Journal of Rawalpindi Medical College (JRMC); 2017;21(4):400-405

Original Article

Histological Effects of Letrozole and Clomiphene Citrate on Albino Rat Ovary after Consecutive 1-4 Estrous Cycles

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Abstract

Background:To observe the histological effects of letrozole and Clomiphene citrate on albino rat ovary after consecutive 1-4 estrous cycles.

Methods: An experimental study was carried out on eighty four adult female Albino rats. The rats were equally divided into three groups. Vaginal smear cytology was performed to determine the phases of estrous cycle. The group A was given normal saline orally and served as control. Group B was given letrozole(Femara) at dose 5mg/kg orally and group C was given clomiphene citrate at dose 100ug/kg orally. Each group was further subdivided into four subgroups on the basis of duration of treatment. Ovarian sections were made and stained with Haematoxylin and Eosin. Number of antral follicle in both ovaries of each subgroups were counted and were analyzed using SPSS 20.0. Mean ± S.D.

Results: Morphometric and statistical analysis revealed that number of antral follicle was significantly increased with both letrozole and clomiphene citrate as compared to control. However marked increase in number of antral follicle and development of cystic follicles was observed in clomiphene citrate group.

Conclusion: Letrozole and clomiphene citrate both have improved folliculogenesis. However letrozole is more effective and safe for ovulation induction.

Key words: Clomiphene citrate, Letrozole,Antral follicle.

Introduction

Infertility is common health problem worldwide affecting approximately 8-10 % of couples.[1] Treatment for ovarian stimulation includes clomiphene citrate, aromatase inhibitors and injectable gonadotropins.[2]Clomiphene citrate is selective estrogen–receptor modulator, acts by inhibiting the binding of estradiol to its receptors in hypothalamus.[3] Letrozole inhibits aromatase enzymes which catalyze the conversion of androgen into estrogen. The release FSH from pituitary is enhanced due to estrogen negative feed back mechanism.[4,5]

Clomiphene citrate failure is due to hypersecretion of LH and antiestrogenic effects on endometrium and cervical mucus.[6] Clomiphene citrate induces different types of structural and numerical aberration in human lymphocytes such as, fragment sister chromatid union, chromatid break, chromatid exchange and polyploidy.[7] Pregnancy and ovulation rates are much better in patients treated with letrozole and FSH. Letrozole found to be first line of treatment in clomiphene citrate resistant patients.[8] Image analysis of endometrial sections revealed significant increase of optical density of VEGF immunostaining in letrozole group than clomiphene citrate. This was due to improved vascularity and endometrial thickness.[9]

Letrozole is considered as much better then clomiphene citrate for treatment of anovulatory infertility in patients with the polycystic ovary syndrome.[10] Known complication of clomiphene citrate use include multifetal pregnancy and ovarian hyperstimulation syndrome(OHSS).[11] The incidence of clinically significant ovarian hyperstimulation syndrome (OHSS) is 2-3% however milder form of ovarian hyperstimulation syndrome (OHSS) is 20-30% of all IVF patient.[12] Different theories are proposed to explain pathogenesis of ovarian cancer with fertility drugs. Fallopian tube theory, incessant ovulation theory and gonadotropin theory. According to Incessant ovulation theory, fertility drugs stimulate the ovulation induction and this incessant repetitive trauma to ovarian epithelium results into DNA damage and enhance the risk of ovarian cancer. Type of medication , dosage and number of cycles administered are very important parameter to investigate the reproductive risks of fertility drugs.[13] Discripencies between ovulation and pregnancy rates and high rates of abortion with clomiphene citrate use

suggests the side effects of clomiphene citrate on oocyte, endometrium and cervical mucus.[14]

Material and Methods

Eighty four adult female albino Wister rats were obtained from the colony raised at University of Health Sciences Lahore. Animals were 20 weeks old and weighing about 150-170 gms. These animals were divided into three main groups A, B and C of 28 animals each, which were further divided into subgroups. Group A constituted as control and was given 2ml normal saline orally. Group B was given Letrozole 5mg/kg of body weight daily as a single dose orally.Group C was given Clomiphene Citrate 100ug/kg of body weight as a single dose orally.Animals were housed in separate cages at animal house of Postgraduate Medical Institute, Lahore and were kept in controlled environment at room temperature of 23 ± 2 ^{o}C, humidity of 50± 5% and light and dark cycles of 12 hours each. Rats were given chow and water ad libitum. Vaginal smear cytology was done to check the regularity of estrous cycle and to determine the different phases of estrous cycle. Dose was given at diestrus phase and dissection was done on estrus phase. Each group was further divided into four subgroups (1, 2, 3 and 4) based on the time of dissection . Animals were sacrificed at estrus phase of cycle under deep anaesthesia. Ovaries were dissected and fixed in 10 %neutral buffered formaline. Serial section 7um thick were cut and stained with haematoxylin & Eosin. Antral follicles were counted using ocular grid and stage micrometer. Mean ± SD was calculated for quantitative variables (number of graafian follicles, number of antral follicles and corpus luteum). One way ANOVA was used to compare the quantitative histological parameters among the sub groups. Post hoc Tukey's test was applied to show which group's mean differs.*p- value ≤ 0.05 is statistically significant.

Results

The histological ovarian section in control group A showed normal cortex and medulla (Figure 1). Number of antral follicle was significantly increased with both letrozole and clomiphene citrate as compared to control. Marked increase in number of antral follicle and development of cystic follicles was observed in clomiphene citrate group (Table 1-6)The cortex showed numerous follicles in various stages of development (primary, antral and graafian follicle) and corpus luteum. The medulla was highly vascular (Figure 2). Ovarian section of group B (treated with letrozole)revealed multiple follicular development. Matured graffian follicles, antral follicle and corpus luteum were also seen (Figure 3). Multiple follicular development and normal cortex medulla histology was seen (Figure 4). Ovarian section of rats treated with clomiphene citrate for 1 estrous cycle (C1) showed normal cortex and medulla. Healthy graafian follicles with its histological features was evident (Figure 5). Necklace sign was seen in experimental group C4 where cystic follicles were arranged along the periphery of the ovary (Figure 6) . Cystic follicles with degenerating stratum granulosum was seen in experimental group C 4(Figure 7). When clomiphene citrate was given to the albino rats for four consecutive estrous cycles, number of antral follicle was increased and cystic change was observed in antral follicles (Figure 8).

Table1: Comparison of antral follicles after 1-4 estrous cycles.

Animal group	Control A	Letrozole B	Clomiphen citrate C
One cycle(1)	13.00±0.57	16.57±.97	14.85±2.67
Two Cycles(2)	13.57±.97	17.85 ± .89	18.85±.069
Three Cycles(3)	13.71±1.1	17.85± .89	23.42±0.97
Four Cycles(4)	13.14±0.6	21.00± 1.52	26.57±1.27

Table 2: Post hoc test showing comparison of number of antral follicles in both ovaries at the end of experiment after one estrous cycle.

(I) Groups minor	(J) Groups minor	Mean difference (I-J)	p-vlaue
A1	B1	-3.57143	.002*
A1	C1	-1.85714	.124
B1	C1	1.71429	.164

*p- value ≤ 0.05 is statistically significant

Table 3: Post hoc test showing comparison of number of antral follicles in both ovaries between groups at the end of experiment after two estrous cycles

(I) Groups minor	(J) Groups minor	Mean difference (I-J)	p-vlaue
A2	B2	-4.285	0.000*
A2	C2	-5.285	0.000*

B2	C2	-1.00	.167

*p- value ≤ 0.05 is statistically significant

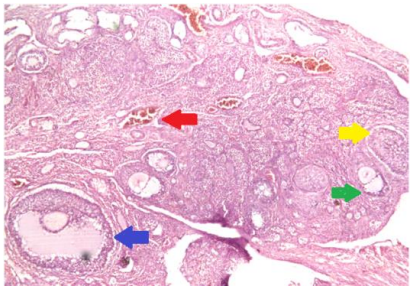

Fig .1.Histological ovarian section from control group showing Graafian follicle(blue arrow), antral follicle (green arrow), corpus luteum (yellow arrow) and blood vessels of medulla (red arrow)

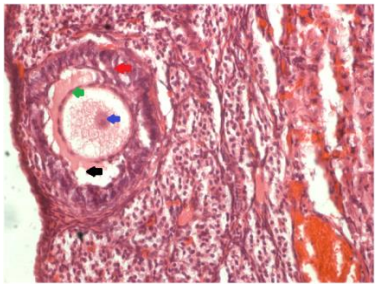

Fig: 2 Histological section of ovary from control group, showing features of antral follicle , nucleated oocyte (blue arrow) , zona pellucida (green arrow) , antrum (black arrow), stratum granulosum (red arrow)

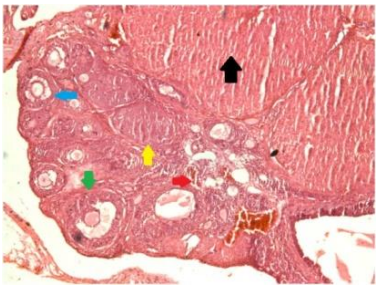

Fig 3.Histological ovarian section of Letrozole group Graafian follicle (green arrow), antral follicle(blue),corpus luteum(black yellow arrow)

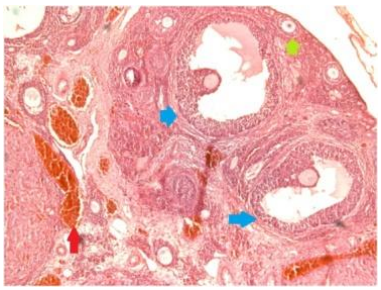

Fig.4.Histological section of ovary from Letrozole group (B4) showing multiple follicular development , Graafian follicles (blue arrow), antral follicle (green arrow)

Colour page

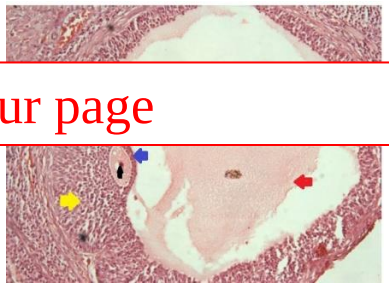

Fig 5.Histological section of ovary from Clomiphene citrate group (C1) showing histological features of graafian follicle, nucleated oocyte (black arrow), large sized antrum (red arrow), stratum granulosum (yellow arrow), corona radiate (blue arrow) , theca interna and theca externa (green arrow).

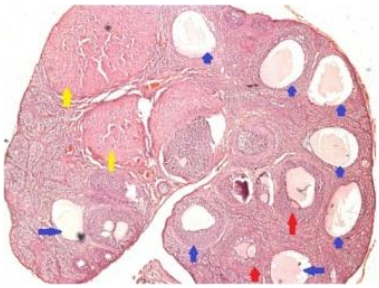

Fig.6.Histological section of ovary from Clomiphene citrate group (C4) showing cystic follicles (blue arrow), graafian follicle (red arrow) and corpus luteum (yellow arrow)

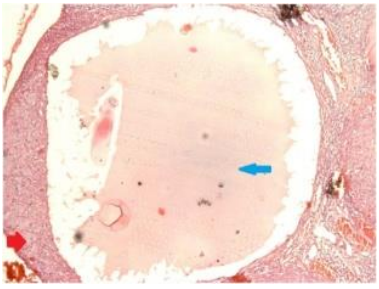

Fig 7.Histological section of ovary from Clomiphene citrate group (C4) showing cystic follicles (blue arrow) and degenerating stratum granulosum (red arrow)

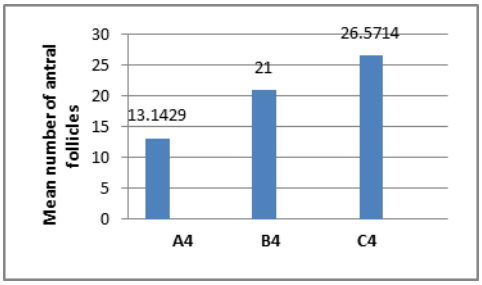

Figure 8:Bar chart showing comparision of antral follicles after 4 estrous cycles.

Table 4: Comparison of number of antral follicles in both ovaries between groups at the end of experiment after three estrous cycles (Post hoc test)

(I) Groups minor	(J) Groups minor	Mean difference (I-J)	p-value
A3	B3	-7.000	0.000*
A3	C3	-9.714	0.000*
B3	C3	-2.714	0.000*

*p- value ≤ 0.05 is statistically significant

Table 5: Comparison of number of antral follicles in both ovaries between groups at the end of experiment after four estrous cycles (Post hoc test)

(I)Groups minor	(J) Groups minor	Mean difference (I-J)	p-vlaue
A4	B4	-7.8571	0.000*
A4	C4	-13.428	0.000*
B4	C4	-5.571	0.000*

*p- value ≤ 0.05 is statistically significant

Table 6: Comparison of number of antral follicles among groups at the end of experimental period (Post hoc test)

Groups	Groups	Mean difference	p-value
Group A	Group B	-5.678	0.000*
	Group C	-7.571	0.000*
Group B	Group C	-1.892	0.062

*p- value ≤ 0.05 is statistically significant

Discussion

Medical induction of ovulation in women with WHO type II anovulation with clomiphene citrate has been practiced but this treatment modality has proven to be ineffective with multiple complication in most of the patients Now a days IUI and IVF techniques are considered as alternative treatment but increased risk of ovarian hyperstimulation, multiple pregnancy and significant cost of treatment highlight the need of improvement of ovulation induction strategies.[15,16]

Morphometric and statistical analysis of antral follicles showed that clomiphene citrate and letrozole stimulate the folliculogenesis. After one estrous cycle , number of antral follicles in both experimental groups B1 & C1 were significantly more as compared to control group A1. However there was insignificant difference in number of antral follicles between B1 &C1. Ultrasongrafic findings after use of letrozole and clomiphene citrate in PCOS were reported. The number of antral follicles were comparable with letrozole and Clomiphene citrate and there was no significant difference between them.[10]

After two estrous cycles, increase number of antral follicles was observed in both experimental groups. however insignificant difference was observed between experimental groups. It is reported that number of antral follicle predict the response of ovary. Multiple follicular development is basis of ovarian hyperstimulation syndrome(OHSS). Evaluation of ovarian reserve is one the important strategy to reduce the incidence of ovarian hyperstimulation syndrome (OHSS).Regarding prediction of hyper-response the study suggests that AFC > 14 antral follicles gave the highest sensitivity (82%) and specificity(89%).[12]A retrospective cohort study was performed to compare IVF and pregnancy outcome with letrozole and gonadotropin in patients with poor ovarian reserve. It was concluded that antral follicle count was comparable after minimal stimulation and high dose stimulation.[17]

Nandi et al (2011) have studied the effects of aromatase inhibitor letrozole among 106 women with PCOS. 2.5 mg tablet of letrozole was given from 3-7 days of menstrual cycle in B.D dose and their parameters were follicular development, endometrial thickness, pregnancy and miscarriage. The results showed 67.9% ovulation rate and 27.3% pregnancy rates in anovulatory infertile patients due to PCOS.[18]

After four estrous cycles, number of antral follicles were more in both experimental groups B4 &C4 as compared to A4. Significant difference was not only present between control and experimental groups however significant difference was also observed between both experimental groups B4 &C4.It was reported that necklace sign is an important hallmark of ovarian hyperstimulation syndrome (OHSS). USG visualization of an ovary showed antral follicles located around the periphery of ovary.[19]It was notified that no literature was found regarding incidence of of severe ovarian hyperstimulation syndrome (OHSS) with letrozole.However necklace sign was seen in current study with experimental group(clomiphene) C4 where cystic follicle were arranged along the periphery of the ovary . When clomiphene citrate was given to the albino rats for four consecutive estrous cycles, number of antral follicle was increased and cystic change was observed in antral follicles. Cystic follicles with degenerating stratum granulosum was seen in experimental group C4.

Perven et al (2012) studied the variation in size of graafian follicle and number of ovarian follicle in

Bangladeshi women with age. The histomorphometric research was done in Anatomy department of Dhaka medical college Dhaka, methodology used for the measurement of ovarian follicle will be considered as reference point for any other study.[20]

Papanikolaou et al suggested ovarian hyperstimulation syndrome (OHSS) as a reason for multiple follicular development. Antral follicle count is one of the predictor of hyper-response of ovary and its cut off level > 14 antral follicles shows the highest sensitivity (82%) and specificity(89%) .[12]

Usually the medicines which are used in ART includes clomiphene citrate , FSH, GnRH agonist and HMG . These medicines induce short and long term side effects, like ovarian hyperstimulation, adverse pregnancy outcome (ectopic pregnancy , multifetal pregnancy and abortion), GnRH agonist induced bone loss and cancer development.[7] Rossing et al raised a question on poteintal risks of fertility drugs. The study showed five out of nine ovarian cancer patients have taken clomiphene citrate for more than 12 cycles and they came to conclusion studies that prolong use of clomiphene citrate enhance the risk of invasive ovarian cancer.[21]

Usually infertile couples are used for experimentation. Women are considered as Test sites or Living Laboratories.[22]Kar, S et al (2013) notified the advantages of letrozole over clomiphene citrate firstly hypothalamic pituitary axis is kept intact by letrozole, secondly letrozole has no antiestrogenic effects throughout the body and thirdly short half life of letrozole.[23]Requena emphasize the use of letrozole for the preservation of fertility in cancer patients .[24]

Brinton et al (2014) has focused on long term relationship of ovulation –stimulating drugs to breast cancer risk. In this cohort study there were 9,892 infertile women with mean age at first evaluation is 30 years. A total of 38.1% of patients has been exposed to clomiphene citrate and 9.6% to gonadotropin , after 30 years of followup 749 breast cancer cases were identified with the mean age of 52.7 years. The results showed the relationship between increasing risk of breast cancer and increasing clomiphene citrate cycles, the patients who received clomophene citrate >12 cycles the risk rises.[25]

Conclusion

1.Letrozole and clomiphene citrate both has enhanced folliculogenesis and follicular maturation.
2.Development of cystic follicles and marked increase in antral follicle is indicative of hyper-response of ovary in clomiphene citrate groups after 3-4 consecutive estrous cycles.
3. Ovarian hyperstimulation syndrome (OHSS) is one life threatening complication of ovulation induction.
4.Letrozole can be considered as a workable option fro ovulation induction.

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