

Effect of Clomiphene Citrate on the Ultrastructure of Testis in Albino Rats

Efecto del Citrato de Clomifeno sobre la Ultraestructura de los Testículos en Ratas Albinas

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SUMMARY: The aim of the present work was to study the closer effect of clomiphene citrate on the ultrastructure of the testis of adult albino rats to provide a basis for optimizing this drug in the treatment of male infertility. The testes were removed from both groups under anesthesia and then prepared for examination by light using hematoxylin and eosin stains and a transmission electron microscope. Semithin sections were cut into 1 μm thick sections, stained with toluidine blue, and examined by light microscopy for a survey. The desired areas were placed in the center, and other areas were trimmed. Primary spermatocytes showed marked nuclear changes (pyknosis), and their nuclear membranes were ill-defined and disrupted. The cytoplasm showed widespread degeneration of mitochondria and lysosomes and focal degeneration of the rough endoplasmic reticulum compared with the control group. The spermatids were pale, and the two phases of spermatogenesis were distinctly identifiable in the control group but were confused in the treated group. Some spermatids had interrupted nuclear membranes, also containing degenerated mitochondria, focal fragmentation of rough endoplasmic reticulum, and free ribosomes. Spermatozoa in the treated group appeared deformed compared to the control, where they had deformed head caps. Leydig cells of the treated group have an irregularly shaped nucleus, with focal chromatin aggregation and peripheral chromatin condensation on the inner surface of the nuclear membrane. The observations of the present work indicate a possible causal relationship between testicular affection and ingestion of clomiphene citrate, which can be avoided by close medical observations using ultrasonography, semen analysis, or testicular biopsy to detect early malignant changes. Furthermore, the drug should not be used for more than three to six cycles and should be stopped for at least three cycles before reuse. When clomiphene citrate is ineffective in the treatment of male infertility, human menopausal gonadotropin (hMG) administration is typically selected. However, high-dose hMG therapy is associated with a variety of adverse effects. In this work, we report the success of a modified clomiphene citrate regimen in increasing sperm count without any hazards to the testicular tissue.

KEY WORDS: Clomiphene citrate; Testis; Albino rats; Spermatogenesis; Electron microscope.

INTRODUCTION

Clomiphene citrate is structurally related to stilbesterol, which is a weak estrogen that exhibits less activity than natural estrogens and antagonists. Clomiphene citrate blocks hypothalamic estrogen receptors so that the negative feedback of natural estrogens is prevented and the pituitary gland responds by increased secretion of gonadotropins. Clomiphene citrate has been used in male infertility, although the treatment must be continued for two to three months, as this is the time required for the maturation of spermatozoa, especially those with a normal or high rate of production of endogenous estrogens (Brown & Chakraborty, 2009). Clomiphene citrate is widely used to treat infertility in both male and female patients and was assumed to induce and stimulate spermatogenesis in human gonads (Brown & Chakraborty, 2009). There are abundant

studies on the clinical and biochemical effects of the drug on the male and female reproductive systems. However, there is a paucity of information regarding the effect of the drug on the histology and ultrastructure of the gonads. Thus, the aim of the present work was to study the closer effect of clomiphene citrate on the ultrastructure of the testis of adult albino rats to determine the best way to use the drug in males or to avoid its possible hazards.

MATERIAL AND METHOD

Animals. Forty adult male albino rats were used in this study. The average weight ranged from 180 mg to 220 mg. The animals were divided into the following groups:

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- A. Control group: This group consisted of 20 rats that received distilled water.
- B. Treated groups consisted of 20 rats that received clomiphene citrate. The tablets were crushed and dissolved in 5 ml of distilled water, and 0.18 ml was administered orally by a dropper daily for 40 days (corresponding to 3 spermatogenic cycles). The dose was calculated according to the formula of (Paget & Barnes, 1964). All animals were kept in the animal house under the same environmental conditions and were allowed to move freely in their cages. The rats were fed a daily diet composed of milk powder, bread, and vegetables.

Method

Excision of testes. Each group of animals was anesthetized with ether, their abdomen was opened through the incision, and their testes were excised and immersed immediately in 1/2 Karnovsky's fixative and Bouin solution. The rats in the control and treated groups were weighed, the testes of the control and treated groups were extracted and weighed on a digital electronic balance, and their volumes were measured using the fluid displacement method.

Preparation of testis for light microscopy. The tissues were placed in Bouin's solution for 24 h and then removed and placed in 80 % alcohol until the color disappeared. Then, the tissues were processed as previously described (Bancroft & Gamble, 2002). The sections were dewaxed, stained with hematoxylin for 10 min, and washed in running tap water for 3 min. Then, the sections were stained with aqueous eosin for 3 min. The sections were washed with water, dehydrated in ascending grades of alcohol, and cleared in xylene.

Preparation of the testes for electron microscopy. The testes were immersed immediately in 1/2 Karnovsky's fixative containing 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The testes were processed as previously described (Tizro *et al.*, 2019).

Semithin sections were cut into 1-micrometer-thick sections, stained with toluidine blue, and examined by light microscopy for a survey. The desired areas were placed in the center, and other areas were trimmed. Ultrathin sections ranging from 70-90 nm were cut and stained doubly with uranyl acetate for 15 minutes and lead citrate for 3 minutes. The grids were left to dry and examined by a JEOL electron microscope at an accelerating voltage. The cross-sectional diameter of 20 randomly rounded selected seminiferous tubules from both groups was measured; a Leitz MPV Compact cytophotometer was used for this purpose (Müller *et al.*, 2008).

Statistical analysis. The mean of these diameters was taken for each group. For statistical analysis of the results, the mean value was used as a representative value for each group. The standard deviation was used as an index for the variability among each group. The paired T test was used as a test for the statistical significance of the changes occurring in each group. The difference was considered significant when the P value was ≤ 0.05 . A highly significant difference was indicated by a P value ≤ 0.01 (Mishra *et al.*, 2019).

RESULTS

Gross Appearance. No apparent changes were detected in the control group, while the treated group showed a loss of appetite (anorexia), lethargy, indolence, recurrent attacks of diarrhea, and diminished movement with a moderate reduction in the mean body weight of the treated group (Table I). There was a moderate decrease in testicular weight, as the statistical analysis revealed a significant difference ($P \leq 0.05$) between the mean value of the testicular weight of the treated group and the control group (Table II). A marked decrease in the testicular volume of the treated group could also be detected, as the statistical analysis revealed a highly significant difference ($P \leq 0.01$) between the mean value of the treated group and the control group (Tables III and IV).

Table I. Diameters of 12 randomly selected samples showing the weight in grams for each group.

	Mean inter pupillary diameter	Mean crossed axon numbers
Group N	54.213±1.945mm	(120±11).10 ³ /mm ³
Group Nr	52.465±3.324mm	(123±14).10 ³ /mm ³
Group W	56.124±4.043mm	(116±13).10 ³ /mm ³

Table II. Effect of clomiphene citrate on the weight in grams of rats and testicular weight and volume.

	Weight	WRT	WLT	VRT	VLT
1	190	0.81	0.83	1.0	1.0
2	200	1.01	1.02	1.2	1.2
3	190	0.86	0.87	1.1	1.1
4	180	0.96	0.97	1.2	1.2
5	200	1.01	1.02	1.3	1.3
6	190	1.02	1.01	1.2	1.2
7	200	1.05	1.03	1.3	1.3
8	210	1.04	1.03	1.2	1.2
9	190	0.91	0.99	1.1	1.1
10	210	1.06	1.08	1.2	1.2
11	220	1.17	1.24	1.3	1.3
12	200	1.26	1.31	1.4	1.4
X	200	1.02	1.03	1.2	1.2

WRT = weight of right testis in grams, WLT = weight of left testis in grams, VRT = volume of right testis in cubic ml, VLT = volume of left testis in cubic ml.

Table III. Comparative statistical study of the effect of clomiphene citrate on the weight of rat testes.

	Control group	Treated group
Number	20	12
Range	1.11-1.79	0.83-1.31
Mean value	1.33	1.02
S. D.	0.22	0.19
S. E.	0.06	0.04
T - value		1.93
P		<0.05
Significance.		Si

S. D. = standard deviation, S. E. = standard error, T. Value = two-tailed Student's t test, P = paired t test

Table IV. Comparative statistical study of the effect of clomiphene citrate on the volume of rat testes.

	Control group	Treated Group
Number	20	12
Range	1.5-1.9	1.0-1.4
Mean value	1.75	1.20
S. D.	0.15	0.20
S. E.	0.06	0.02
T - value		3.62
P		<0.01
Significance		H. S.

S. D. = standard deviation, S. E. = standard error, T. Value = two-tailed Student's t test, P = paired t test.

Light microscopic findings. Semithin sections of the testis in the treated group showed shrinkage of seminiferous tubules (Table I) and very wide interstitial spaces between seminiferous tubules. The stratified epithelium lining seminiferous tubules was formed of two or three rows of spermatogenic cells, including sustentacular cells (Sertoli cells) (Figs. 1A and B and Fig. 2A and B), denoting the arrest of spermatogenesis compared to the control group.

Spermatogonia type "A" dark and type "A" pale in addition to primary spermatocytes could be identified inside the seminiferous tubules. The dark type "A" spermatogonia were distinguished by ovoid-shaped nuclei with dark nucleoplasm. The type "A" pale spermatogonia were distinguished by ovoid nuclei and pale nucleoplasm. Both spermatogonia type "A" had swollen nuclei compared to the control group (Figs. 1C and Fig. 2C). Sustentacular cells were lying upon the basement membrane, and they appeared triangular with bases lying on the basement membrane, while apices were directed toward the lumen. Sustentacular cells have large nuclei and can be identified as a single row of cells.

The primary spermatocytes were hardly found inside the seminiferous tubules; some had a nuclear ghost, and others had ruptured nuclei with consequent scattering of their chromatin content. No spermatids or spermatozoa were

observed in most of the seminiferous tubules, denoting the arrest of spermatogenesis (Fig. 1 C and Fig. 2 C). The interstitial spaces were very wide and contained collagen fibers, blood vessels, and interstitial cells. Fibrosis of intertubular spaces could be identified (Fig. 1 D and Fig. 2 D) but in small numbers compared with the control group.

Electron microscopic findings. The testes of the treated group showed pale type "A" spermatogonia with an ill-defined nucleus; the nuclear membrane was ill-defined, while its nucleolus was evident and their cytoplasm contained few cell organoids. The basement membrane appeared very thick compared with that of the control group, and myoepithelial cells were resting on its outer surface (Fig. 3A and Fig. 4A).

Spermatogonia type "A" dark were resting on a thick basement membrane, and have an irregularly shaped nucleus with a peripheral nucleolus, dark chromatin granules and focal rough endoplasmic reticulum (Fig. 3 B and Fig. 4B), while type "B" spermatogonia were resting on a thick basement membrane and have an oval nucleus, with a central nucleolus and moderately dense nucleoplasm. Its cytoplasm contained a few normal mitochondria, degenerated mitochondria, and free ribosomes.

The primary spermatocytes showed marked nuclear changes; these changes were in the form of pyknosis, and the nuclear membrane was ill-defined and disrupted. The cytoplasm showed widespread degeneration of mitochondria and lysosomes and focal degeneration of the rough endoplasmic reticulum compared with the control group (Fig. 3 C and Fig. 4C).

The nucleoplasm and cytoplasm of the spermatids were pale. The two phases of spermatogenesis were clear in the control group but confused in the treated group; the first was the Golgi phase, which was ill-defined compared to the control group, and the second was the cap, and the well-defined cap-phase spermatids had interrupted nuclear membranes, degenerated mitochondria, focal fragmentation of rough endoplasmic reticulum, and free ribosomes (Fig. 3 D and Fig. 4D).

Spermatozoa appeared deformed compared to the control, where they had deformed head caps. The other remaining spermatozoa appeared normal (Fig. 3 E and Fig. 4E).

Interstitial cells of the treated group had an irregularly shaped nucleus, with focal chromatin aggregation and peripheral chromatin condensation on the inner surface of the nuclear membrane, cytoplasm containing ill-defined cytoplasmic granules except for free ribosomes, and stitial tissue containing fibroblasts (Fig. 3 F and Fig. 4F).

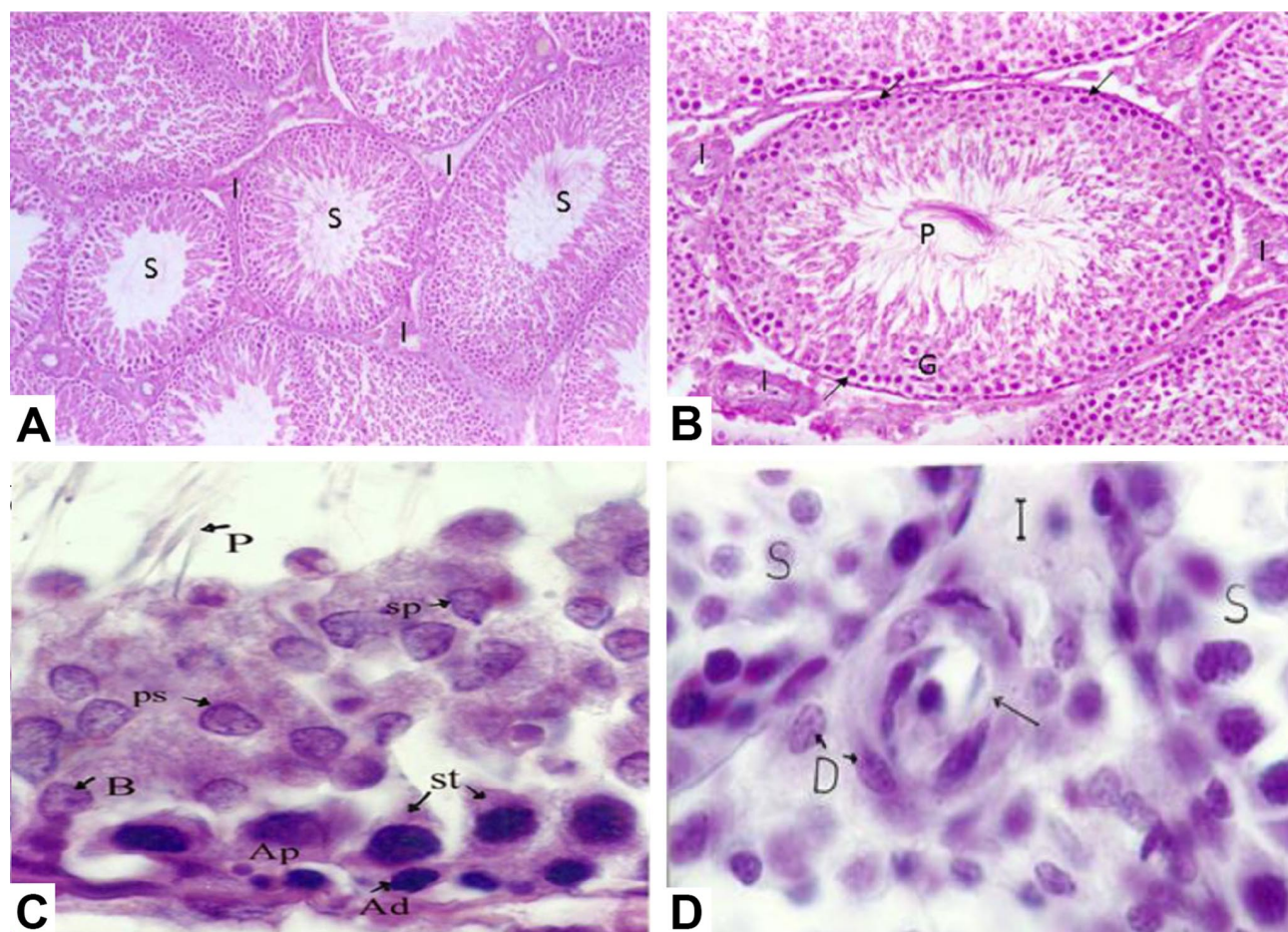


Fig. 1. Testes of the control group stained with hematoxylin and eosin. A. A photomicrograph of the testis of the control group showing seminiferous tubules (S). The interstitial spaces (I) are identified between the seminiferous tubules. The seminiferous tubules are lined by stratified epithelium showing various stages of spermatogenesis. H&E; (125X). B. A photomicrograph of the testis of the control group showing one complete seminiferous tubule surrounded by interstitial spaces (I). The seminiferous tubule is lined by stratified epithelium, which consists of spermatogenic cells (G) at various stages of spermatogenesis and sustentacular cells (arrows), with the presence of spermatozoa (P) in the lumen of the seminiferous tubule. H&E; (250X). C. A photomicrograph of the testis of the adult rat control group showing a sector of seminiferous tubule containing various stages of spermatogenesis and sustentacular cells (St). Spermatogonia A dark (Ad), spermatogonia A pale (Ap), spermatogonia B (B), primary spermatocytes (ps), spermatids (sp) and spermatozoa (P). H&E; (1000X). D. A photomicrograph of the testis from the adult rat control group showing the interstitial tissue (I) between the seminiferous tubules (S) containing interstitial cells (D) and blood vessels. H&E; (1000X).

DISCUSSION

In the present work, the animals were exposed to some general signs of ill health, including loss of appetite (anorexia), diminished movement (lethargy), recurrent attacks of diarrhea, vomiting, and reduction of weight. All these signs appeared in the treated group only.

In the present work, the testes of treated rats were reduced in size, and the seminiferous tubules appeared shrunken and separated by wide interstitial spaces. Some of

the seminiferous tubules were highly affected, showing collapse or degeneration of spermatogenic cells with consequent arrest of the spermatogenic process and the absence of spermatozoa in the center.

The less affected tubules showed thickening of their basement membrane with some degenerated and abnormal cells with a low number of spermatozoa in the center of tubules, while the other tubules appeared to have normal

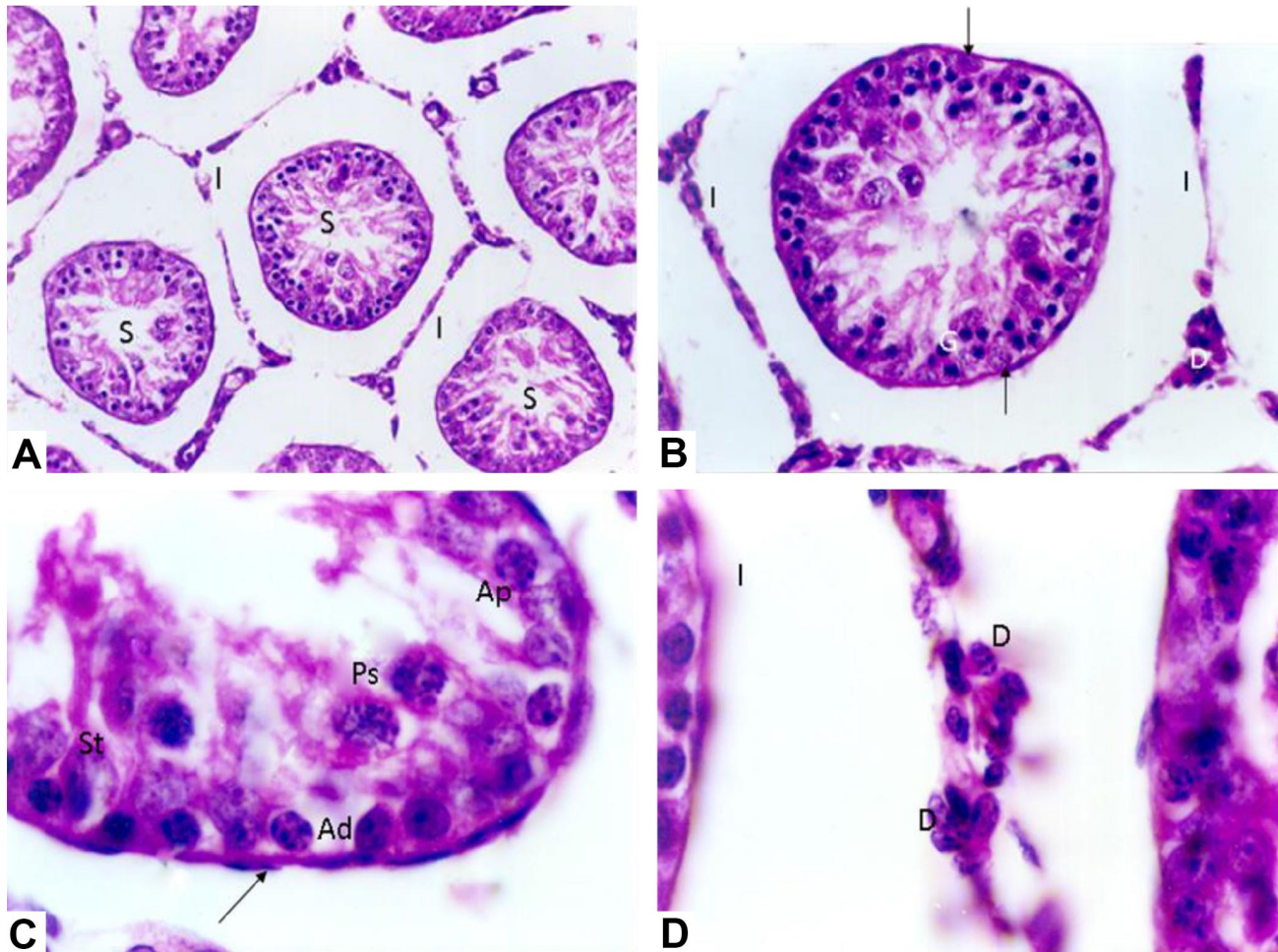


Fig. 2. Testes of the treated group stained with hematoxylin and eosin. A. A photomicrograph of the testis of the treated group showing shrunken seminiferous tubules (S), with a widening of the interstitial spaces (I). The spermatozoa were absent in its lumen. H&E; (125 X). B. A photomicrograph of the testis of the treated group showing one seminiferous tubule surrounded by wide interstitial spaces (I) containing interstitial cells (D). The stratified epithelium lining the seminiferous tubule consists of spermatogenic cells (G) and sustentacular cells (arrows). The spermatogenic cells show maturation arrest. H&E; (250 X). C. A photomicrograph of the testis of the treated group showing the sector of seminiferous tubule containing sustentacular cells (St), spermatogonia (A) pale (Ap), spermatocytes (A) dark (Ad) and primary spermatocytes (Ps). Spermatids and spermatozoa could not be identified. The basement membrane appeared thick (arrow). H&E (1000X). D. A photomicrograph of the testis of the adult rat posttreatment group showing widening of the interstitial space (I), which contains the interstitial cells of Leydig (D) H&E; (1000X)

architecture. The spermatogenic and sustentacular cells appeared normal in these tubules, while the interstitial cells appeared few in number and atrophied compared with the control group. The primary spermatocytes of the treated group showed marked nuclear changes where their membranes were ill-defined and disrupted; there was evidence of nuclear changes in pyknosis. Primary spermatocytes were the most affected cells after spermatids, and spermatozoa appeared deformed.

The present study revealed that spermatids were the most affected cells, followed by spermatocytes, spermatogonia and sustentacular cells. These results were

in agreement with those of Hamid *et al.* (2021), who attributed this effect to the cumulative damage to older cells and the resistance developed in sustentacular cells.

Kerr & Sharpe (1985) reported that FSH increases interstitial cell size and number in rats. As clomiphene citrate suppresses gonadotrophin hormones (FSH & LH) in rats, while (Brown & Chakraborty, 2009), suppression of FSH by clomiphene citrate leads to a decrease in the number and size of interstitial cells; this clearer study.

In the present study, the testes of the treated group showed shrunken or collapsed seminiferous tubules. These

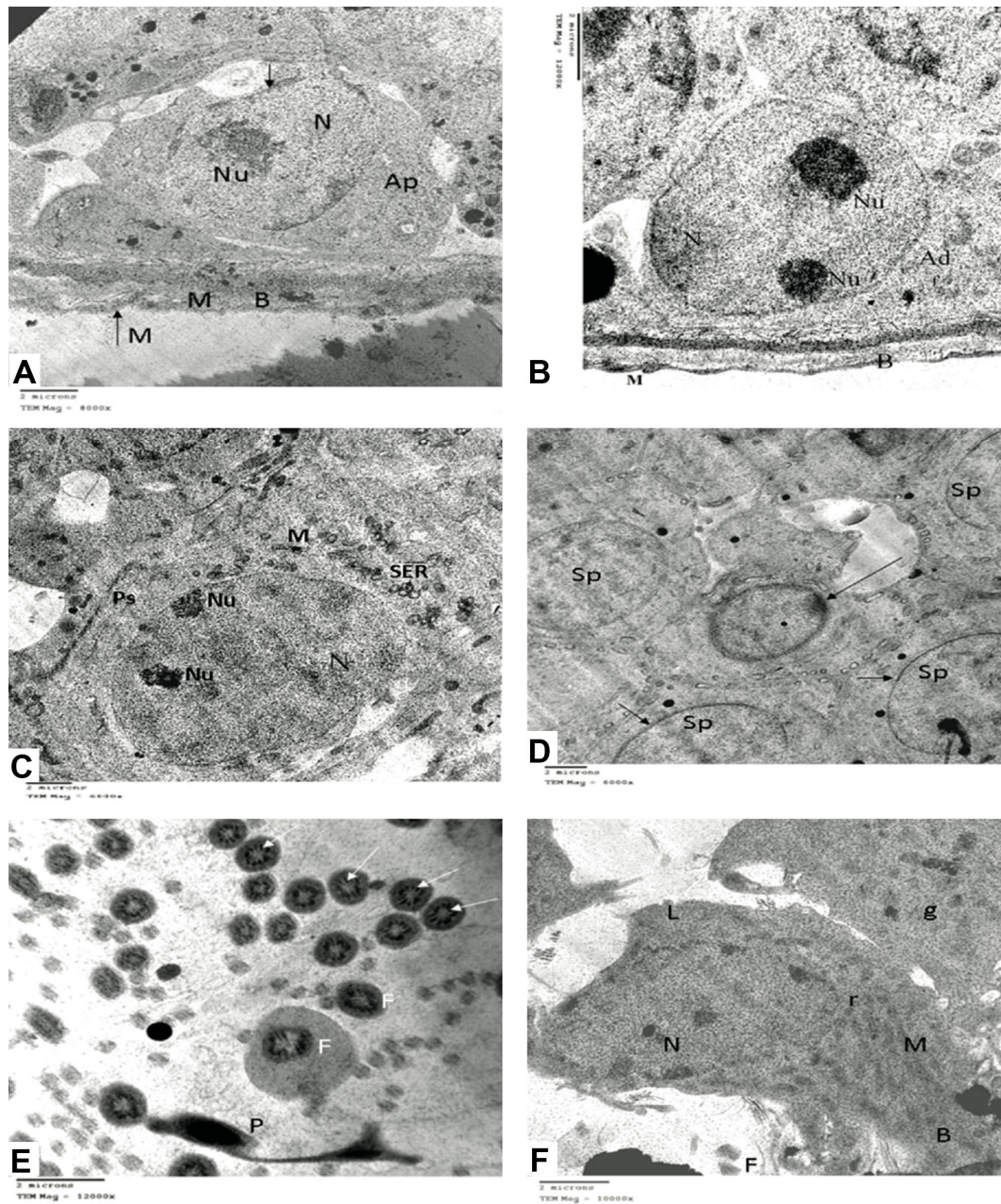


Fig. 3. Testes of the control group examined by electron microscopy. A. Electron micrograph of the testis of the control group showing the spermatogonium (A) pale (Ap) resting up on a basement membrane (B), which is covered by myoepithelial-like cells (M). The spermatogonia (A) are pale and have an ovoid-like nucleus (N) with an eccentric nucleolus (Nu) and a slightly pale nucleoplasm. (8000X). B. Electron micrograph of the testis of the control group showing spermatogonia (A) dark (Ad) resting on a basement membrane (B), which is covered by myoepithelial-like cells (M). The dark spermatogonium (A) has an ovoid nucleus (N) with double nucleoli (Nu) and dark nucleoplasm. The cell has a rim-like cytoplasm. (12000X). C. Electron micrograph of the testis of the control group showing a primary spermatocyte (Ps) with an ovoid-shaped nucleus (N) and two nucleoli (Nu). Its cytoplasm is rich in mitochondria (M) and smooth endoplasmic reticulum (SER) (6000X). D. Electron micrograph of the testis of the control group showing many spermatids (Sp). The spermatids show the Golgi phase of spermiogenesis. The Golgi phase (arrow) is characterized by the formation of membrane-limited acrosomal granules bound to the nuclear membrane. (6000X). E. Electron micrograph of the testis of the control group showing, ahead of a sperm (P), transverse sections of many sperm showing different parts, some in the midpiece (F) and others in the tail (arrows). (12000X). F. Electron micrograph of the testis of the control group showing interstitial cells of the interstitial tissue between the seminiferous tubules. Ledge cells have an oblong-shaped nucleus (N) with dark nucleoplasm. The nuclear membrane has condensed peripheral chromatin. Its cytoplasm has many mitochondria (M) and ribosomes (r). Notice the presence of fibroblasts (F). (10000X).

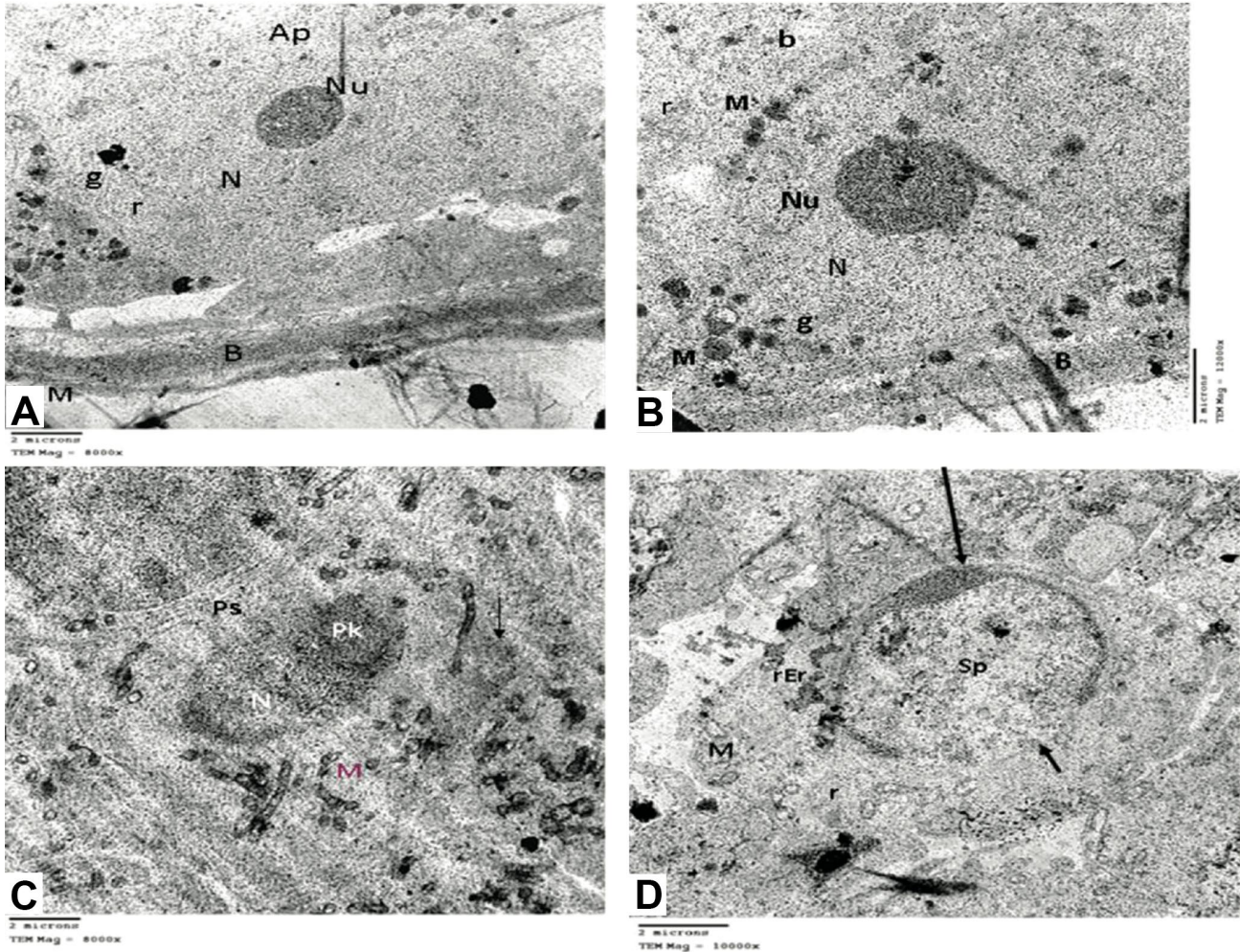


Fig. 4. Testes of the treated group examined by electron microscopy. A. Electron micrograph of the testis of the treated group showing; spermatogonium (A) pale (Ap) which has an ill-defined nucleus (N) where its nuclear membrane is ill-defined, but its nucleolus is evident (Nu). Its cytoplasm contains few cell granules (g) and few ribosomes (r). The basement membrane (B) appeared thick, with myoepithelial-like cells (M). (8000X). B. Electron micrograph of the testis of the treated group showing the spermatogonium (B) (b), which has an oval-shaped nucleus (N) with a central nucleolus (Nu) and moderately stained nucleoplasm. Its cytoplasm contains mitochondria (M), degenerated mitochondria (M), free ribosomes (r), and granules (g). The basement membrane appeared thick and intact (B). (12000X). C. Electron micrograph of the testis of the treated group showing primary spermatocytes (Ps). Its nuclear membrane is ill-defined and disrupted, with evident nuclear pyknosis (Pk). Its cytoplasm shows widespread degenerated mitochondria (M). (8000X). D. Electron micrograph of the testis of the treated group showing a deformed spermatid (Sp) with interrupted nuclear membrane (short arrow) and limited acrosomal phase (long arrow). The cytoplasm is pale and contains degenerated mitochondria (10000X). E. Electron micrograph of the testis of the treated group showing deformed spermatozoa. The deformed head is covered by a deformed transverse section (N2) compared with the control group. (20000X). F. Electron micrograph of the testis of the treated group showing interstitial cells (D) in the interstitial tissue with an irregularly shaped nucleus (N) with focal chromatin aggregation and chromatin condensation on the inner surface of the nuclear membrane. Its cytoplasm contains ill-defined cytoplasmic organelles. Notice the presence of fibroblasts (F) (15000X).

results are in agreement with those of Harman *et al.* (2006); they stated that treatment with clomiphene citrate was associated with atrophy and decreased testis weight and suppression of spermatogenesis in rats and attributed these changes to gonadotrophin inhibition.

The present study found that the primary spermatocytes of the treated group showed marked nuclear changes where the nuclear membrane was ill-defined and disrupted. These changes were explained by spermatogenic arrest at the primary spermatocyte level. Similar results were

obtained by Heller & Heller (2000) and Ameli *et al.* (2019). These changes were due to the increased number of abnormal and degenerated cells in male and rat testes as a result of the systemic toxicity of the drug. Another suggestion by the same authors was that alteration in the basement membrane structure and/or function could lead to blockage of the free flow of nutrients across the barrier, thus adversely affecting the germinal cells, and this was observed in our study.

The interstitial cells appeared few in number and atrophied compared with the control group. Our results were in disagreement with the results obtained by Milon *et al.* (2019), who reported changes in the testes in an adult man in the form of a testicular tumor after treatment with clomiphene citrate, which proved to be a interstitial cell tumor.

Our results showed that the abnormal forms of sperm increased and that the total number of sperm decreased following the administration of clomiphene citrate, which was in disagreement with Huijben *et al.* (2022), who found that the administration of clomiphene citrate leads to functional changes in sustentacular cells; this was associated with an increase in sperm count of %, while in rats, another result was obtained with clomiphene citrate treatment in the form of atrophy of the testes and reduction of their weight, and which leads to oligospermia. This result was in agreement with that of Brown & Chakraborty (2009), who stated that treatment with clomiphene citrate was associated with atrophy and decreased weight of the testis and suppression of spermatogenesis in rats; they attributed these changes to gonadotrophin inhibition, which agrees with our results.

CONCLUSION

The observations of the present work indicate a possible causal relationship between testicular affection and ingestion of clomiphene citrate, which can be avoided by close medical observations using ultrasonography, semen analysis or testicular biopsy to detect early malignant changes. Furthermore, the drug should not be used for more than three to six cycles and should be stopped for at least three cycles before reuse.

When clomiphene citrate is ineffective in the treatment of male infertility, human menopausal gonadotropin (hMG) administration is typically selected. However, high-dose hMG therapy is associated with a variety of adverse effects. Here, we describe the success of a modified clomiphene citrate regimen in increasing sperm count.

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RESUMEN: El objetivo del trabajo fue estudiar el efecto del citrato de clomifeno sobre la estructura de los testículos de la rata albina adulta, con la finalidad de determinar la mejor manera de utilizar este fármaco en el tratamiento de la infertilidad masculina. Los testículos se extrajeron bajo anestesia y para su análisis a través de microscopio de luz se tiñeron con HE. Además, las muestras fueron preparadas para su examen con microscopía electrónica de transmisión. Por otra parte, se cortaron secciones semifinas de 1 µm de espesor, se tiñeron con azul de toluidina y se examinaron mediante microscopía óptica. Los espermatoцитos primarios mostraron cambios nucleares marcados (picnosis) y sus membranas nucleares estaban mal definidas y alteradas. En el grupo experimental las células presentaban el citoplasma con degeneración generalizada de las mitocondrias y de los lisosomas y una degeneración focal del retículo endoplásmico rugoso en comparación con el grupo control. Las espermátidas estaban pálidas y las dos fases de la espermatogénesis eran claramente identificables en el grupo control, pero se confundían en el grupo tratado. Algunas espermátidas tenían membranas nucleares interrumpidas, y también contenían mitocondrias degeneradas, fragmentación focal del retículo endoplásmico rugoso y ribosomas libres. Los espermatozoides del grupo tratado se presentaban deformados en comparación con el control. Las células de Leydig del grupo tratado presentaban un núcleo de forma irregular, con agregación focal de cromatina y condensación de cromatina periférica en la superficie interna de la membrana nuclear. Las observaciones del presente trabajo indican una posible relación causal entre la afección testicular y la ingestión de citrato de clomifeno, que puede evitarse mediante observaciones médicas minuciosas a través de ecografía, análisis de semen o biopsia testicular para detectar cambios malignos tempranos. Además, el medicamento no debiera ser usado durante más de tres a seis ciclos y debe suspenderse durante al menos tres ciclos antes de volver a usarlo. Cuando el citrato de clomifeno es ineficaz en el tratamiento de la infertilidad masculina, normalmente se selecciona la administración de gonadotropina menopáusica humana (hMG). Sin embargo, la terapia con hMG en dosis altas se asocia con una variedad de efectos adversos. En este trabajo, informamos el éxito de un régimen modificado con citrato de clomifeno para aumentar el recuento de espermatozoides sin riesgo para el tejido testicular.

PALABRAS CLAVE: Citrato de clomifeno; Testículos; Ratas albinas; Espermatogénesis; Microscopio electrónico.

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