
Effects of *Tribulus Terrestris* (Quttub) and Clomiphene Citrate on Ovaries of Female Mice; Histological and Histochemical Study

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Abstract:

Background: *Tribulus terrestris* increases levels of various hormones in steroid family including testosterone, DEHA, and estrogen and for this reason improves sport performance, fertility in men and women, sexual function in men and women. There is, at present, lack of scientific confirmation of these supposed benefits. Therefore, this study aims to investigate the possible effect(s) of *Tribulus terrestris* on the mouse ovarian morphology and function, alone and in combination with other ovulation modulator agent (clomiphene citrate).

Materials & Methods: A total of 49 sexually mature healthy Norway albino female mice were used in this study; 25 for pilot study and 24 for the experimental study. Experimental animals were divided into 4 groups, each contained 6 animals. The 1st group was given *Tribulus terrestris*, the 2nd group was given clomiphene citrate, the 3rd group was given the two agents together, and the 4th group was control. Treatment was given daily for 10 days via orogastric intubation. Histological, histochemical, cytological and morphometrical studies were carried out.

Results: demonstrated that *Tribulus terrestris* alone causes an increase in the number and size of the mature follicles, with no significant change in the total follicular number, with obvious progesterone and some estrogen effects on the otherwise normal vaginal smears. On the other hand, clomiphene alone revealed no mature follicles and no corpora lutea, with strong estrogen-like effect and minimal progesterone effects on the vaginal smears. When *Tribulus terrestris* and clomiphene were given together, mature follicles and corpora lutea appeared in relatively large numbers, with estrogen and progesterone effects on the vaginal cytology.

Conclusion: *Tribulus terrestris* can stimulate ovulation when given alone, and oppose the anti-ovulatory effect of clomiphene and resume ovulation when given in combination with it.

Keywords: *Tribulus Terrestris* (Quttub), Clomiphene Citrate, Mice Female Ovaries

Introduction:

Tribulus terrestris is a tap-rooted flowering plant that grows wild throughout China, India, western parts of Asia and southern parts of Europe and Africa. The medicinal part is the dried herb harvested after the flowering season, the fruits and the seeds^[1]. The main active constituent of *Tribulus* is a furostanol saponin called "protodioscin". Other saponins present in it include dioscin, diosgenin, hecogenin, etc^[1,2].

In folk medicine of ancient India and eastern Europe, wide range of sexual problems have been treated by *Tribulus terrestris*, for example: impotence^[3], infertility, decrease libido (in male and female), ... etc^[4].

Bulgarian studies performed in the early 1980s showed that *Tribulus terrestris* significantly improved libido and erectile function in men and increased serum LH and serum testosterone, and caused stimulation of spermatogenesis with increased concentration of sperms, sperm motility and viability^[5,6,7].

Recent animal studies showed that *Tribulus terrestris* caused a statistically significant increase in prostate weight and intracavernous pressure (ICP) and improvement of the sexual behavior parameters in male rats^[8,9,10,11].

In male mice, histological and histochemical studies demonstrated that *Tribulus terrestris* increases the number of Leydig cells and the androgens produced by these cells are directly responsible for enhanced spermatogenesis^[12].

Tribulus terrestris supported sex drive, ovulation and sexual reproduction functions through elevated levels of FSH. *Tribulus* aided ovulation in more than

60% of infertile women. European research showed *Tribulus terrestris* helps with a feeling of wee being at the menopause.^[13]

Tribulus terrestris increases levels of various hormones in steroid family including testosterone, DEHA, and estrogen and for this reason improves sport performance, fertility in men and women, sexual function in men and women^[14] and symptoms of menopause such as hot flashes^[15].

Unfortunately the design of these studies appears to fall far short of modern scientific standards and there has not been any trustworthy scientific confirmation of these supposed benefits. Therefore, this study aims to investigate the possible effect(s) of *Tribulus terrestris* on the mouse ovarian morphology and function, alone and in combination with other ovulation modulator agent (clomiphene citrate) using histological, cytological, and histochemical and morphometrical means.

Materials & Methods:

A total of 49 healthy virgin sexually mature Norway albino mice (*Mus musculus*), 8-12 weeks age, and were used in this study. They were housed in the Animal Breeding Center, College of Medicine, University of Baghdad, under normal diurnal lighting system. They were fed ordinary mice pellet diet, kept in room temperature and housed individually in wire-meshed stainless steel cages, with free access to food and water.

In a pilot study, twenty-five virgin sexually mature female mice divided into five groups, were used to assess the appropriate dose of *Tribulus terrestris* to be given in the experimental study^[9].

The dose for the 1st four groups was as follows: 2 mg/kg/day, 5 mg/kg/day, 10 mg/kg/day and 20 mg/kg/day. The control group was given distilled water. For experimental study, twenty – four virgin adult female mice were used. Their average weight ranged between 16 to 38 gm. The animals were divided into four groups each contained six animals. The dose of *Tribulus terrestris* given to group 1 and group 3 was 5 mg/kg/day (chosen depending on the results of the pilot study). The dose of clomiphene citrate (Clomid, 50 mg/tablet, Aventis) given to group 2 and group 3 was 0.75 mg/kg/day^[16].

Control animals were given distilled water (through an orogastric tube). For pilot and experimental studies, the herb and/or clomiphene was given daily by orogastric tube for ten successive days. Daily vaginal smears were performed for staining with Papanicolaou method^[17].

After 10 days of treatment, the ovaries were excised while the animal was anaesthetized by open ether anesthesia {diethyl ether, (C₂H₅)₂O, BDH chemicals Ltd). The ovarian samples, fixed by 10% formalin (Fluka AG chemicals, Buchs) were processed for routine paraffin-wax embedding (Bancroft and Stevens, 1975). Serial 5 microns thick sections were cut using mechanical microtome (Leitz). The collected ribbons (from each tissue block) were divided into two groups: one was stained with hematoxylin-eosin stain^[17] and the other group was used for the combined alcian blue–PAS technique^[18,19].

Morphometry was used to study the following parameters in ovarian sections: follicular diameter of mature, secondary and large multilaminar primary follicles was measured and the number of follicles per section.

Measurements were obtained using micrometry eyepieces: grid-type to count the follicles and scale-type to measure the diameters. Calibration was assessed by using calibration slide.

The measurements data collected during this study were analyzed using the software statistical package of SPSS (Statistical Package for Social Sciences, Version 15, September 2006). The results were presented in simple measures of mean \pm S.D. (standard deviation).

Results:

A. Pilot study:

Haematoxyline & Eosin stain showed the following results:

Control group: Ovaries showed different stages of follicular development. Primary follicles were the most numerous types. Secondary (antral) follicles were fewer than primary follicles and mature follicles were the least type. Some slides showed multiple corpora lutea while others contained no corpora lutea.

2 mg/kg group: In most sections, no significant changes were noticed regarding the total follicular

number and the number of follicles in each stage and the number and size of corpora lutea. However, some histological sections showed a single giant ovarian cyst (fig. 1-A).

5 mg/kg group: There was a moderate increase in the number of large secondary follicles when compared with the control group, mature follicles were slightly more than control and no corpora lutea were found (fig. 1-B).

10 mg/kg group: A large number of secondary (antral) follicles were noticed occupying nearly the whole ovary with no one reaching full maturity. Ovarian medulla was small in size (fig. 2-A).

20 mg/kg group: Here, the predominating type of follicles was the primary, with single or double layer(s) of follicular cells (fig. 2-B). Few secondary (antral) and no mature follicles were seen.

PAS-alcian blue stained sections revealed the same histological findings of hematoxylin-eosin stained sections (fig. 3-A & fig. 3-B). Alcian blue stain the antral fluid of large antral follicles (fig. 4-A), and the cytoplasm of the granulosa cells lining the antral cavities (fig 4-C). Granulosa cells of mature, secondary and multilaminar primary follicles showed strong positive PAS reaction. Cells of corpora lutea (granulosa lutein cells) showed milder PAS reaction (fig. 4-B).

Zona pellucida of each visible oocyte demonstrated a very bright magenta colour, as well as the remnants of zonae pellucida that were found as distorted layers inside atretic follicles (fig. 4-D). The results of **vaginal cytology** of the five groups of pilot study were as follows:

The control group and the 2 mg/kg group: Daily smears of these two groups showed the typical estrus cycle of mice, consisting of four phases with five days duration (Fig. 5 A, B, C&D).

The 5 mg/kg group and the 10 mg/kg group showed the following results:

Estrus: lasted for one day, this phase showed decreased percentage of cornified cells, with nucleated and anucleated cells in approximately the same proportion (fig. 6-A).

Metestrus: This phase was increased in duration to cover two days instead of one. It showed clusters of nucleated cornified cells with edge enfolding (fig. 6-B), in addition to high percentages of intermediate and parabasal cells, together with large numbers of PMN leukocytes infiltration and amorphous substance.

Diestrus: The duration of this phase was reduced to one day. It showed variable numbers of degenerated PMN leukocytes with fewer epithelial cells in clusters (fig. 6-C).

Proestrus: lasted for one day, this phase showed predominancy of intermediate and parabasal cells with some PMN leukocytes and superficial cells and mucus (fig. 6-D).

The 20mg/kg group: This group showed a six-day estrus cycle with the following findings:

Estrus: lasted for one day, this phase showed a minimal percentage of cornified individual cells, mostly nucleated (fig. 7-A).

Mtestrus: This phase was increased in duration for two days. It showed clumped epithelial cells with marked edge enfoldment (fig. 7-B), high percentage

of intermediate and parabasal and PMN cells, with relatively high amounts of mucous material

Diestrus: like in control group, this phase lasted for two days and showed high percentage of PMN cells, degenerated or fragmented epithelial cells and abundance of amorphous material (fig. 7-C).

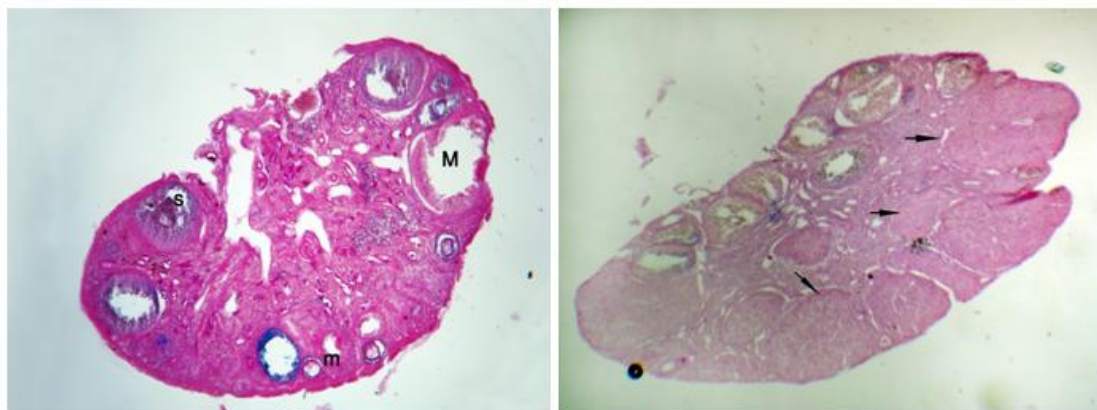


Fig.1: The ovary of a pilot mouse given (A): 2mg/kg *T. terrestris*, note the gaint ovarian cyst, (B): 5mg/kg *T. Terrestris*, note large number of secondary follicles(PAS-alcian blue stain,x100)

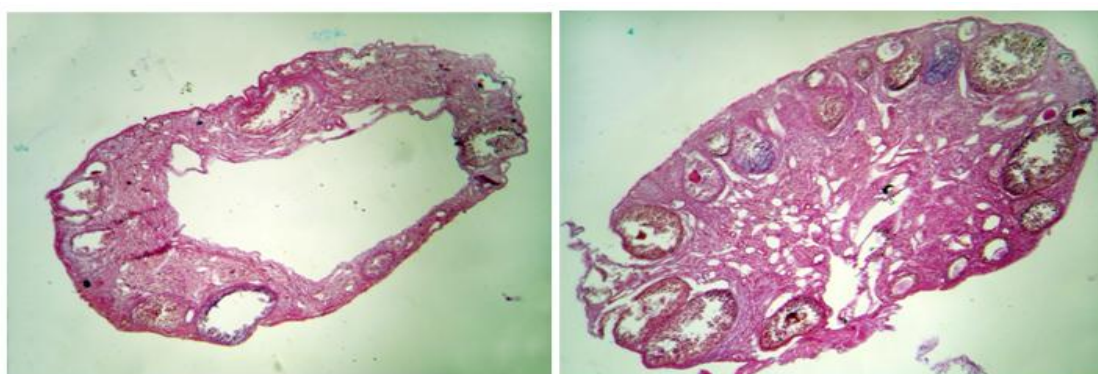


Fig.2: The ovary of a pilot mouse given (A) 10mg/kg *T. Terrestris*, note the huge number of secondary follicles (H&E stain x100), (B) given 20mg/kg *T. Terrestris*, note multilaminar primary follicle (H&E stain, x400).

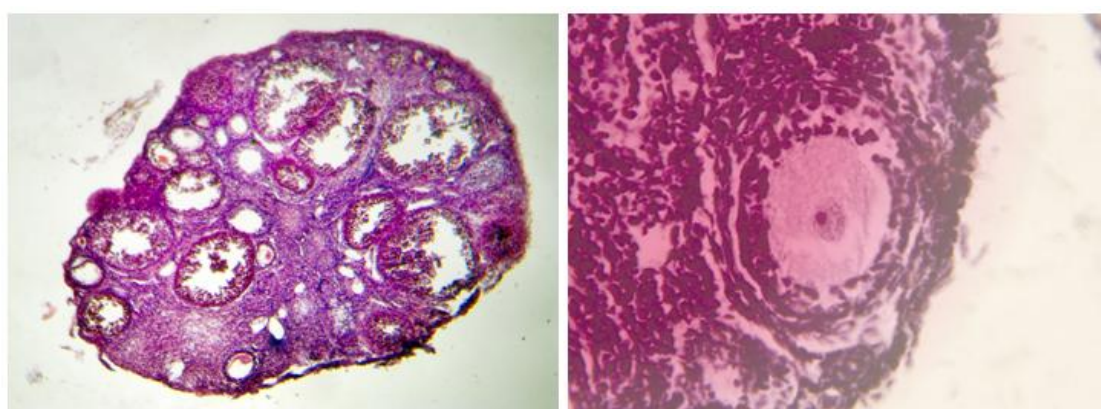


Fig. 3: The ovary of a control mouse (pilot study) shows: (A) multilaminar primary (m), secondary(s) and mature (M) follicles; (B): multiple corpora lutea (arrows) (PAS-alcian blue stain,x100)

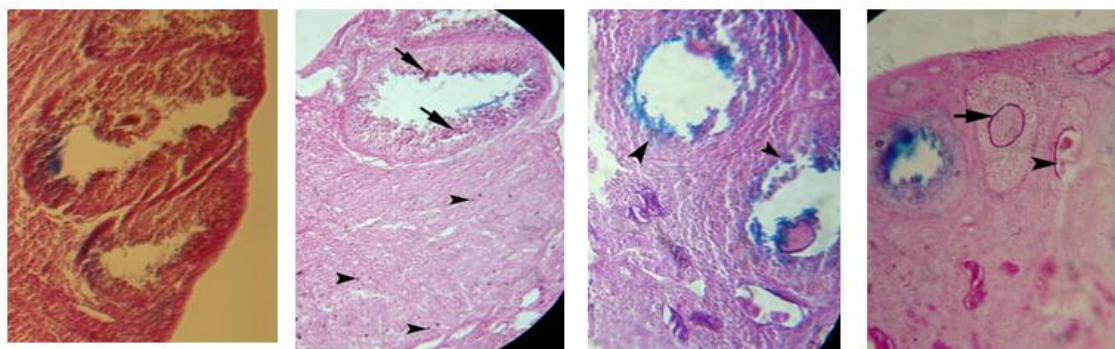


Fig.4. PAS-alcian blue stain of a mouse ovary showing: (A) large secondary follicle with aggregation of alcian blue-stained material in the antral cavity (x200), (B) strong PAS reaction in the granulosa cells (arrows) and weaker reaction in granulosa lutein cells (arrow heads) (x200), (C) alcian blue staining the cytoplasm of some granulosa cells (arrow heads)(x400), (D) intact zona pellucida (arrow) and zonal remnants in atretic follicle (arrow head) (x400) (PAS-alcian blue stain).

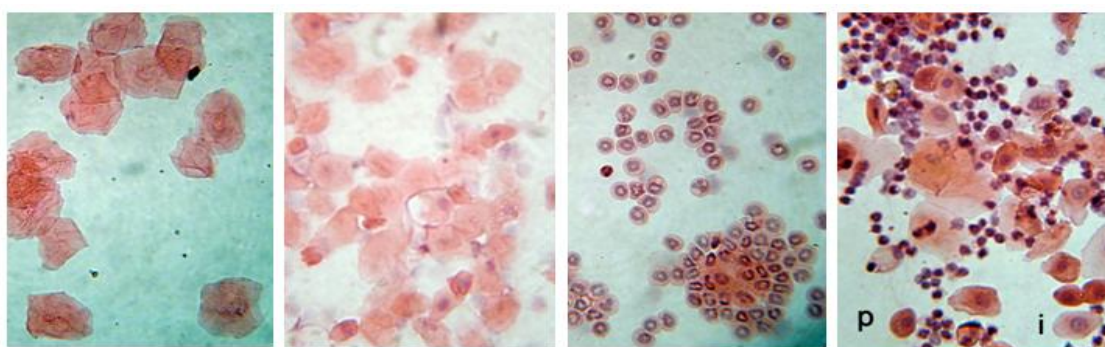


Fig.5: Vaginal smear of a control group showing the typical estrus cycle of a mouse with 4 phases: estrus (A), metestrus (B), Diestrus (C) and proestrus (D) (A&C x400), (B&D x200) (papanicolaou stain).

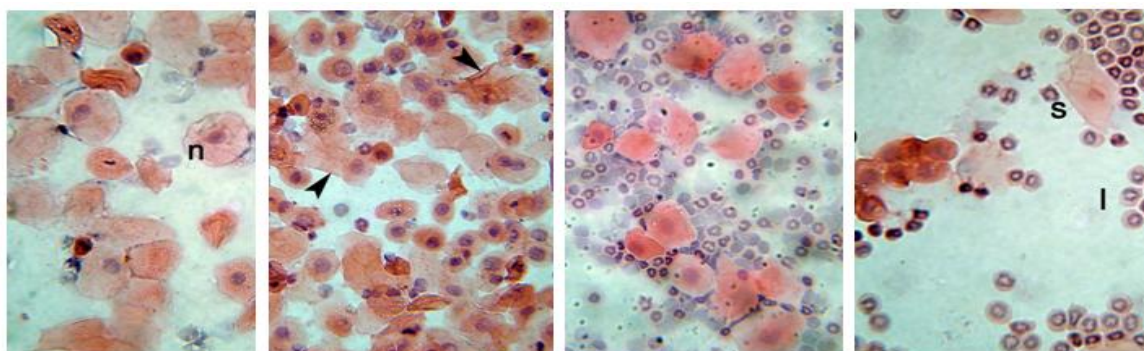


Fig.6: Vaginal smear of a pilot mouse given 5mg/kg *T. Terrestris* shows estrus cycle of 5 days duration with 4 phases: (A) estrus -1 day. (B) metestrus- 2 days, (C) diestrus- 1 day and (D) proestrus - 1 day. (A, B&C X 200, D X 400) (papanicolaou stain)

Proestrus: lasted for one day, this phase showed large number of degenerated PMN cells (fig. 7-D), intermediate and parabasal cells with some cornified epithelial cells.

B. Experimental study: the dose (5 mg/kg/day) was chosen to be given to the experimental animals because it is the only dose that gave a slight increase in the number of mature follicles than controls.

Tribulus-treated and combined treated animals were more active, they were climbing the top of

their cages all the time, fast in movement, difficult to be caught and forcefully resisting vaginal swab and orogastric intubation.

All the animals in the three experimental groups showed some increase in body weight during the treatment period. No significant difference in weight gain was seen between control and treated groups, neither among treated groups. The appetite was generally good, and slightly higher in *Tribulus* treated group.

Control group: showed the same histological findings that reported in histological texts [20, 21, 22, 23].

***Tribulus* treated group:** Concerning follicular numbers, no significant change was noticed in unilaminar primary follicles. The numbers of multilaminar primary follicles and secondary (antral) follicles were significantly less than those seen in the control group, causing a significant reduction in the total number of follicles. The major difference seen in this group was the statistically significant increase in the number of mature follicles, and the size of these follicles were very large (more than 500 micron diameter) (figs.8 A & B). No corpora lutea were found. Interstitial glands were present in relatively large amounts scattered throughout the ovary (fig. 9-A). Under magnification, the cells of those glands were large, polyhedral with abundant vacuolated cytoplasm containing many lipid droplets of varying sizes (fig. 9-B). Atretic follicles were significantly less than those seen in control and clomiphene treated groups.

Clomiphene treated group: In this group, there was a statistically significant decrease in the total follicular numbers, which was mainly due to a significant reduction in the numbers of unilaminar

primary follicles (fig. 17). The most common type seen was the multilaminar primary follicles, followed by secondary follicles. Strangely, neither mature follicles nor corpora lutea were seen (figs. 10 A&B). Interstitial glands were few or absent. Atretic follicles were seen in large numbers, nearly similar to those in control group and significantly more than what was seen in *Tribulus* treated group (fig. 10 B).

Combined treated group: Here, the changes that were seen with *Tribulus* group and clomiphene group were both found together. The total follicular numbers were slightly less than controls (statistically insignificant decrease) (table III, fig. 17). Unilaminar primary follicles were nearly similar to control, but significantly more than what was seen in clomiphene group. Multilaminar primary follicles numbers were also close to the control group, as well as to the clomiphene group. Significant decrease in the numbers of secondary (antral) follicles and significant increase in the numbers of the mature follicles in comparison with the control group were seen (figs. 11- A & B and figs. 12- A&B, fig. 17).

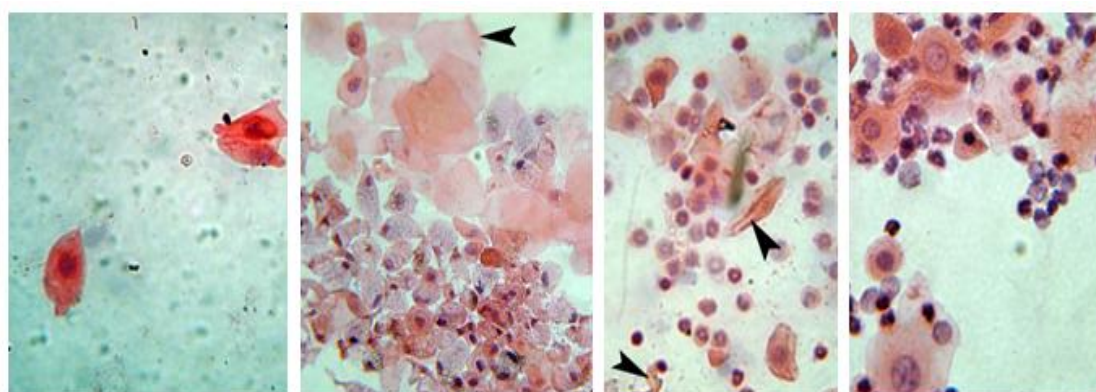


Fig.7: Vaginal smear of a mouse given 20mg/kg *T. Terrestris* shows a 6 days estrus cycle with 4 phases (A)estrus -1 day, (B) metestrus-2 days, (C) diestrus-2 days ,(D) proestrus -1day (X200) (papanicolaou stain)

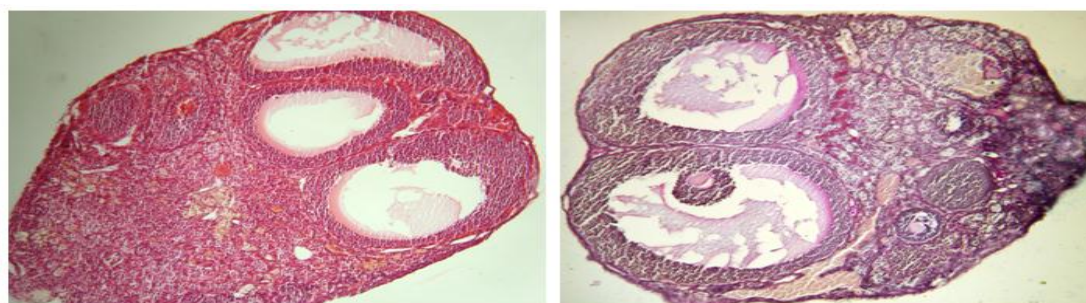


Fig.8: Ovary of a *Tribulus terrestris* –treated mouse, note the large mature follicles. (A) H&E, (B) PAS-alcian blue stain (X100)

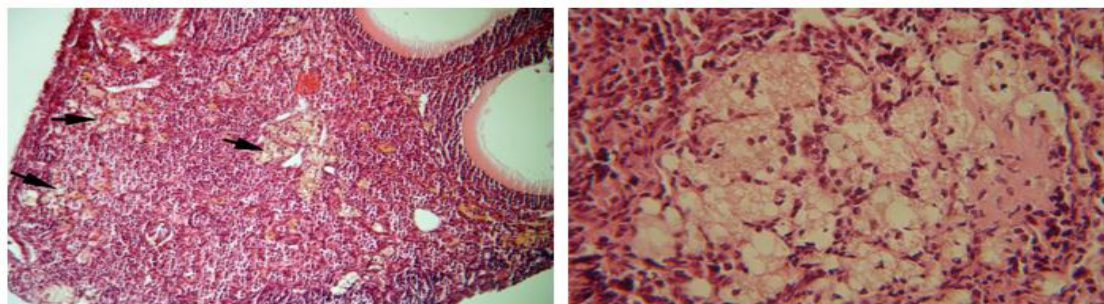


Fig. 9: Ovary of a *Tribulus terrestris* –treated mouse, note (A) the large amount of interstitial glands (arrows) ; (B) under magnification interstitial cells are large polyhedral with abundant vacuolated cytoplasm containing different –size lipid droplets (H&E) (A X200) (B X 400)

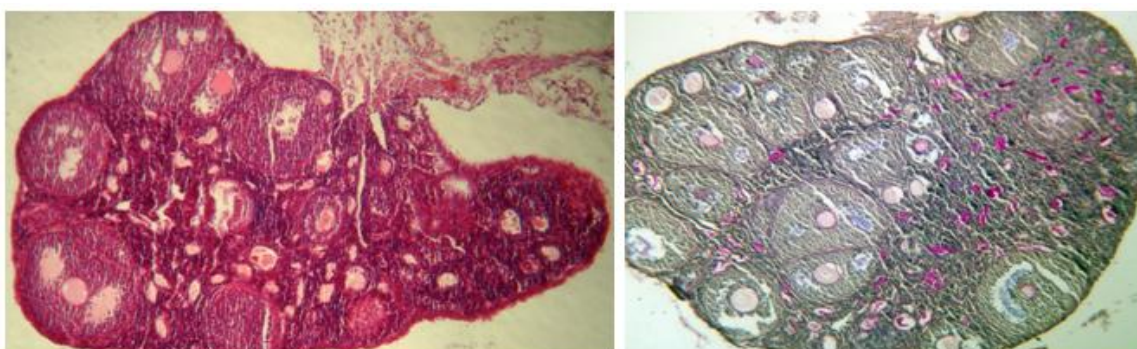


Fig. 10: Ovary of a clomiphene-treated mouse .Note the large number multilaminar primary follicles and large number of atretic follicles.(A) H&E , (B) PAS-alcian blue stain , X100

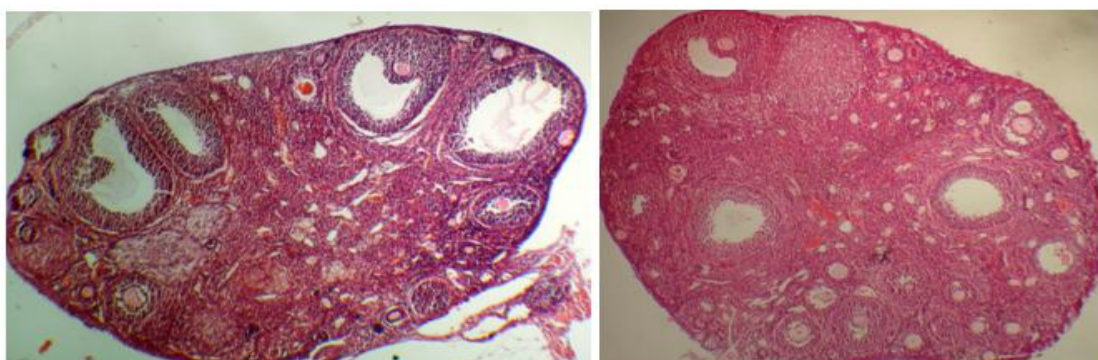


Fig.11: Ovary of a mouse with combined treatment. Note the large number of mature follicles (A) and large numbers of variable follicular stages(B) (H&E X100)

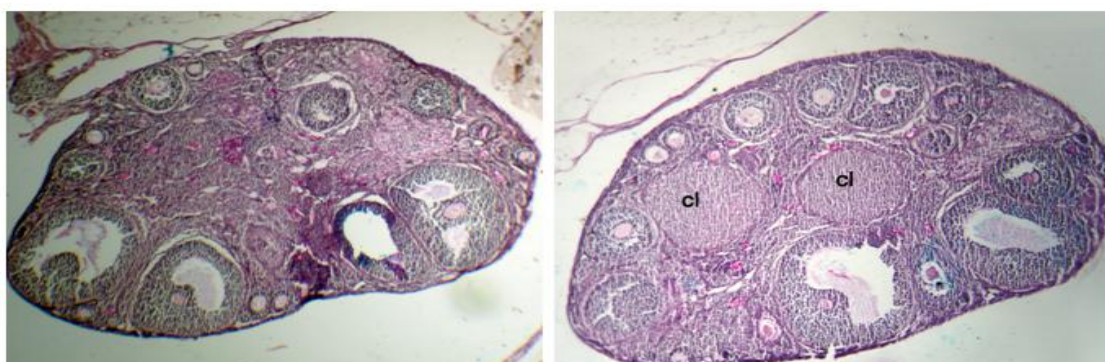


Fig.12: Ovary of a mouse with combined treatment. Note the two corpora lutea (cl) (PAS-alcian blue stain, X100)

As seen in the control animals, corpora lutea were present in some sections and absent in others. The difference here was that in many animals, corpora lutea were present together with mature follicles, a phenomenon that was not seen in control group (fig. 12-B). Interstitial glands were present in small amounts or absent. Atretic follicles were present in variable numbers, and their inverse relation with the presence of corpora lutea seen in control group was not clear here.

PAS-alcian blue stained sections revealed the same features seen by hematoxylin-eosin stained sections. Alcian blue stain the antral fluid of the secondary follicles (fig. 13-A).

PAS positive reaction was maximum in the cytoplasm of granulosa cells of mature and secondary (antral) follicles, followed by multilaminar and unilaminar primary follicles' follicular cells, then granulosa lutein cells (fig. 13-B). The antral fluid in mature follicles of *Tribulus* treated mice stained lightly pink.

Zonae pellucidae appeared as bright magenta coloured rings around the oocytes, especially in the clomiphene treated group. Early atretic changes were seen as wrinkled zonae pellucidae with spaces separating them from the shrunk oocytes, surrounded by disintegrated follicular cells.

Established atretic follicles appeared either as empty small spaces (cystic changes) or small spaces filled with the remnants of zona pellucida, that appear -in this case- as a very bright magenta-

coloured wrinkled concentric membranes (fig. 13-C).

Vaginal Cytology:

Control group: This group demonstrated typical five-day duration estrus cycle, showing the same phases and finding seen in the control group of the pilot study.

***Tribulus* treated group (Fig. 14):** Here, estrus cycles were of six-days duration, with two days **Estrus**, two days **Metestrus**, one day **Diestrus** and one day **Proestrus**.

Clomiphene treated group (Fig. 15): Smears of this group revealed extended (6days) estrus cycle with two days **Estrus**, one day **Metestrus**, two days **Diestrus** and one day **Proestrus**.

Combined treatment group (Fig.16): Smears of this group showed extended (6days) estrus cycle with one day **Estrus**, two days **Metestrus**, two days **Diestrus** and one day **Proestrus**.

Morphometrical Study:

The mean of follicular numbers counted in each section in this study are shown in (fig. 17). Concerning the follicular diameters, mature follicles of the *Tribulus* treated group were quite larger than those seen in control and combined treatment groups.

Multilaminar primary follicles of the clomiphene-treated group were generally larger than those seen in the other groups.

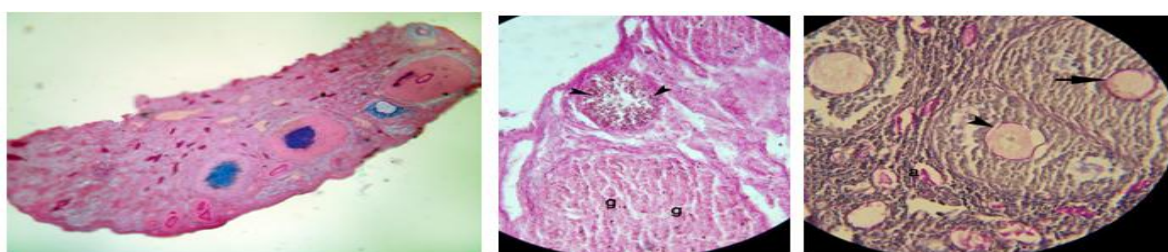


Fig.13 Ovary of a mouse stained with PAS-alcian blue . Note : (A) the alcian blue in the antral cavities , (B) positive PAS reaction in the granulosa cells (arrowheads) and granulosa lutein cells(g), (C) intact (arrow), degenerating (arrow head) zona pellucida (x100).

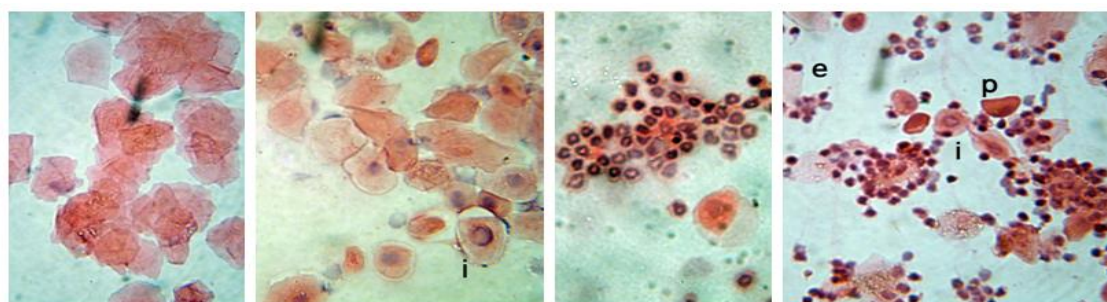


Fig.14: Vaginal smears of a mouse treated with *T. Terrestris* show a 6days estrus cycle: estrus (A) -2days; metestrus (B)-2days; diestrus (C)- 1day and proestrus (D)-1day (papanicolaou stain) (A,B&C x400) (D x200)

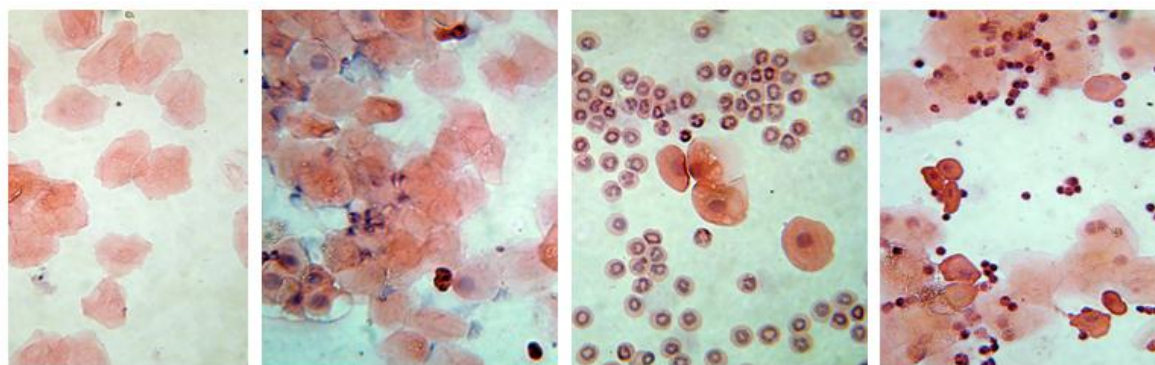


Fig.15: Vaginal smears of a mouse treated with clomiphene show a 6 days estrus cycle: estrus (A) – 2 days, metestrus(B)-1day, diestrus(C)- 2 days and proestrus (D)–1 day(papanicolol stain)(A,B&Cx400)(Dx200)

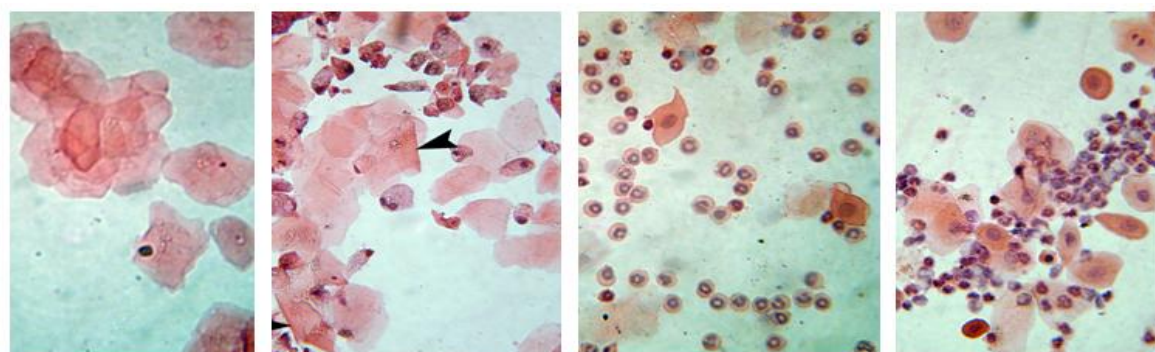


Fig.16 : Vaginal smears of a combined – treatment mouse showing a 6 days estrus cycle: estrus (A)- 1 day; metestrus(B)-2 days ; diestrus(C)-2 days and proestrus (D)- 1 day(papanicolaou stain)(X400)

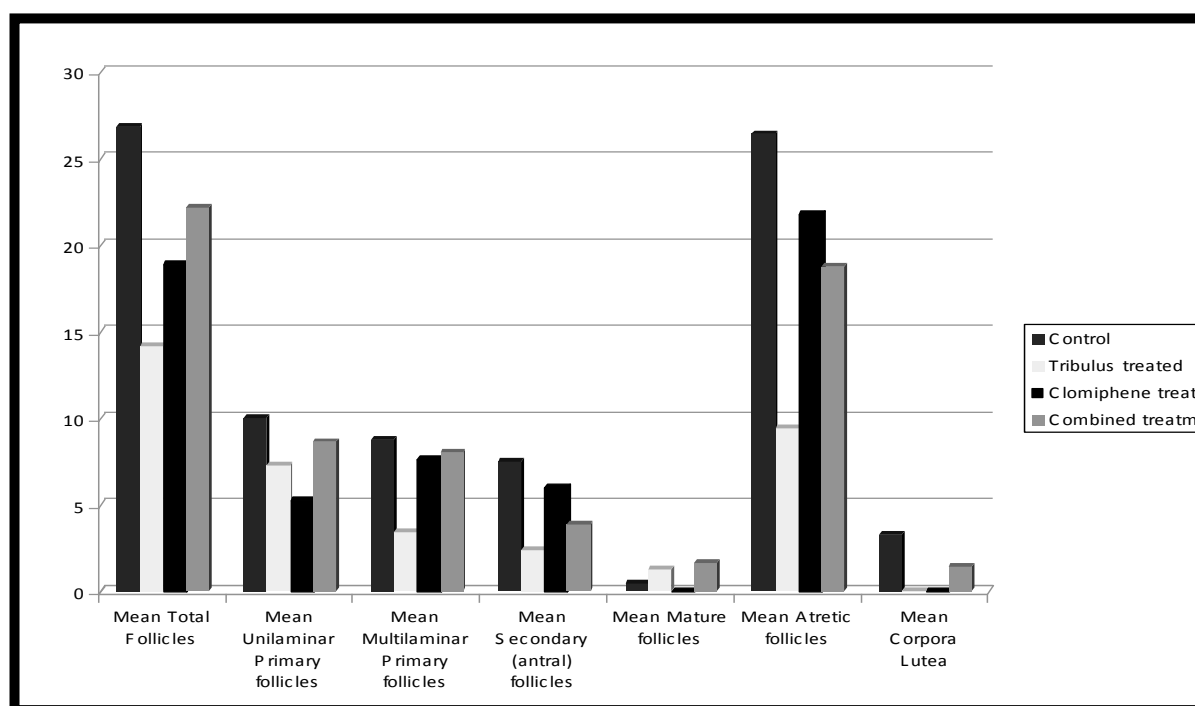


Fig.17: The mean numbers of different follicular stages and corpora lutea in different experimental groups (Significance of difference: $P < 0.05$)

Discussion:

The increase in the activity of the animals that have been fed on *Tribulus terrestris* alone or combined with clomiphene was probably due to the herb itself, as many previous studies claimed that *Tribulus terrestris* increases the body testosterone level^[24, 25, 26].

The mechanism of this increase could be due to an increase in the luteinizing hormone (LH) level secondary to *Tribulus terrestris* intake^[24].

The weight gain that was noticed in all the animals (treated and control) was proportional to the expected weight gain according to the weight/age table described by Lane-Petter^[27]. The slight increase in the appetite seen in *Tribulus terrestris* treated mice could be due to its anabolic effect described by many authors^[24, 25, 28].

Histological study of control group is supported by many old and recent studies as there is an inverse relation between the follicular numbers of a certain stage of follicular development and the progress of that stage. Thus, any ovarian section will be found to contain primordial follicles in the highest number, followed by unilaminar primary follicles, then multilaminar primary follicles, secondary (antral) follicles and finally mature follicles which are the least common type^[29].

In the present study, *Tribulus terrestris* seemed to increase LH level, causing an increase in the growth and maturity of the secondary follicles, resulting in large numbers of large mature follicles. As LH receptors start to appear on the follicular cells in the secondary stage of follicular development, elevated LH level induced the growth and activity of granulosa cells, and hence an increase in estrogen secreted by them^[24, 26, 30].

High estrogen level causes high-frequency GnRH pulse secreted by the hypothalamus^[31], and this in turn, induces more LH secretion, creating a vicious cycle. The mature follicles seen in this group were very large compared to the control group. This is probably due to the high serum levels of LH and estrogen. This explanation is supported by the results of vaginal smearing of this group.

Negative feedback effect of the high estrogen levels causes decreased FSH level, resulting in a reduction in the number of multilaminar primary follicles and secondary follicles. Hence, total follicular number reduction. The presence of corpora lutea indicates that ovulation had occurred^[31].

Many studies have been made to investigate the effect of clomiphene on the ovulatory function of different laboratory animals. Authors suggested that in many species, clomiphene citrate has two opposite effects on estrogen receptors: in low doses it binds to estrogen receptors blocking the negative feedback of endogenous estrogen resulting in gonadotropin secretion and hence, ovulation, while in high doses (0.5mg/kg or more) clomiphene binds

to those receptors and acts like estrogen (i.e., agonist) blocking gonadotropin secretion^[32, 33, 34, 35, 36]. Other authors suggested that the above opinion is not true in rats^[37] and mice^[38].

The latter two suggested that the effect of clomiphene depends on the species, tissue type and experimental design (dose used) to exert its action. Martin et.al.^[39] and Martin^[40] claimed that clomiphene has no antagonistic activity in mice (i.e., does not cause ovulation). Recent studies showed that clomiphene and its isomer enclomiphene can cause ovulation and superovulation in mice in very low doses (1 and 10 µg/kg) and only in combination with human chorionic gonadotropin (hCG)^[41].

In the present study, clomiphene was found to inhibit ovulation in mice. The explanation is that the dose used (0.75 mg/kg) was high enough for clomiphene to act as estrogen agonist in the hypothalamus, pituitary, ovary and vaginal epithelium. This is strongly supported by the early 1970s studies (above) and by Pakrasi and Kumar^[41].

In the hypothalamus and pituitary gland, estrogenic effect of clomiphene induced the negative feedback inhibition resulting in decreased levels of FSH and LH. Low FSH level caused a reduction in the total follicular number, and low LH level caused failure of maturation of the secondary follicles. Therefore, no mature follicles neither corpora lutea were found in any section of this group. These results are close to those described by Martin et al^[39] and Martin^[40].

On the other hand, estrogenic effect of clomiphene on the follicular cells of the already present multilaminar primary follicles has probably caused an increase in the sensitivity of these cells to the low level of FSH resulting in further proliferation and their growth and the appearance of many large multilaminar primary follicles and early antral follicles. However, no one of these follicles could mature and ovulate due to the low LH level.

The absolute absence of corpora lutea, and the disappearance of any progesterone effects on the vaginal cytological examination indicate that no corpus luteum had formed and, by conclusion, ovulation had not occurred. The relative increase in atretic follicular numbers could be due to the low FSH level that made large number of follicles fail to grow and mature and eventually pass through follicular atresia.

The findings of the combined – treated group can be considered as a summation of the findings seen in the other two treated groups, with the following explanation: clomiphene has acted as an agonist to the estrogen receptors in the hypothalamus and pituitary gland, activating the negative feedback inhibition of gonadotropin secretion. Low FSH level caused a reduction in the growth of unilaminar primary follicles.

On the other hand, the estrogenic effect of clomiphene on the follicular cell level has increased

their sensitivity to the low level of FSH. As one function of estrogen is to up-regulate FSH and LH receptors on follicular cells^[42].

This has led to the resumption of the normal growth rate of the unilaminar primary follicles and their transformation into multilaminar primary follicles.

The effect of *Tribulus terrestris* here was manifested in the appearance of significantly large numbers of mature follicles, even larger than those of the control group. This point was obviously due to the role of high LH level, which has resumed the formation and maturation of mature follicles that failed to develop when clomiphene was given alone. Moreover, the number and size of these mature follicles were significantly larger than those seen in the control group.

The presence of corpora lutea in many sections of the combined-treated group indicates that ovulation took place. The presence of corpora lutea in the sections that contained mature follicles may indicate that ovulation had occurred in many follicles at different times.

The alcian blue stain distribution reveals that the antral fluid of the large secondary and mature follicles is very rich in acid mucins, which are also present in lesser amounts in the granulosa cells adjacent to the antral cavity^[19].

Strong PAS reaction that was seen in certain areas is most probably due to the presence of glycoproteins and/or 1, 2 glycoles of high molecular weight^[17,43].

Finding the maximal PAS-positive reaction in the granulosa cells is supported by previous studies^[44, 45].

The magenta colour exhibited by the majority of sections is due to the presence of neutral mucins. In fact, most large antral follicles revealed gradual shift from magenta colour peripherally to blue colour centrally. This gives an idea that as one move from the outer granulosa cells towards the antral cavity, the proportion of acid mucins increases.

The very bright magenta colour exhibited by zona pellucida supports the opinion suggesting the predominance of hyaluronic acid and sulphated glycosaminoglycans in its structure^[29,44].

Mice of **Control Group** showed a normal five-day estrus cycle, with the four stages present in their normal duration and cytological picture. The typical estrus phase reflected normal follicular development in the ovary, evidenced by the estrogen effects on the vaginal epithelium where keratinized anucleated cells predominated in the estrus phase. In a similar way, progesterone effects were obvious during the metestrus and early diestrus phases, with typical enfoldment of epithelial cell edges and intermediate and parabasal cells in mucous material seen in these phases^[46, 47, 48, 49].

When the last vaginal smear of each animal was compared to its ovarian section, the follicular stage

of the ovaries matched the corresponding phase of the estrus cycle. This indicates that control animals had normal hormonal state with alternating estrogen and progesterone predominance on the vaginal epithelium^[20].

In ***Tribulus*-treated Group**, the high estrogen level secreted by the granulosa cells of the large mature follicles was manifested as an increase in the percentage of cornified epithelial cells and an increased duration of the estrus phase to cover two days. However, progesterone effects were obvious in the extension of metestrus phase to two days also on the extent of diestrus, which was reduced to one day.

This is expected as large mature follicles form large corpora lutea after ovulation, leading to high levels of progesterone. The cycle, thus, was extended to cover six days.

In **Clomiphene-treated Group**, the picture was nearly absolute predominance of estrogenic effects, manifested as an increase in the percentage of cornified anucleated epithelial cells and extension of estrus to cover two days. On the other hand, the progesterone effects were minimal or absent, as metestrus-I resembled estrus and metestrus-II was absent. This can obviously be explained by the minimal levels of progesterone due to the absence of corpora lutea secondary to the low LH levels that was caused by the clomiphene treatment.

In **Combined-treated Group**, the estrogenic effects (from clomiphene estrogenic activity on the vaginal epithelium) were manifested by the high percentage of cornified epithelial cells during estrus. However, estrus here was not extended in duration.

This may be because progesterone was not absent as in clomiphene treated group. Furthermore, progesterone effects (from multiple corpora lutea secondary to the large number of mature follicles formed after *Tribulus* intake) were more predominant as the metestrus phase was extended to cover two days, with high numbers of intermediate and parabasal cells and typical edge enfoldment of the epithelial cells.

In conclusion, the present study has demonstrated that *Tribulus terrestris* can stimulate ovulation when given alone, and oppose the anti-ovulatory effect of clomiphene and resume ovulation when given in combination with it.

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