

ORIGINAL ARTICLE

Effect on ovarian weight of female albino rat with histological changes after prolonged ovulation induction

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ABSTRACT

Background: Infertility is becoming global health issue, which affects multiple aspects of human life in both genders . Medical induction of ovulation with clomiphene citrate has been first line of treatment of infertility since 1962 . Discrepancy in ovulation rate and pregnancy rate with clomiphene citrate along with multiple complication in most of the infertile patients raises the question for alternative treatment . Increased risk of (OHSS) ovarian hyperstimulation syndrome is associated with prolonged ovulation induction. Letrozole was found to be an effective alternative ovulogen.

Aim: To study the effects on ovarian weight of female albino rat with histological changes after ovulation induction with letrozole and clomiphene citrate .

Method: An experimental study was conducted at animal house of PGMI with 84 female Wistar albino rats having weight between 150-250gm. The study was carried out after approved by Ethical Committee of Postgraduate Medical Institute, Lahore. Eighty four rats were equally divided into three groups (one control group A and two experimental groups B & C). Normal saline was given orally to the group A , letrozole (Femara) at dose 5mg/kg and clomiphene citrate at dose 100ug/kg were given orally to B & C groups respectively . Major groups were further subdivided into four subgroups according to duration of treatment (1-4 estrous cycle). Ovaries of female albino rats were removed after dissection and weight was calculated. Ovarian sections were made and stained with Haematoxylin and Eosin, to study the histology of ovaries.

Results: Experimental study showed statistically significant difference in the weight of ovary among control and experimental groups after 1-4 estrous cycles but insignificant difference was observed between letrozole and clomiphene citrate group. This showed that both drugs have enhanced the folliculogenesis as increase in ovarian weight is due to enhanced gonadotropic activity . Normal histological features were observed with letrozole even after 4 estrous cycle. But in clomiphene citrate group after 4 estrous cycles, significant increase in ovarian weight with cystic follicles arrangement at the periphery of ovary showing

Practical implication: As ovary is an important reproductive & endocrine organ and plays vital role in female reproductive life , this study showed the hazardous effects of commonly used clomiphene medicine on ovary . Practically letrozole should be used as first line of treatment as it is safe & does not damage the normal histology of ovary .

Conclusion: Present study concluded that there is increase in ovarian weight after ovulation induction with Letrozole and clomiphene citrate. But Significant increase in ovarian weight along with cystic changes in ovarian histology with Clomiphene citrate after prolonged use might be hallmark of OHSS (Ovarian hyper stimulation syndrome), still lots of work is required in this regard.

Keywords: Ovarian weight, Clomiphene citrate, letrozole, OHSS, Cystic follicles .

INTRODUCTION

Infertility is becoming global health issue , one in seven couples is infertile. 25% of the cases are suffering from ovulatory dysfunction. Clomiphene citrate has been in gynecological practice for the treatment of ovulatory dysfunction, recently Aromatase inhibitor (Letrozole) is emerging as alternate modality for ovulation induction¹.

Clomiphene citrate is selective estrogen receptor modulator, it inhibits binding of estrogen at level of hypothalamus and enhances release of FSH, LH from pituitary by negative feedback mechanism. Release of follicular stimulating hormone induces ovulation and stimulates follicular development. Drawbacks of clomiphene citrate treatment including clomiphene resistance and clomiphene failure, multiple pregnancy rates and OHSS (ovarian hyper stimulation syndrome). Over all poor efficacy was found in study as 22% rate of live birth with upto six cycles of clomiphene citrate treatment². Discrepancy in Ovulation rate and live pregnancy was found with clomiphene citrate which due 2 weeks half life and antiestrogenic effects on endometrium. 60-85% ovulation rate with clomiphene citrate treated infertile patients but pregnancy rate is low 10-20% however 20-25% women are clomiphene resistance also as reported by Radwaan³

Letrozole was first introduced in 2000 as alternative medicine for the treatment infertile patients. Letrozole is aromatase

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enzymes inhibitor, It inhibits the aromatase enzyme by binding to the heme part of cytochrome P450 hence there is decrease in estrogen level. Through negative feedback mechanism it stimulates the release FSH from pituitary. It has been used in gynecological practice more than 20 years for medical ovulation induction⁴. Letrozole has been in use for multiple clinical conditions such as anovulatory infertility, PCO, endometriosis and clomiphene resistant unexplained infertility. Short half life , lack of antiestrogenic effects and improved live births has proven superior alternative role of letrozole treatment as compared to clomiphene⁵.

Letrozole treatment is associated with thick endometrium and higher pregnancy rate but clomiphene citrate reduces endometrial receptivity due to anti estrogenic effects. Letrozole might be good alternative because of less cost , side effects and patient inconvenience⁶. Ovarian hyperstimulation syndrome is an iatrogenic complication of medical ovulation induction that results in broad range of sign symptoms such as enlarged ovaries, nausea, vomiting, abdominal pain pleural effusion, abnormal renal function, cardiac arrhythmias and sepsis .Severe OHSS (Ovarian hyperstimulation syndrome) may lead to fatal complication⁷.

Recent research was conducted on female albino rat ovary to study the effects of commonly used clomiphene citrate and letrozole (previously used for breast cancer treatment) on ovarian weight with histological changes. As no such research was done to investigate the changes in ovarian weight with histology after prolonged ovulation induction and its correlation to Ovarain

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hyperstimulation syndrome(OHSS) which is one of the common complication of medical ovulation induction .

MATERIAL METHOD

An experimental study was conducted at animal house of PGMI with 84 female Wistar albino rats having weight between 150-250gm. The study was carried out after approved by Ethical Committee of Postgraduate Medical Institute, Lahore. Eighty four rats were equally divided into three groups (one control group A and two experimental groups B & C). Normal saline was given orally to the group A, letrozole (Femara) at dose 5mg/kg and clomiphene citrate at dose 100ug/kg were given orally to B & C groups respectively. Determination of estrus phase of each rat done by vaginal smear cytology. Major groups were further subdivided into four subgroups according to duration of treatment & time of dissection (1-4 estrous cycle). Female albino rats were sacrificed at estrus phase after 1, 2, 3 & 4 estrous cycles under deep anaesthesia. After careful dissection of ovaries, the weight of ovary was recorded on electronic scale (Sartorius Precision balance germany). Ovaries were fixed in 10 %neutral buffered formaline, 7um thick serial sections were cut and stained with haematoxylin & Eosin. One way ANOVA was used to compare the weight 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.

Fig 1: Photograph of a dissected female Albino rat showing ovary in yellow.



Ovary: The left and right ovaries of all rats were grossly examined. They were pink in color, showing no abnormality in the color and texture. Mean ovary weight ± SD of rats in grams of all subgroups A1, B1 and C1 were 0.06 ± 0.007, 0.102 ± 0.0138 and 0.125 ± 0.0139 gm respectively (Fig 2). Mean ovary weight ± SD of rats in grams of all subgroups A2, B2 and C2 were 0.06 ± 0.007, 0.101 ± 0.014 and 0.114 ± 0.01 gm respectively (Fig 3). Mean ovary weight ± SD of rats in grams of all subgroups A3, B3 and C3 were 0.09 ± 0.016, 0.10 ± 0.013 and 0.142 ± 0.0048 gm respectively (Fig 4). Mean ovary weight ± SD of rats in grams of all subgroups A4, B4 and C4 were 0.07±0.009, 0.10±0.013 and 0.132±0.016 gm respectively (Fig 5).

Analysis of ovarian weight: Statistically significant difference was observed in the weight of ovary among control and experimental groups after one to four estrous cycles. After applying post hoc Tukey's test a significant difference was observed between subgroups A1 and B1, B1and C1 and A1 and C1. Significant difference was observed between subgroups A2and B2 and A2 and C2. While insignificant difference was observed between subgroups B2 and C2. Significant difference was observed between subgroups A3 and C3 and B3 and C3. While insignificant difference was observed between subgroups A3 and B3. Significant difference was observed between subgroups A4 and B4, B4 and C4 and A4 and C4 (Table 1). After applying post hoc

tukey's test on major groups significant difference was observed between A and C, B and C and A and B (Table 2).

Fig 2: Comparison of mean ovarian weight (gm) at the end of experiment after one estrous cycle.

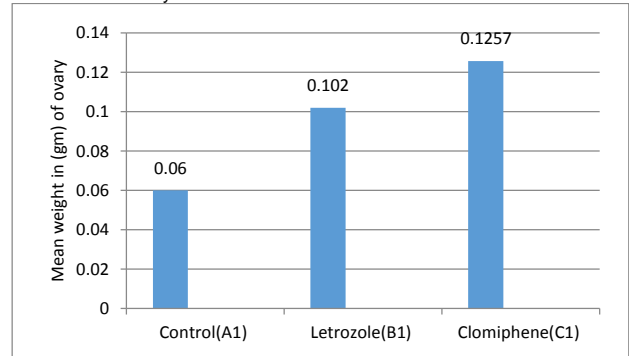


Fig 3: Comparison of mean ovarian weight (gm) at the end of experiment after two estrous cycles.

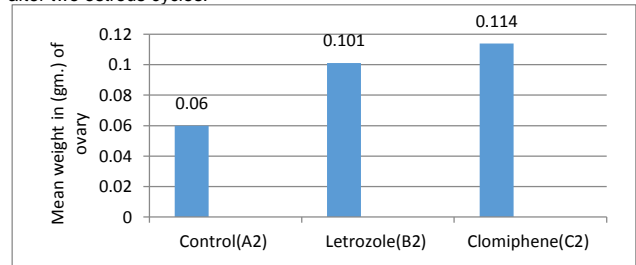


Fig 4: Comparison of mean ovarian weight (gm) at the end of experiment after three estrous cycles.

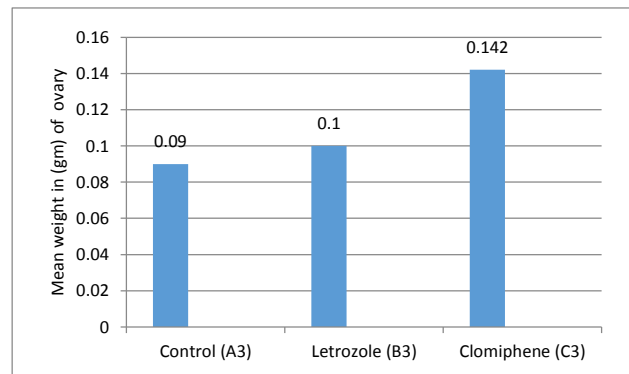


Fig 5: Comparison of mean ovarian weight (gm) at the end of experiment after four estrous cycles.

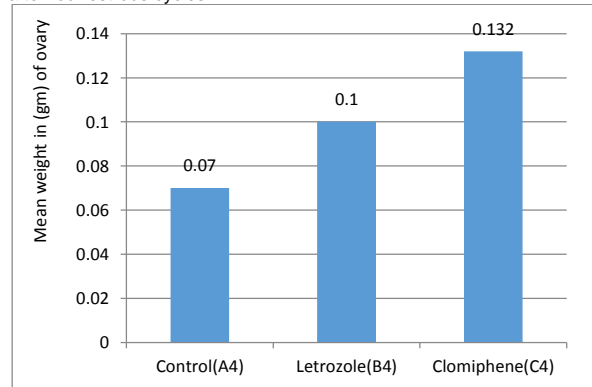


Table1: Post hoc test showing comparison of mean weight of ovary in grams between groups at the end of experiment after one ,two ,three & four estrous cycle.

Groups minor (J)Groups minor		Mean difference(I-J)	p-value
A1	B1	-.0400	0.000*
A1	C1	-.0628	0.000*
B1	C1	-.0228	0.007*
Groups minor (J)Groups minor			
A2	B2	-.0385	0.000*
A2	C2	-.0514	0.000*
B2	C2	-.0128	.178
Groups minor (J)Groups minor			
A3	B3	-0.008	.428
A3	C3	-0.048	0.000*
B3	C3	-0.0400	0.000*
Groups minor (J)Groups minor			
A4	B4	-.028	0.002*
A4	C4	-.058	0.000*
B4	C4	-.030	0.002*

Table 2 Post hoc Tukey test showing comparison of ovarian weight among groups at the end of experimental period.

Groups	Groups	Mean difference	p-value
Group A	Group B	-0.028	0.000*
	Group C	-0.055	0.000*
Group B	Group C	-0.026	0.000*

*p- value ≤ 0.05 is statistically significant.

Histological features of ovaries of control and experimental groups:

The histological section of ovary of the animal in control group A showed normal cortex and medulla (Fig.6). The cortex showed numerous developing follicles , mature graafian follicle and corpus luteum as well .The central zone of the ovarian stroma, the medulla was highly vascular showing blood vessels and ground substance (Fig.6).

Histological section of rats treated with letrozole for three consecutive estrous cycles (B3) showed multiple follicular developments . Number of antral and graafian follicles were increased (Fig. 8)

Ovarian section of rats treated with Letrozole for 4 consecutive estrous cycles (B4) showed multiple follicular developments. Normal cortex and medulla patten of ovarian section (Fig. 9).

Histological section of albino rats treated with clomiphene citrate for three consecutive estrous cycles (C3) showed multiple follicular developments. Numerous cystic follicles were seen in histological sections (Fig .10). Ovarian section of female rats treated with Clomiphene citrate for four consecutive estrous cycles (C4) showed multiple follicular development. Cystic follicles were arranged on periphery of ovarian section . Cystic follicles with degenerating stratum granulosum was evident (Fig.11, 12), Necklace Sign was observed in clomiphene citrate group.

Fig 6: Photomicrograph of histological section of ovary from control group, showing medulla in the center with blood vessels (red arrow) and connective tissue. Cortex at periphery showing multiple growing follicles (green arrow) corpus luteum(yellow arrow).H&E stain X 40.

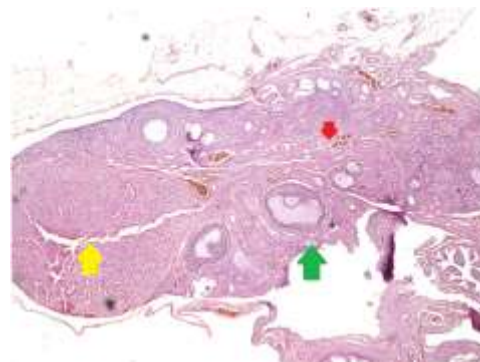


Fig 7 : Histological ovarian section of control group showing features of Graafian follicle, nucleus of oocyte (red arrow), zona pellucida (blue arrow), corona radiata (green arrow), antrum (black arrow), stratum granulosum (yellow arrow) and theca cells (light purple arrow). H&E X 400

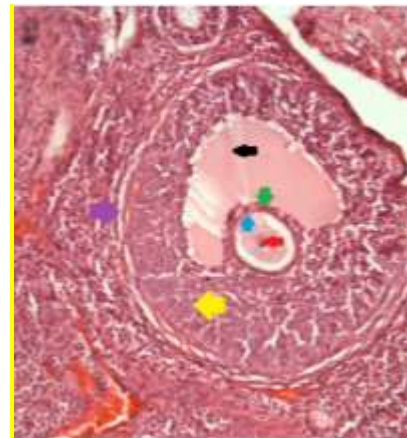


Fig 8 Histological ovarian section of Letrozole group (B3) showing normal cortex and medulla pattern. In cortex Graafian follicle (black arrow) .Medulla showing blood vessels (orange arrow). H&E X 200.

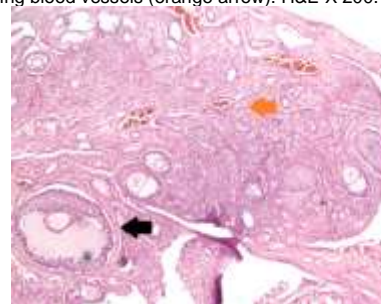


Fig 9:Histological section of ovary from Letrozole group (B4) showing multiple follicular development , Graafian follicles (black arrow). H&E stain X 200.

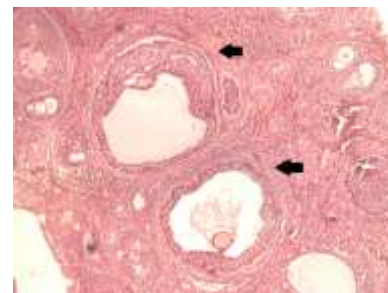


Fig 10: Histological section of ovary from Clomiphene citrate group (C3) showing antral follicles (blue arrow), cystic follicle (black arrow) and atretic follicle (yellow arrow) H&E stain X40.



Fig 11: Histological section of ovary from Clomiphene citrate group (C4) showing cystic follicles (blue arrow), graafian follicle (red arrow) and corpus luteum (yellow arrow) H&E stain X40.

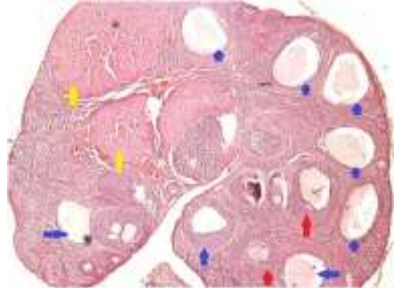
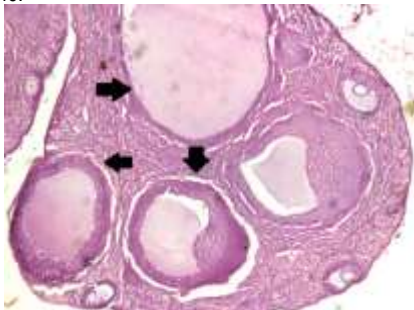


Fig 12: Histological section of ovary from Clomiphene citrate group (C4) showing cystic follicles (black arrow), with disruption of cortex and medulla .H&E stain X40.



DISCUSSION

Infertility is a medical condition that affects emotional, spiritual and medical health of patient. Ovary is female reproductive organ having dual role in female body. It periodically releases the ovum during ovulation but also acts as endocrine gland which is crucial in enhancing fertility outcomes⁸. In ovary after ovulation, the residual follicular wall cells of mature follicle become corpus luteum. It is a transient endocrine unit of ovary. The main role of corpus luteum is the production of estradiol, progesterone and androgen⁹. Women are considered as living laboratories, medicines are given to them for experimentation but it horrifying truth. Clomiphene citrate, a drug has been used for conventional infertility treatment and on IVF techniques world wide. It is dangerous for females because of severe side effects and may cause cancer. Health hazards in women due to clomiphene citrate use are possibility of multiple birth and hyperstimulation of ovaries. Hyperstimulation is related with swelling of ovaries, cyst formation, Meigs like syndrome, thrombosis and ectopic pregnancy¹⁰.

Present study was focused on weight of ovary with histological correlates so that we can analyze the changes in the ovary after use of letrozole and clomiphene citrate for consecutive

1- 4 estrous cycle. Weight of the ovary is usually considered as parameter to analyze gonadotropic activity of ovary. Selvi, A et al studied the effect of ghrelin on ovary, the reproduction is regulated by endocrine factors produced by the brain, pituitary gland, and gonads. Reduction in weight of ovaries with ghrelin-treated rats might be due to suppression of ovarian sex hormone secretion and reduced follicular development¹¹. The weight of the ovary was also studied to investigate antiinfertility effects of drugs. It was concluded that decrease in the weight of the ovaries might be due to antigonadotropic activity of the drugs¹². These studies showed that weight of the ovary was considered to be an important parameter. In current study increase in ovarian weight in both experimental groups B & C was observed which might be due to enhanced secretion of ovarian sex hormone with letrozole and clomiphene citrate. Chattopadhyay, A. et al, studied effect of gold chloride on female reproductive system in immature rats. Gold chloride stimulated serum estradiol level and ovarian delta5-3beta-hydroxysteroid dehydrogenase (delta5-3beta-HSD) activity. Weight of the ovary and uterus was significantly increased. Development of mature graafian follicle was found on histological study of ovary¹³. Sakhavar et al (2013) reported that increase in ovarian weight was also observed after 1 week treatment of letrozole. The results go with the current study in which weight of the ovary was increased with letrozole¹⁴.

After three estrous cycles, increase in weight of the ovary was observed in letrozole and clomiphene citrate groups B3 & C3, however there was insignificant difference in weight between experimental group B3 and control group A3. This showed that letrozole has enhanced gonadotropic activity but increase in weight is controlled. As we compared the histological features of ovary in letrozole and clomiphene citrate group, there was significant change in cortex medulla pattern of ovary in clomiphene citrate group with follicular cysts (Fig .10)

It was observed that there was significant increase in weight of the ovary in both experimental groups B4 & C4 after **four estrous cycle** as compared to control group A4. Significant difference was observed between B4 & C4 indicates that increase in ovarian weight with letrozole is controlled. Histological correlation also showed that letrozole group has maintained normal histology but clomiphene citrate group there was disruption of cortex medulla pattern along with cystic follicles were also more in number. It was concluded that letrozole and clomiphene citrate might be related with increase in ovarian weight. However further research has to be done to investigate the relation between marked increase in ovarian weight with cystic changes with clomiphene citrate and incidence of ovarian hyperstimulation syndrome (OHSS).

Ovarian hyperstimulation syndrome is one of the common complication of ovulation induction, Women with OHSS are mainly characterized by enlarged ovaries and massive fluid shift and ascities in the peritoneal cavity. Clinical features of severe OHSS are similar to shock such as hypovolemia, oliguria and renal impairment, hydrothorax, hemoconcentration, even death as a result of electrolyte imbalance. Research conducted by C Zheng to study the association between LH receptors regulation and ovarian hyperstimulation syndrome in rodent model. Ovarian weight and diameter was studied as parameter which was significantly increased in OHSS group¹⁵.

Khaja M, et al., published a case report of 29 years old female with OHSS, multiple enlarged follicles were found in both ovaries on pelvic ultrasound with 3.5cm & 4.3cm diameter simple cyst in right & left ovary respectively¹⁶. These findings correlates with present study in which clomiphene citrate group showed cystic follicles at periphery as necklace sign (fig .11&12).

Mitchell SY et al reported a case of moderate to severe hyperstimulation syndrome in patient who was treated with clomiphene citrate for ovulation induction. She presented with amenorrhoea, lower abdominal pain and a positive pregnancy test. Diagnostic laparoscopy was done followed by laparotomy. Oophorectomy was performed because of enlarged ovary with

complex solid areas¹⁷. 33% patients having IVF may suffer from mild OHSS, presenting with enlarged ovaries with abdominal pain. The condition may become serious because of renal, respiratory & CVS complications¹⁸. According to the SUCRA (surface under the cumulative ranking curve) results, the rankings in terms of efficacy of different drugs in preventing moderate-to-severe OHSS. Few of them are as follows: 92.4% calcium, 61.3% letrozole, 58.8% metformin and glucocorticoids 18.9%¹⁹.

Tsiami AP et al., conducted a study on infertile women with polycystic ovarian syndrome. After comparison of ovulation rate with letrozole and clomiphene citrate, it was found better ovulation rate with letrozole and higher percentage of live births. According to the latest guidelines of WHO, letrozole should be referred as the first-line of treatment for PCOS infertile women²⁰. Potential association of ovarian cancer and medicines used for ovulation induction is a serious health issue. Worldwide 13 cases of ovarian carcinoma have been reported to occur in women with the history of medical ovulation induction (clomiphene citrate and/or gonadotropins). Questions have been raised to study the side effects of these drugs used for medical ovulation induction²¹. Ovarian hyperstimulation syndrome (OHSS) is still a frustrating life threatening syndrome occurring during medical ovulation induction. Clinical presentation of OHSS is with cystic change with significant increase in ovarian weight²². These findings correlate with present study in which after 4 estrous cycle clomiphene citrate group showed significant increase in ovarian weight with cystic changes in ovary. Luyan Fang et al., found that letrozole with prednisone may be helpful in decreasing the rate of severe OHSS in women during IVF taking prolonged gonadotropin-releasing hormone agonist²².

A study conducted on rat OHSS model to investigate the effects of metformin. Weight of the body, ovary and uterus, serum hormone levels and ovarian histology was studied. Weight of body, ovary and uterus was elevated in OHSS group but treatment with metformin didn't change these parameters²³. Meloxicam was given to study its effects on OHSS rat model, result showed reduction in excessive increase in weight of the ovary and VEGF expression. Meloxicam is COX-2 inhibitor which helps to control significant increase of ovarian weight the OHSS rat model²⁴. Liu A et al compared Letrozole with clomiphene citrate for treatment of infertile patients, result showed that letrozole proved to be effective as clomiphene in infertile patients with unexplained infertility. 2.5mg letrozole is recommended dose for effective results, more research is required to study the effectiveness of letrozole²⁵.

CONCLUSION

Present study concluded that there is increase in ovarian weight after ovulation induction with Letrozole and clomiphene citrate. But Significant increase in ovarian weight along with cystic changes in ovarian histology with Clomiphene citrate after prolonged use might be hallmark of OHSS (Ovarian hyper stimulation syndrome), still lots of work is required in this regard. Development of simple, effective and safe treatment for infertility is global health goal. Letrozole is cheap, patient friendly and effective ovulogen, it may be better choice.

Conflict of interest: Nil

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