ORIGINAL ARTICLE

Effect of Clomiphene Citrate on Rectus Femoris Histology and Spermatogenesis in rats

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ABSTRACT

Aim: Clomiphene citrate, used to treat PCOS in female, has both antiestrogenic and estrogenic properties. It has also been used by athletes to counteract the testosterone induced estrogenic effects and increases endogenous testosterone level. Present study was designed to observe the effect of clomiphene citrate on skeletal muscle histology and spermatogenesis in rats.

Methods: Twenty four rats were divided randomly into three groups. Group A, control, received 100µl of corn oil for 6 days. Group B received 10mg/kg body weight clomiphene citrate intramuscularly everyday for 6 days. Group C received the same dose as group B but every 5th day for 30 days. Animals were sacrificed 24 hours after last injection. Testes and muscle were removed, weighed. H&E sections were obtained and studied.

Results: No significant difference was found in diameter and number of muscle fibers, Johnsen's score and serum testosterone level in all groups. In group B, the diameter of leydig cells and serum LH level were high.

Conclusion: Clomiphene citrate significantly increased LH level in group receiving clomiphene citrate, however, no significant decrease in level of serum testosterone was seen in both treated groups, a probable reason for no effect on muscle mass.

Keywords: Clomiphene citrate, estrogen receptor modulator, hpothalamo-pituitary-gonadal axis, serum testosterone, serum leutinizing hormone.

INTRODUCTION

Clomiphene citrate (CC) is a chlorethylene derivative with chemical name of 1-[*p* (β diethylaminoethoxy) phenyl]-1, 2-diphenylchloroethylene)[1]. It has both estrogenic and antiestrogenic properties and hence is termed as estrogen receptor modulator[2]. These properties are due to the presence of two isomers namely: enclomiphene and zuclomiphene. The former is estrogen antagonist and the latter is estrogen agonist[3]. It is absorbed well when taken by oral route and has a half life of 5-7 days[2].

Clomiphene citrate is used for treatment of polycystic ovarian syndrome (PCOS) and infertility due to anovualtory cycles[1]. Being similar to estrogen in structure, clomiphene citrate binds to estrogen receptors in hypothalamus for a longer period of time[4] and increases the concentration of gonadotrophin releasing hormones (GnRH) in the blood. GnRH in turn stimulates secretion of FSH and LH[5].

Common side effects of this drug are hot flushes, constipation, headache and reversible hair loss. It has also been seen to cause depression, fatigue, weight gain and increased urinary frequency[2]. Visual symptoms such as diplopia, blurred vision, scotoma are uncommon but requires stoppage of treatment[6].

Clomiphene citrate has also been used by athletes, who use exogenous androgens as dietary supplements to enhance muscle strength[7,8]. Clomiphene citrate, in these cases was used to reverse the adverse effects of exogenous androgens that lead to decrease in serum testosterone levels and caused hypogonadism[7,8]. Clomiphene citrate, acting as an estrogen antagonist, inhibits the negative feedback loop of exogenous testosterone on hypothalamus and leads to a rise in serum testosterone level[7].

When testosterone levels are high, they inhibit the release of LH and to some extent FSH[9], which are the main hormones that regulate spermatogenesis[10]. Leydig cells produce testosterone under the influence of LH. Sertoli cells, under the influence of FSH, produce aromatase which converts testosterone to estradiol in the testes. LH causes conversion of pregnenolone, an endogenous steroid, to testosterone in leydig cells[11]. Estradiol, locally produced in the testis, is also thought to be involved in this negative feedback loop known as hypothalamic-pituitary-testicular axis[9]. In men, estradiol (produced by aromatization of testosterone) inhibits secretion GnRH from

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hypothalamus and Gonadotrophin from pituitary[12]. In view of these effects, it was decided to study the effect of clomiphene citrate on muscle and testicular physiology in albino rats.

MATERIAL AND METHODS

Twenty four (24) healthy adult male Wistar rats 6-8 weeks of age, weighing between 200-250gm, were procured from animal house, University of Health Sciences, Lahore. Rats were caged and kept under controlled temperature of 23±2ºC and humidity of 55±5% and light-dark cycles of 12 hours each. Animals were given standard rat chow and water ad libitum and allowed to acclimatize for 3-4 days before the start of the experiment. All procedures were carried out in a clean and aseptic environment approved by ethical committee of University of Health Sciences. Weight of all the rats was recorded at the beginning and at the end of experimental period.

Animals were divided randomly into three groups A, B and C of eight rats each.

Group A was given 100µl corn oil intramuscularly in quadriceps femoris muscle everyday for 6 days.

Group B was treated with 10mg/kg clomiphene citrate[4] in 100µl of corn oil intramuscularly in quadriceps femoris muscle everyday for 6 days (acute effect).

Group C was treated with 10mg/kg clomiphene citrate[4] in 100µl of corn oil intramuscularly in quadriceps femoris muscle every 5th day for 30 days (chronic effect).

The drug was obtained from Sigma (USA). The required dose was prepared by dissolving appropriate amount of clomiphene citrate in the minimum volume of isopropyl alcohol and this solution was later suspended in 100µl of corn oil and a homogenous mixture of required dose was prepared. The mixture was kept overnight so that alcoholic portion evaporated as much as possible.

Rats in groups A and B were sacrificed on 7th day and group C one day after the last injection after being anesthetized with ether-soaked cotton. Each animal was weighed and blood drawn through cardiac puncture. Testes and Quadriceps femoris muscle were removed weighed to the nearest mg and were fixed in Bouine's solution for 48 hours. The tissues were washed three times with 50% alcohol and kept in it for 24 hours. They were processed in an automated processor, cleared in xylene and embedded with paraffin. Five micron thick sections were cut using Leica automatic microtome. Slides from each animal were stained with hematoxylin and eosin (H&E). Microscopic parameters included diameter of muscle fibers, number of muscle fibers, diameter of leydig cells and Johnsen's scoring. Johnsen scoring was done in ten tubules from each block (animal) and mean calculated. Diameter of leydig cells was also taken[13]. Diameter was calculated along two axes right angle to each other for 20 different cells from a slide and mean was calculated. Diameter of ten muscle fibers was calculated in three different fields along minor and major axis as done by Aughesteen et al. (2006)[14]. Mean value was calculated from these measurements. Similarly, muscle fibers were counted in the three non overlapping fields before calculating the mean. LH and testosterone were measured by ELISA using kits for rat serum (Glory, USA). Data were analyzed using SPSS 21. One way ANOVA followed by post-hoc tukey test was applied to assess the difference between groups. Kruskal Wallis test was applied for data that were not distributed normally e.g., Johnsen scoring, serum testosterone level and serum LH levels. A p-value of ≤0.05 was considered statistically significant.

RESULTS

No change in gross features of testes was observed in any of the three groups (A, B and C) of rats. Mean weight of animals in group A at start and end of experiment was 227.75±8.87 and 240.87±12.05 gm respectively; that of group B was 221.62±5.15 and 234±12.60 gm respectively and in group C was 224.75±4.09 and 233.87±6.2 respectively. There was no statistically significant difference ($p>0.05$) in weight of animals at start and end of experiment.

Combined weight of testes at the end of experiment in groups A, B and C was 2.51±0.43mg, 2.29±0.12mg and 2.39±0.15mg respectively. These weights were not different from each other (ANOVA; NS). Similarly, combined weight of quadriceps femoris in groups A, B and C was 3.56±0.15mg, 3.54±0.12mg and 3.42±0.14mg respectively were not different (ANOVA; NS).

No effect on spermatogenesis was seen after treatment with clomiphene citrate in groups B and C as compared with control. Johnsen score was calculated as follows[14]:

10 Complete spermatogenesis.
9 Many spermatozoa but germinal epithelium disorganized.
8 few (5-10) spermatozoa.
7 No spermatozoa but many spermatids.
6 No spermatozoa but few (5-10) spermatids.
5 No spermatozoa or spermatids but many spermatocytes.
4 Few spermatocytes (5-10), no spermatids.
3 Only spermatogonia.
2 No germ cells but only setoli cells.
1 No cells.

Based on these parameters, no difference in Johnsen's score (10.0±0.46) was observed in any of the group of the study.

Mean diameter of leydig cells in three groups was 13.43±0.30µm, 13.93±0.39µm and 13.37±0.49µm respectively. One way ANOVA showed a significant difference among the mean diameter of three groups (p = 0.02). Post Hoc Tuckey test revealed that diameter of leydig cells in group B was greater than that in group C.

Mean diameter of muscle fibers in group A, B and C was 28.03±0.84µm, 28.21±1.72µm and 27.09±1.03µm respectively. These values were not different from each other (ANOVA, NS).

Similarly, no difference was observed in number of muscle fibers counted at X40 in three (28.41±3.02, 28.96±2.04 and 28.90±2.10 respectively) study groups.

Mean serum testosterone level in group A, B and C was 0.10±0.18ng/ml, 0.72±0.09ng/ml and 0.39±0.44 ng/ml respectively. The difference in serum testosterone was not significant statistically.

Mean serum LH level in group A, B and C was 6.62±4.73mIU/ml, 11.11±9.90mIU/ml and 2.40±2.96mIU/ml respectively. One way ANOVA (p = 0.02) showed statistically significant difference among the groups. Post-hoc test showed that group B values were significantly higher than that of group C values. No difference was seen between groups A and B and A and C.

Figures:

Fig. 1: Photomicrograph of transverse section of testis from group A showing seminiferous tubules lined by spermatogonia (SG), primary spermatocytes (PS), spermatid (SD), spermatozoa (SZ). leydig cells (LC) are seen scattered between tubules in interstitium and nuclei of leydig cells (LN) are also seen. Diameter of leydig cells was calculated by measuring diameter along two axes right angle to each other and calculating the mean. H&E stain. X400.

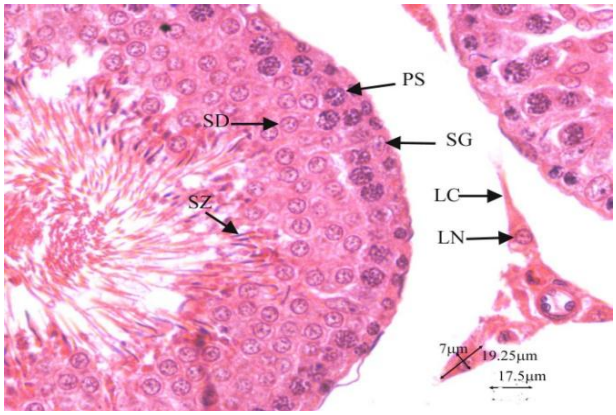


Fig. 2: Photomicrograph of transverse section of testis from group B showing leydig cells (LC) having greater diameter than group A and C, scattered between tubules in interstitium, nuclei of leydig cells (LN) are also seen. Seminiferous tubules were seen lined by germinal epithelium consisting of spermatogonia (SG), primary spermatocytes (PS), spermatid (SD) and spermatozoa (SZ). H&E stain. X400

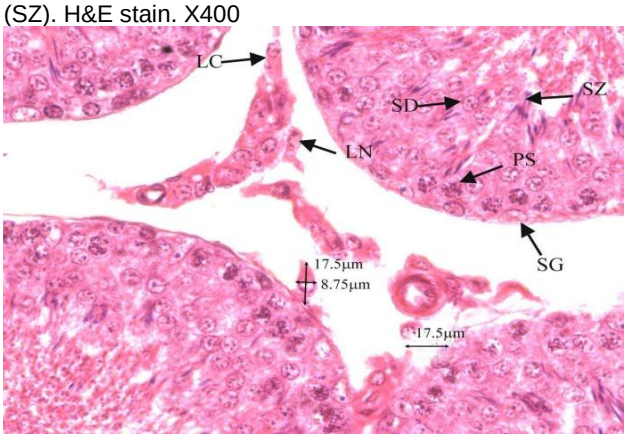
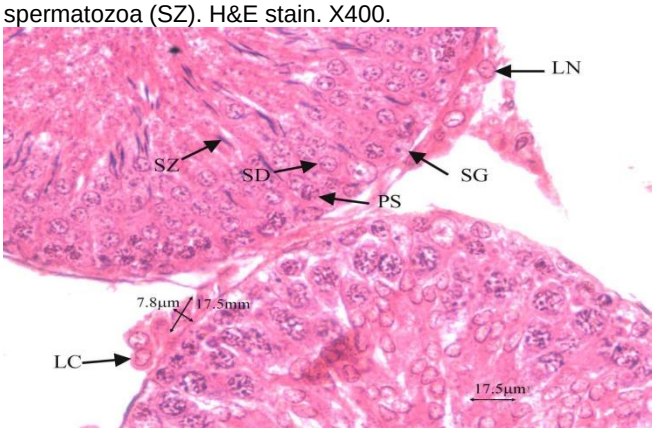


Fig. 3: Photomicrograph of transverse section of testes from group C showing leydig cells (LC) having same diameter as group A but smaller than group B, scattered between tubules in interstitium. Nuclei of leydig cells (LN) are also seen. Seminiferous tubules are seen lined by germinal epithelium consisting of spermatogonia (SG), primary spermatocytes (PS), spermatid (SD) and spermatozoa (SZ). H&E stain. X400.



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DISCUSSION

In the present study intramuscular injections of clomiphene citrate in oil at a dose 10mg/kg body weight per day for 6 days (60mg total dose) consecutively or every 5th day did not change the animal (p=0.34), testes (p=0.29) and muscle weight (p-value=0.14).

Microscopic examination of testes showed normal spermatogenesis in experimental groups. Johnsen scoring was comparable among the groups and all the layers of germinal epithelium were seen and were comparable with lumen filled with spermatids. These findings, are different from the results of Bharti et al[15], who reported impairment of spermatogenesis after treating rats with 5mg clomiphene citrate every 5th day for 30 days. This difference was probably due to high dose[4] of drug used by them.

Treatment with clomiphene citrate increased the diameter of leydig cells in group B that received daily dose of clomiphene citrate (10mg/kg) for 5 days, while in group C the leydig cell diameter was comparable to that of control group. Flickinger et al[16]. observed a decrease in the size of leydig cells after daily treatment with 2.5, 3.5 and 5mg/100gm body weight per day clomiphene citrate for a long duration of 12 weeks. As such our results are also different from the results of Flickinger et al[16]. This may be due to the long duration of treatment and the higher dose of clomiphene citrate used or rat strain was different in the two studies.

Analysis of serum testosterone and LH level estimated by ELISA revealed an increase in serum LH levels in group B as compared with control and group C. These results are in agreement with the results of Santen et al.[17] who treated men with 100mg clomiphene citrate (dose) daily for 7 days and observed an increase in serum LH levels. Increased serum LH levels in these two studies, in men and rats after treatment with clomiphene citrate are presumably due to the antagonistic action of clomiphene citrate on hypothalamus-gonadal axis. Although, serum LH level decreased in group C of the present study, however, the difference between control and group C was insignificant. Bharti et al[15] also observed a decrease in serum LH levels of rats after 30 days of treatment with clomiphene citrate but the decrease was significant in their study. According to them, a decrease in LH level in their study was due to potentiation of estrogen agonistic property (negative effect on testis by clomiphene citrate) of clomiphene citrate rather than estrogen antagonist and as described earlier, due to high dose of clomiphene citrate used.

In the present study, serum LH levels were raised only in group B receiving clomiphene citrate on consecutive days (acute effect). This effect was probably due to the two isomers of clomiphene citrate, i.e., enclomiphen and zuclomiphene. Enclomiphene is potent antagonist of E_2 as compared with zuclomiphene which is E_2 agonist. Enclomiphene also has a shorter half life[1]. Group B received daily dose of clomiphene citrate and on premise that it produced a persistent level of enclomiphene, thus increasing serum LH level. The group C, that received drug every 5th day did not show any rise in serum LH level showing enclomiphene in the drug clomiphene citrate was cleared from body due to its shorter half-life.

Statistically insignificant difference in serum testosterone level was observed in both the experimental groups (p=0.28). This finding contradicts the earlier report by Guay et al. (19) who treated hypogonadal men with 50mg clomiphene citrate for 4 months and observed an increase in serum testosterone levels. Bharti et al[15] observed a decrease in serum testosterone levels after treatment of male rats with 5mg clomiphene citrate every 5th day for 30 days. These authors suggested that this decrease was due to estrogen agonistic action of clomiphene citrate at the estrogen receptor level.

In the current investigation, statistically insignificant difference was observed in diameter of muscle fibers (p=0.18) and number of muscle fibers (p=0.88). An increase in muscle mass seen after exogenous testosterone treatment was reported by Bhasin et al[19] who observed increase in muscle mass in men treated with 100mg exogenous testosterone, weekly, intramuscularly for 10 weeks. Since no effect on the serum testosterone was observed in the present study, no changes in the muscle mass or number are expected. However, detailed studies on variable duration and concentration are required to see the effect on muscle related parameters.

CONCLUSION

Present study indicates a rise in serum LH level and leydig cell diameter in adult albino rats with daily treatment with clomiphene citrate for 6 days. However, no rise in serum testosterone level was seen. This can be useful in treating hypogonadism and increasing muscle mass if serum testosterone level increase after treatment for long duration. A long-term experiment may be designed, using different doses and treatment duration of clomiphene citrate to see if statistically significant changes in testosterone level and other hormone are observed which can affect muscle related parameters like mass and muscle strength.

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ERRATUM

The name of third author **MAIRA MAHMOOD** has been wrongly printed as ***Maria Mahmood*** in her original article title ***"Evaluation of Sodium Pumps Activity in Patients of Lahore City Suffering from Diabetes Mellitus Type 1"*** published in Pakistan Journal of Medical & Health Sciences Page 643-645, Vol.10, April-June 2016 issue. This typographical error is regretted.