Histological and Hormonal Study on Effect of Clomiphene Citrate (CC) on Some Reproductive and Embryonic Parameters in Female Mice

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Abstract

In present study, it was investigated the effect of clomiphene citrate on the ovaries reproductive hormones and embryos of mature female mice, were studied. Female mice treated with (5, 10,50 mg/kg) daily for 7 days. Results cleared that cross section showed a significant increase ($p<0.05$) in multi follicular (different numbers)on the right and left ovaries of female mice. In other hand; there were a significant elevation ($p<0.05$) in the follicle stimulation hormone(FSH), estrogens and progesterone concentration, while, no significant changes were noticed in luteinizing (LH).

Conclusions: Clomiphene citrate is the best initial treatment especially in relevance with ovulatory problems (dysfunction), and has a super stimulatory effect on ovulation, thereby increasing the number of recruited follicles in albino female mice rather than affecting the follicular steroidogenic function, and its induce multi follicle ovum forming. Similarly, it has positive affection on gonadotropene hormones, eventually it leads to a twin pregnancy. Combined c with appropriately timed IUI, Clomiphene citrate treatment also increases cycle fecundity in couples with unexplained infertility.

Keywords: **clomiphene citrate, hormones, ovary,mice.**

Introduction:

Ovulation problems (ovulatory dysfunction), is one of the mos common causes of reproductive failure; (sub fertile and infertile) couples. Where as, about 8 to 10% of women with the polycystic ovary syndrome and this may be considered a major cause of female infertility [(1)]. Women with this syndrome, have hyperandrogenism, morphologic changes in the ovary polycystic, inappropriate gonadotropin secretion (hyperinsulinemia) [(2)]. Successful ovulation induction often back normal fertility. Clomiphene citrate; is a first drug use to treatment of the polycystic ovary syndrome [(3)].

Clomiphene citrate (CC); is the best treatment for infertile women with ovulatory dysfunction, such as, females with anovulation or oligo ovulation. Further that, its use was accompanied with many useful effects [(4)]. CC, its a selective estrogen receptor modulator that increases production of gonadotropins, by inhibiting negative feedback on the hypothalamus and is used mainly in female infertility, mainly as ovarian stimulation to reverse oligoovulation or anovulation, as well as being used for ovarian hyperstimulation, such as part of an *in vitro* fertilization procedure [(5)].

Clomiphene citrate (CC) is a synthetic non steroidal drug that acts on an anti estrogens and competitively binds to estrogens receptors in the hypothalamus and pituitary glands. It is a combinations with human chrioonic gonadotrophin (HCG) , human menopausal gonadotrophin (HMG) and sometime with FSH and LH [(6)]. Clomiphene has also been shown to sensitize pituitary gonadotrophins to LHRH action both *in vivo* and *in vitro*, thus causing an excess secretion of FSH and LH in response to LHRH [(7)].

Moreover; some reports indicated that clomiphene citrate has changed in the physiological action of estrogens and influences on the tissues of ovary that sensitive to estrogens singling in female reproductive tissues, including rodent ovary and human endometrium [(8)]. Also (CC) has some side effects which can be bypassed like as ovarian hyper stimulation ovaries, thick cervical mucus, abdominal pain and hair loss also a physiological or corpora lutea cyst [(9)]. Lastly, the present work aims to study the effects of Clomiphene citrate on mice histological and hormonal states of mature female mice including: ovaries and percentage of twins embryo, and LH, FSH, estrogens, progesterone hormones.

Materials and Methods:

The study was carried out in department of pathological analyses/ Institute of technical /AL-Kut city between December -2016 and February 2017. Adult female albino mice with an average weight of 23±25 g were obtained from the animal house unit of the Institute of technical of AL-Kut. The 40 mice used for the study were included into four group as: the control group, group given 5 mg/kg/day, group given 10 mg/kg/day and group given 50 mg/kg/day, for one week.

Animal grouping

The 40 animals were divided into four groups:

Group1: Control (1 ml double distilled water)

Group2: Given CC (5mg/kg day)
Group3: Given CC (10 mg/kg/ day).
Group4: Given CC (50 mg/kg/ day)
The treatment lasted for a period of one week.

Preparation and administration of clomiphene solution:

A stock solution of Clomiphene citrate, was prepared by dissolving Clomiphene citrate in distilled water; three concentrations of 5, 10, and 50 mg/ml were prepared. Each concentration of Clomiphene citrate was given to the mice orally by intra gastric intubation. Each dose administered for limited group of the female mice..

Histological study:

Reproductive organs (ovaries) of freshly scarified mice were fixed with formalin 10% for 12hr, and dehydrated through progressive increasing concentrations of ethanol alcohol, then cleared with xylene for 30 minutes, then replaced by other paraffin over night in oven. Sections were made from paraffin block (serial sections), then stained with alum haematoxylin and eosin stain. Slides were examined with light microscope using (4X). All serial sections of the ovaries were counted for the various stages of follicle development as described [(10)]. The parameter, number each of primary follicles, growing follicles, Grafian follicle, and weight of ovaries, number of single embryos, and number of twins embryos.

Statistical analysis:

Statistical analysis was performed using SPSS (Statistical Package for Social Science; Version 15.0). Crude data analysis was done using student's t-test so called paired sample t-test for tables with mean and standard error of mean (S.E.M.) to compare between pre-and post-treatment for all groups [(11)].

Results:

Histological study of ovaries after treating mice with 5mg/kg Clomiphene citrate for one week caused d a highly significant increased ($P<0.01$) in the number of (primary follicle growing follicles, and grafian follicle), after one week of treatment as compared to control group (table1), also high significant elevation were assessed ($P<0.01$) in the number of previously parameters after orally treated with(10mg/kg) CC compared with control group**.** Same results, were obtains when treated with Clomiphene citrate (50mg/kg) concentration in the number of different follicles after compare with control, except with grafian follicles.

Table 1. Effect of Clomiphene citrate on the number of different ovarian follicles

Treatment	primary follicles	growing follicles	grafian follicle
Control	5.766±0.247	3.467±0.230	3.645 ±0.117
5 mg/kg Clomiphene citrate	9.133*±0.301	7.165*±0.466	5.580*±0.625
10 mg/kg Clomiphene citrate	12.112* ±0.972	9.140* ±0.6003	5.331* ±0.770
50 mg/kg Clomiphene citrate	12.481* ±0.252	8.140* ±0.235	4.393. ±0.614

Values mean + S.E.M
***: Significant differences between treated groups and control group.**

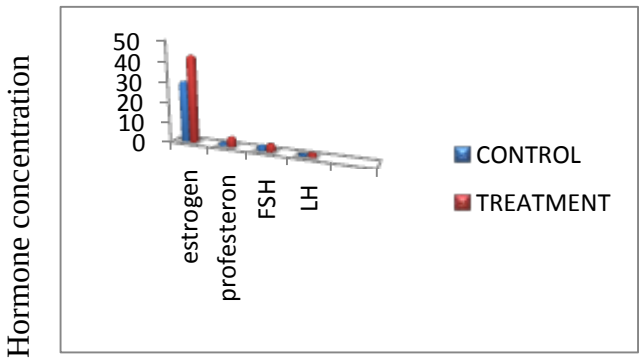
Table2. Effect of Clomiphene citrate on the weight of ovaries (mg) and number of embryos and twins

Treatment	Ovaries weight	Number of embryos	Number of twins embryos
Control	4.246±0.310	7.502* ±0.368	0.045 ±0.100
5 mg/kg Clomiphene citrate	10.08 * ±0.150	12.634* ±0.702	2.360*±0.024
10 mg/kg Clomiphene citrate	12.055* ± 0.325	16.563* ±0.865	5.331* ±0.513
50 mg/kg Clomiphene citrate	9.720 * ± 0.131	14.363* ±0.676	2.204* ±0.134

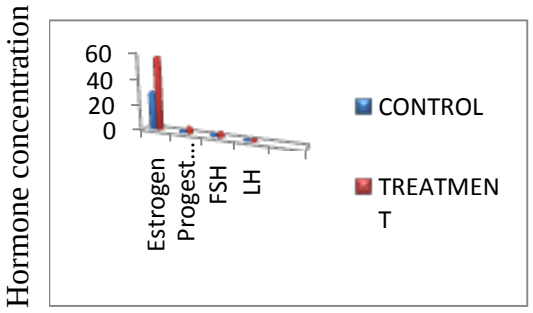
Values mean + S.E.M.
***: Significant differences between treated groups and control group.**

However, (table2) showed; a high significant increases (P<0.01) in the weight of ovaries were assessed for all doses within one week of treatment when compared with control group.

In addition, number of embryos was a highly significantly increased (P > 0.01) in all treated groups for one week with all different doses of colmid citrate (CC)(5, 10, 50 mg/kg) as compared to the control group after treatment. Furthermore, significant differences (P<0.05) was noticed in number of twins embryo, when compared the results of the control groups for week of treatment.



Figure(1) Effect of clomiphene citrate(CC) (5mg/kg) dose on hormones in female mice.



Figure(2) Effect of clomiphene citrate(CC) (10mg/kg) dose on hormones in female mice.

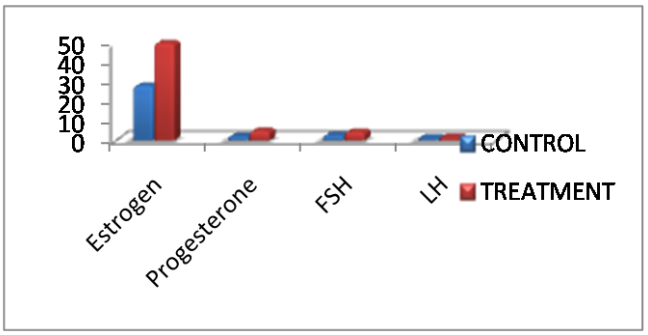


Figure (3): Effect of clomiphene citrate (CC) (50mg/kg) dose on hormones in female mice.

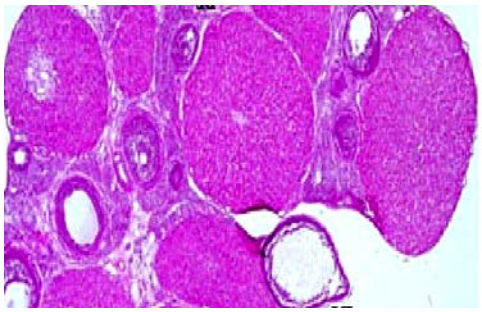
Results of this study demonstrated the effect of drenching clomiphene citrate at a doses of (5mg/kg) to mature female mice. Figure (1) indicates that there are significant increase ($p<0.05$) in the estrogen , progesterone, FSH, and LH hormones concentrations of treated as compared with control. Same results appeared with other doses (10, 50mg/ kg), also highly significant elevation($p<0.001$) for previous hormones(figure 2,3)

Histological Effect of clomiphene citrate on ovaries:

The histological sections of control groups (Figure 4), showed normal histology and no significant pathological features were showed in the transverse sections of ovary like congestion, atrophy, degeneration, and sloughing.

Similar observations were noticed in the histological sections of all treated groups for each CC doses, and appear clearly increasing in all stage of follicles development showed multi ovum follicles in addition weight of ovaries in treated groups compared to control (Figures 5 ,6 and 7).

Figure (4): transverse section in ovary of a mature female mouse for control group, showing number of follicles and corpus leutum (H & E , 4 X) .



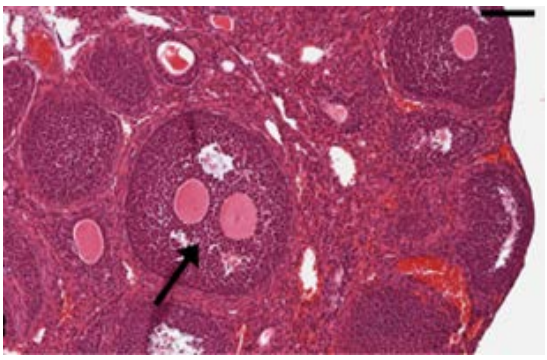


Figure (5): transverse section in ovary of a mature female mouse for (5mg/kg of CC)treated group, showing number of follicles (multiovum follicles) and corpus leutum (H & E , 4 X) .

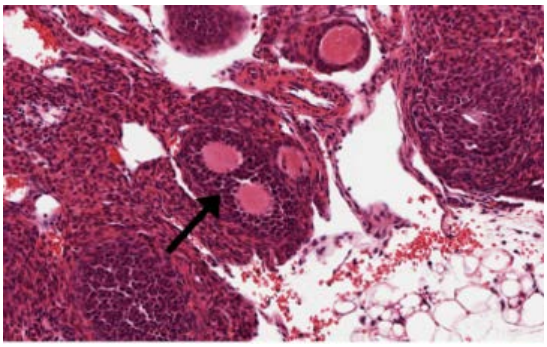


Figure (6): transverse section in ovary of a mature female mouse for (10mg/kg of CC)treated group, showing number of follicles(miltovum follicles) and corpus leutum (H & E , 4 X) .

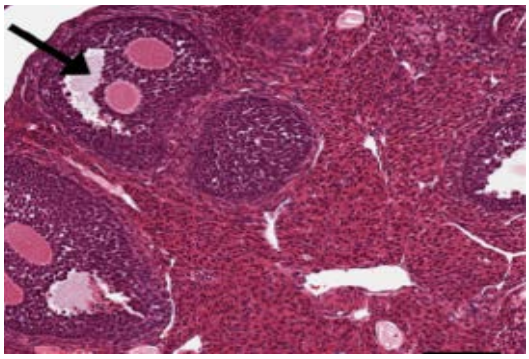


Figure (7): transverse section in ovary of a mature female mouse for (50mg/kg of CC)treated group, showing number of follicles(miltovum follicles) and corpus leutum (H & E , 4 X) .

Discussion:

Clomiphene citrate (CC); has been considered as the most effective drug to treat female infertility which is highly effective in inducing ovulation in females with anovulation or oligo-ovulation [12]. In successful routine cycle, one or more dominate follicles emerge and mature with one oocyte [13]. While in our study, histological sections appearance there are many follicles (multiples' ovum) that shown in figures(1,2,3,4) compared to control. This discraped in table (1 and 2), that shown significant increment in number of all follicles with different stages of development (primary, growing, grafian follicles), number embryos and twins after treated with all CC doses(5,10,50 mg /kg) compare with control.

This illustrates role of Clomiphene citrate in ovulation induction that can be attributed to actions at the hypothalamic level [14]. Given, its structural similarity to estrogen allows CC to bind to estrogen receptors (ER) throughout the reproductive system [15]. Clomiphene citrate a selective estrogen-receptor modulator, presumably works to induce ovulation by inhibiting negative, endogenous, estrogen-feedback on the hypothalamic-pituitary axis resulting in increased FSH secretion, generating a rising tide of E_2 that ultimate triggers the mid cycle LH surge and ovulation follicular[16], and these results are similar to those found in my study: table (1),and figures(1,2,3,4).

Moreover, to support this concept CC reduced levels estrogen negative feedback trigger normal compensatory mechanisms that alter pulsatile hypothalamic GnRH secretion to stimulate increased pituitary gonadotropin release that, in turn, drives ovarian follicular activity in ovulatory woman CC treatment increases GnRH pulse frequency [17]. In anovulatory woman with polycystic ovary syndrome (PCOS) in whom the GnRH pulse frequency is already abnormally high , CC treatment increases pulse amplitude , but not frequency [18] .

Further, the effects of 5,10,50 mg /kg CC on serum levels of estrogen , progesterone and FSH , LH hormones are significantly increased ($p<0.05$) in mentioned hormones respectively Figures(1,2,3). It has been reported that CC acts as a protein kinase C inhibitor and reduces cAMP level in follicular fluid causing both LH and FSH levels to rise [19,20,21]. Clomiphene citrate - induced ovulation in women with polycystic ovarian syndrome is accompanied by increased secretion of LH and FSH with enhanced estrogen secretion. The increased LH pulse amplitude after Clomiphene citrate, together with decreased pituitary sensitivity to GnRH, suggests a hypothalamic effect [22]

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Same trends [23] to support this concept, clomiphene isomers are estrogenic in some species and antiestrogenic in others; these varying properties have been observed in different organs, tissues, and cell types. In intact ewes, clomiphenes inhibited ovulation but induced estrous, which suggested that different regions of the brain respond differently to clomiphene or its isomers [24]. Similar findings were observed in rabbits; CC maintained normal follicular growth and development, but inhibited ovulation in rabbits [25].

However, in women, CC competes with the circulating endogenous E2 for a

binding site in the hypothalamus and blocks the negative feedback of estrogens' and progesterone. This effect induces a significant increase in the secretion of GnRH and subsequent secretions of FSH and LH [26]. Contrary to this, ovarian activities were inhibited in the ewes, i.e., the follicles failed to develop, ovulations did not occur, and the corpora lutea were not formed [27]. No pituitary-stimulatory or gonadotropin-like activity was observed in rats and mice treated with CC [28,29].

We assume, that this could be because CC stimulated the secretion of growth hormone (GH), which increased the number of small-sized follicles(figure 2,3,4) and stimulated LH secretion to levels sufficient to convert small-sized follicles to medium and large-sized follicles also elevation ovaries weight (table2). During the late luteal and early follicular phases, low E2 concentration exerts a negative feedback effect on GnRH secretion. In contrast, during the late follicular phase, high E2 concentration results in increased GnRH secretion [30,31].

The experiment in the current study, superovulatory responses to different treatments during the first follicular wave were more effective in the CC group than in the control group. Our findings were concurrent with the hypothesis stated in a previous studies that superovulatory yields could be increased by initiating the treatment during the early-luteal phase of the estrous cycle [32].

In conclusion, CC is the best initial treatment especially in relevance with ovulatory problems (dysfunction), and has a super stimulatory effect on ovulation, thereby increasing the number of recruited follicles in albino female mice rather than affecting the follicular steroidogenic function, and its induce multi follicle ovum forming. Similarly, it has positive affection on gonadotropin hormones, eventually it leads to a twin pregnancy. Combined CC with appropriately timed IUI,CC treatment also increases cycle fecundity in couples with unexplained infertility.

Therefore, it recommended that treatments with other drugs, which induce follicle maturation and ovulation resulting enhance number of embryos and processing causes of infertility be administered concomitantly with CC.

Conclusions: Clomiphene citrate is the best initial treatment especially in relevance with ovulatory problems (dysfunction), and has a super stimulatory effect on ovulation, thereby increasing the number of recruited follicles in albino female mice rather than affecting the follicular steroidogenic function, and its induce multi follicle ovum forming. Similarly, it has positive affection on gonadotropene hormones, eventually it leads to a twin pregnancy. Combined c with appropriately timed IUI, Clomiphene citrate treatment also increases cycle fecundity in couples with unexplained infertility.

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