [(12)]**.**

Ovulation induction with gonadotropins is the standard second line treatment for clomiphene-resistant women; yet, this method is costly, as well as it has high risks of ovarian hyperstimulation and multiple pregnancies [(13)]**.**

Laparoscopic ovarian drilling can be an alternative second line treatment to gonadotrophins. Although it carries no risk of OHSS and multiple pregnancies, but it is still an invasive surgical intervention with risk of anesthesia, surgery itself and its complications. In addition, there is risk of adhesion and risk of poor ovarian reserve [(14)]**.**

Considering the extent of adverse effects associated with gonadotropins and surgery, the need for continuous monitoring, the inconvenience of daily injections and, the cost, several regimens and therapies have been introduced and studied for the treatment of clomiphene resistant PCOS women, aiming at improving reproductive outcomes with low cost and less side effects.

New discoveries of some of the underlying patho-physiological abnormalities had led to an array of new treatment options such as different insulin sensitizing agents, letrozole, dexamethasone, intermittent CC, prolonged duration of CC treatment and, statins.

Till now and owing to the low quality of evidence and the wide confidence intervals, no recommendation could be made for ovulation-induction in patients with clomiphene resistant PCOS [(15)]**.**

It has been found that insulin resistance and impaired growth hormone (GH) secretion play a role in the pathogenesis of PCOS. In several studies, the GH response during stimulation tests was found to be lower in PCOS patients when compared with normal controls [(16)] [(17)] [(18)]

Some investigators have suggested that insulin- like growth factors (IGFs) are involved in the physiology of developing ovarian follicles, and that IGF-1 regulates aromatase activity and augments FSH action in the granulosa cells of the ovary [(8)] [(9)] [(19)] ..

It has been suggested that GH is a major regulator of IGF-1 production and may increase IGF-1 production in granulose cells in vivo; moreover, it may induce ovarian granulosa cell differentiation by itself [(10)] [(20)].

It is generally accepted that acetylcholine regulates GH secretion by producing a tonic inhibition of hypothalamic somatostatin release[(21)]**.**

Pyridostigmine is an acetylcholinesterase inhibitor and, thus, may activate the cholinergic pathway, leading to inhibition of somatostatin release in the brain and increasing GH secretion[(7)].

By reviewing the literature we didn't find any study evaluating pyridostigmine use in PCOS. Only one published study by ***Kim et al*** [(7)] evaluating the use of pyridostigmine as a cotreatment for controlled ovarian hyper-stimulation (COH) in low responders undergoing in vitro fertilization–embryo transfer was found.

Kim et al [(7)] in their RCT suggest that pyridostigmine cotreatment for COH could affect the serum and intrafollicular GH and insulin-like growth factor-1 concentrations and, hence, improve the ovarian response to COH

These data together with the high cost of GH injections prompted us to evaluate the use of pyridostigmine cotreatment, as a cheaper alternative, to improve the ovarian response to clomiphene citrate treatment.

This is the first study to evaluate the use of pyridostigmine as a cotreatment in clomiphene citrate resistant PCOS women.

In this study using pyridostigmine cotreatment with clomiphene citrate had led to ovulation in 126 women out of 246 one (51.2%).

This is comparable to ***Elnashar et al.*** [22] who investigated the effect of letrozole in CC resistant PCOS women and found ovulation rate of 54.6%.

This may appear in disagreement with ***Parsanezhad et al.*** [23]**,** who conducted a RCT to compare the effect of adding dexamethasone to placebo effect when added to CC in CC resistant PCOS women. Ovulation occurred in 88 % of women in dexamethasone group. But it is to be noted here that this ovulation rate is the cumulative rate as they included 230 women in their trial randomized into two groups, each woman had up to a maximum of 6 treatment cycles unless pregnancy occurred. They didn't mention the total number of treatment cycles nor the ovulation rate per treatment cycle.

Elnashar et al. [24] reported higher ovulation rate (75%) in his RCT to investigate the effect of dexamethasone in CC PCOS women. But this difference may be due to less number of patients included in his trial (40 women in dexamethasone arm).

Vandermolen et al. [25] investigated the effect of metformin in clomiphene citrate resistant PCOS and compared it to placebo effect. Ovulation occurred in 75% of participated women (9/12). There were 28 ovulatory cycles in metformin group (12 patients) but they didn't mention the total number of treatment cycles. Number of treatment cycles was not equal in all patients as each patient was considered to complete the study when she had six ovulatory cycles, became pregnant, or experienced anovulation while receiving 150 mg of CC.

Abu Hashim et al. [26] reported higher ovulation rate with letrozole (64.9% of treatment cycles) and metformin (69.6%). This may be due to higher dose of clomiphene citrate (150mg) or more treatment cycles as each patient could have up to 3 treatment cycles if ovulation and/or pregnancy didn't occur.

As regard pregnancy rate this study showed chemical pregnancy rate of 16.7% (41 patients) which decreased a little bit to 15.4% (38 patients) when ultrasonograghy was done.

Although ***Abu Hashim et al.*** [26] reported higher ovulation rate, but our results regarding pregnancy rate are comparable to their results. They reported clinical pregnancy rate of (14.7%), (14.4%) for letrozole and metformin, respectively.

Elnashar et al. [22], ***Parsanezhad et al.*** [23]**,** ***Vandermolen et al.*** [25] reported higher pregnancy rates with letrozole (25%), dexamethasone (40.5%), and metformin (55%), respectively. This can be explained by smaller sample size and more treatment cycles.

The mean number of mature follicles ≥18 mm on the day of hCG administration was 1.3 (range 1–2; 85 patients (67.5%) developed one mature follicle and 41 patients (32.5%) developed two mature follicles). This limited number of mature follicles with pyridostigmine decreases the risk of multiple pregnancy and ovarian hyperstimulation syndrome. There were three cases of twin pregnancy with no reported cases of OHSS.

The results of this study showed no difference in clinical characteristics between pyridostigmine responders, i.e. patients who had ovulation, chemical pregnancy or clinical pregnancy, and non-responders except in age and BMI. Pyridostigmine responders were younger and this difference was statistically significant P-value = 0.020 in patients who had ovulation, 0.008 in patients who had chemical pregnancy, 0.002 in patients who had clinical pregnancy. They had also statistically significant lower BMI. P-value was < 0.001 in patients who had ovulation, 0.011 in patients who had chemical pregnancy, 0.003 in patients who had clinical pregnancy. This can be explained by the general fact of fertility and ovarian function being affected by age and obesity.

Comparing pyridostigmine responders and non-responders with regard to laboratory characteristics, there were no significant differences in LH, FSH, LH /FSH ratio nor free testosterone.

This is expected as the proposed mechanism of action of pyridostigmine is not related to LH, FSH, or testosterone. It works through enhancing the effect of GH and IGFs.

Thus, pyridostigmine can be given to wider group of PCOS women with CC resistance, because its efficacy is not limited to a specific abnormality. Pyridostigmine can be used in patients with hyperandrogenism, and there is no need to add dexamethasone to reduce adrenal androgen production. Dexamethasone can worsen diabetic tendencies and as a result it may not be a suitable treatment for patients with diabetes, insulin resistance, or glucose intolerance, conditions present in many PCOS patients. Pyridostigmine can be used in case of high LH without the need to add oral contraceptives for pretreatment suppression of LH. Oral contraceptive pills exacerbate insulin resistance. Many PCOS patients are overweight, and obesity is a relative contraindication to oral contraceptive pills.

Pyridostigmine was very well tolerated by all patients with no reported side effects.

IV. Conclusion

Induction of ovulation by adding pyridostigmine to CC in CC-resistant PCOS is promising. It is associated with higher ovulation and pregnancy rates in a significant number of patients with no adverse anti-estrogenic effect on the endometrium.

Induction of ovulation with pyridostigmine appears to be independent of period of infertility, serum level of LH, LH/FSH, and free testosterone.

Addition of pyridostigmine to CC is an effective, inexpensive and safe method for stimulating follicular development in CC-resistant PCOS and may be contemplated before gonadotrophins and laparoscopic ovarian drilling.

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