Study of The Effect of Fenugreek Seeds on The Folliculogenesis in Super-Ovulated Female Rats

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Abstract

The increase of the incidence of usage of ovulation induction and assisted reproductive technology (AST) for treatment of the anovulation disorders, causes increase of multiple pregnancy and ovarian hyper-stimulation syndrome as two main complications. These two complications are caused by hyper-stimulation of ovaries. Trigonella foenum-graecum (fenugreek) is an herbal plant that has been studied for

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its anti-fertility activity. The aim of this research was to study the effect of fenugreek seed aqueous extract (FAE) on the folliculogenesis in super-ovulated female rats by quantifying the ovarian follicles; antral (mature follicle) and atretic follicles. Twenty-four 7-8 week-age cyclic Sprague Dawley female rats were divided into three groups (A, B and C). Group A considered as control group, the female rats in group B and C treated with fenugreek seed aqueous extract 500mg/kg and 1000mg/kg respectively. The extract was administering orally into rats in treated groups for 14 days. Vaginl smears were taken and examined daily to determine the different phases of estrous cycle. During diestrus phase of estrous cycle, the female rats in the three groups were treated with subcutaneous injection of decreasing doses of recombinant human FSH (rhFSH) to induce the superovulation. In next day (proestrus phase), the rats were sacrefied and both ovaries of each rat were removed for histological examination. Administering of 500 and 1000mg/kg body weight of the extract caused significant reduction in the number of antral follicles (p-value < 0.005), while the number of atretic follicles was increased significantly (p-value < 0.005) in both treated groups compared to control group. Moreover, the atretic follicles' number was significantly (p-value = 0.01) higher in group C (treated with 1000mg/kg of FAE) compared to group B (treated with 500mg/kg of FAE). As an outcome of this study, FAE showed an effect on the fluculogenesis by reducing the number antral follicles and increasing the atretic follicles' number.

Keywords: *ovulation induction, fenugreek seeds, aqueous extract, antral follicles, atretic follicles, estrous cycle, diestrus phase, rhFSH, superovulation.*

1. Introduction

In common diseases as polycystic ovarian syndrome and hyperprolactinemia, women have anovulatory cycle, and the anovulation disorders represent about 30% of infertility cases (1). Ovulation induction medications have been used since long time to treat anovulation infertility (2). Besides, the usage of outside in-vitro fertilization (IVF) or assisted reproduction technology (ART) as a treatment of infertility was increased in the last two decades. According to the world collaboration report on ART in 2000, the number of babies which conceived through ART was between 197,000 and 220,000 in forty-nine countries (3).

However, the increase in the incidence of ovulation induction and ART causes increase of multiple pregnancies and ovarian hyperstimulation syndrome as two main complications of this therapy (4). The two main complications of ovulation induction are caused by hyperstimulation of ovaries which lead to develop and ovulate of many oocytes as well as hyperstimulation syndrome. Multiple pregnancy carries a high risk to both mother and fetuses. Mother with multiple pregnancy has higher risk of preeclampsia and gestational diabetes (5). The multiple fetuses are at higher risk of growth restriction and prematurity (5) as well as chromosomal anomalies (6).

Fenugreek (*Trigonella foenum-graecum*) is one of the herbs that has been used since long time in medicine as laxative and to stimulate the appetite (7). Many researches have studied the different properties of this herb, and its effect on fertility. Fenugreek seeds have an estrogenic

activity (8). The seeds also found to cause an increase in serum prolactin level (9), and that causes inhibition of the ovulation. This research studies the effect of fenugreek seeds on the ovaries with ovulation induction model in female rats in term of decrease the chance of ovulation induction complications.

The main goal of this study was to study the effect of fenugreek seed aqueous extract on the fulliculogenesis by determine of quantification of antral and atretic ovarian follicles.

2- Material and Methods

2.1 Preparation of Fenugreek Seed Aqueous Extract

The fenugreek seeds were purchased, and the aqueous extract was prepared at the Pharmaceutical Technology laboratory in Faculty of Pharmacy, International Islamic University Malaysia (IIUM).

First the clean dried fenugreek seeds were ground into a fine powder by using electrical grinder. Then, this seed powder was mixed with distilled water, 1 g of seed powder with 20 ml of distilled water. The suspension was agitated continuously for 24 hours at room temperature using a constant shaker (Memmert Shaker, Model 8W, Germany), as shown in fig.1. Then, the mixture of Fenugreek seed powder and distilled water (aqueous extract) was centrifuged (Hettich Universal 32R centrifuge, Germany) at 9000 rpm for 5 minutes, as shown in fig. 2. After that, the supernatant was collected, and the residue was discarded.

Figure 1: Fenugreek seed powder mixed with distilled water in 250mL glass flasks and continuously agitated by the shaker for 24 hours at room temperature.

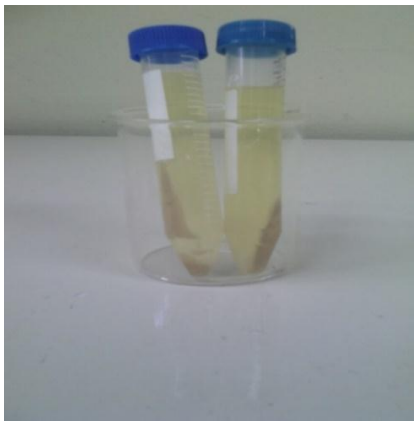

Figure 2: The supernatant and residue after the centrifugation of fenugreek seed powder and distilled water mixture.

Finally, the aqueous extract, the water-dissolved part of fenugreek seed powder, was freeze dried continuously for 5 days (Martin Christ, Model Alpha 1-4, Germany) to a powder form that can be easily stored and later weighted to calculate the dose. The powdered extract was stored in – 20 °C freezer (Sanyo, MDF-U537D, Japan) till it was used (10).

In a study of fenugreek toxicity, feeding fenugreek seeds to rats for 90 days caused no changes in the liver function test, and no histopathological or hematological changes (11). Moreover, a study on the acute toxicity of fenugreek was performed. Where a methanolic extract of fenugreek seeds has been given orally to rats, and the extract found to be safe at dose of 2000mg/kg, no animal mortality. Therefore, oral dose of 2500mg/kg was considered as LD50 (12).

In a study of the effect of three different doses (50, 100 and 200 mg/kg) of the alcoholic extract of fenugreek seeds on female reproductive hormones in mice. The extract was administrated intraperitoneally. They found a significant decrease in FSH, LH and estradiol levels in all study groups, but progesterone level was elevated significantly in the second study group (200mg/kg) only (13). Therefore, in this study the extract was given orally in two doses 500mg/kg and 1000mg/kg to make sure it will have an effect on the ovaries and to compare the effect of these two doses of extract

2.2 Research Animals

Twenty-four healthy Sprague Dawley cyclic female rats were used for this study. The female rats were aged between 8-10 weeks, and weighed between 180-200 g. The rats were kept in the Experimental Animals' room at the Physiology Laboratory in Faculty of Medicine, at a controlled temperature around 25 °C and programmed lighting hours (lights on from 7:00 h to 19:00 h). The female rats were housed in cages, two rats per cage. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the International Islamic University Malaysia. The rats were provided with appropriate facilities

for food and water and the cages were frequently cleaned, as shown in fig. 3.

Figure 3: Housing the Sprague Dawley female rats in animal cages provided with tap water and rat's food pellets.

2.2.1 Experimental Animal Design

After two weeks of acclimatization, the female rats were weighed using (Metter Toledo SB, Switzerland) and randomly divided into three groups, eight female rats each. This sample size was determined by using Sedecor and Cohran equation. The equation is n = 2 + c (SD/d), where < n > is the sample size, < c > is a constant equal to 7.85, SD is the standard deviation, and < d > is the difference of mean number of antral follicles between control and treated groups. The calculation is:

$$n = 2 + c\,(SD/d)$$

$$n = 2 + 7.85(2.4/4)$$

$$n = 6.71 \approx 7$$

$$\text{With 15 \% attribution rate} = 0.15 \times 6.71 = 1.06 \approx 1$$

$$\text{Thus, } n = 7 + 1 = 8 \text{ rats per group}$$

Group A was considered as a control group, the rats in group B were treated with 500mg/kg fenugreek seed aqueous extract, and the rats in group C were treated with 1000mg/kg fenugreek seed aqueous extract. The calculated dose of extract powder for individual rat in group B and C, was dissolved in distilled water and given orally, using syringe with gastric gavage needle, daily for a period of 14 days in a row. The gastric gavage needle was gently passed through the rat mouth and slowly pushed down the oropharynx and the oesophagus. Then the fenugreek extract was expelled out into the stomach, as shown in fig. 4.

Figure 4: Oral administration of Fenugreek Seed Aqueous Extract by using syringe with gastric gavage needle.

2.2.2. Calculation of Fenugreek Aqueous Extract Doses

The two doses of the extract, 500mg/kg b.w. for group B rats and 1000mg/kg b.w. for group C rats were calculated according to the following formulas:

For rats in group B = body weight of rat (g) X 500 / 1000
For rats in group C = body weight of rat (g) X 1000 / 1000

2.3 Determination of The Different Phases of Estrous Cycle

2.3.1 Taking of Vaginal Smear

From the beginning of the experiment, vaginal smears were taken every morning from all rats to detect the different phases of estrus cycle. Disposable plastic loops (COPAN Diagnostics Inc, USA) were used to collect the vaginal fluid with vaginal cells from the female rats. The loop was introduced only half an inch into the vagina and gently rotated inside the vagina in one direction, then it was slowly withdrawn. The collected vaginal fluid on the loop was applied onto a labelled slide with rat's code, as shown in fig. 5. When the smears dried out in air, they were fixed by dipping the slides in 70% ethanol for 3-5 times. Then the fixed smears were stained with 5% methylene blue by flooding them with methylene blue solution for one minute, and then rinsing with tap water. After that the slides were air dried before being examined under the microscope.

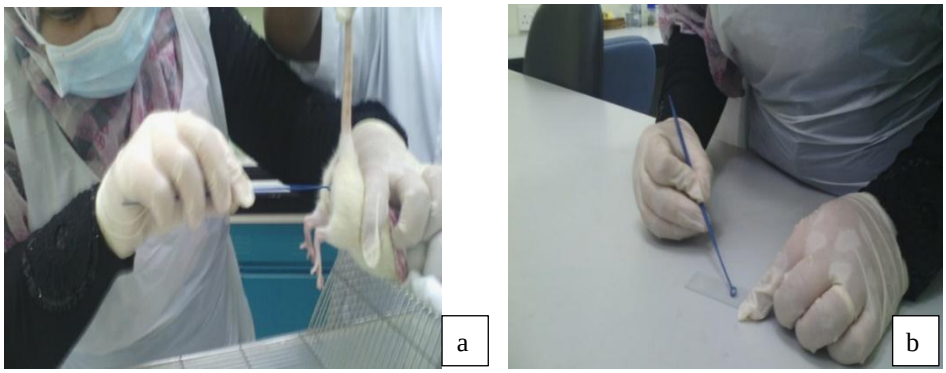

Figure 5: Collecting of vaginal fluid and cells (a), and applying the collected fluid onto a labelled slide (b).

2.3.2 Microscopic Examination

The dried slides were examined under a light microscope (Olympus, Model CX 31, Philippines). There are three types of cell in the

vagina of adult female rats: nucleated epithelial cells, non-nucleated cornified epithelial cells and leucocytes. These cells were identified in the smear by using x40 and x100 objective lenses. The phases of estrous cycle were determined according to the type of cells that were found in the smear.

2.4 Induction of Superovulation

After two weeks from the administration of fenugreek seed aqueous extract, the female rats in the three groups were treated with subcutaneous injection of recombinant human follicle-stimulating hormone (rhFSH) (GONAL-f, Serono, Italy) in the diestrus phase of estous cycle to induce the superovulation. To obtain the required doses of 2.5 IU, 1 IU and 0.5 IU rhFSH needed in this study, the medicine was diluted with sterile water for injection. In the original medicine 300 IU/ 0.5mL. To dial up to the needed dose of medicine, a cap at end of the pen was turning, 50 IU from the drug was obtained, and diluted in 5ml of water for injection, thus each 1ml contained 10 IU rhFSH. Hence, 0.25 ml contained 2.5 IU rhFSH, 0.1 ml contained 1 IU rhFSH and 0.05 ml contained 0.5 IU rhFSH. After finishing the first 5ml, another 5ml was prepared and so on.

The vaginal smear was taken in the early morning. Once it was confirmed that the rat was in the metastrus (Diestrus I) phase, superovulation was induced. The rats were injected with decreasing doses of rhFSH (10), two doses per day for three sequential days of diestrus phase. In diestrus I the rats were injected with 2.5 IU of rhFSH at 10.00 h, and 1 IU of rhFSH at 17:00 h. In diestrus II, the rats were injected with 1 IU of rhFSH at 10.00 h, and 0.5 IU at 17.00 h. Finally, in dioestrus III,

the rats were injected with 0.5 IU of rhFSH at 10.00h, and 0.5 IU at 17.00h.

The sacrificed rat was laid down on its back and a longitudinal abdominal incision was made. The abdominal contents were removed to reach the uterus and the ovaries as shown in fig. 6.

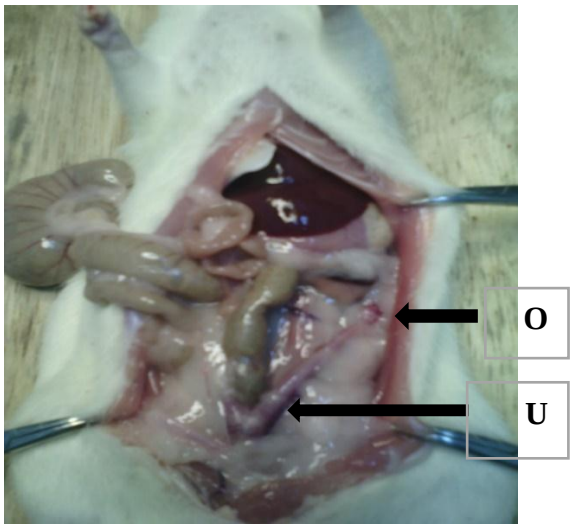

Figure 6: Dissection of a rat to expose the uterus and ovaries, U is the left horn of uterus, and OV is the left ovary at the upper end of uterus.

Both right and left ovaries were dissected and removed from each sacrificed rat. The ovaries were then cleared from adherent surrounding tissues. Then they were immediately put in a labelled coded container filled with 10% formalin as a fixative.

2.5 Histology of The Ovaries

2.5.1 Preparation

The ovaries were carried to the Histology Laboratory in Faculty of Medicine, IIUM. Each ovary was sectioned into two halves, and one half was placed in a histology cassette labelled with the relevant rats' code.

Thus, in this research the histology of both right and left ovaries of each rat was studied.

2.5.2 Tissue Processing

Tissue processing was done using an automated tissue processor (Leica Tissue Processor, Model TP1020, Singapore). The tissue cassettes were placed in the processor at 12.30 pm and the process completed next day at 10.30 am. In this machine, the tissues were sequentially passed through various chemicals required for, fixation, dehydration, clearing and finally paraffin infiltration.

Tissue processing started with fixation, where the ovaries were put in 10 % formalin as a fixative. The goal of fixation is to prevent any further changes that might happen on tissue protein. Then the tissue cassettes were passed in the processor through several increasing concentrations of alcohol; 50%, 70%, 80%, 95% and 100%. These alcohol concentration for dehydration. Dehydration of tissue is necessary because the paraffin is immiscible with water.

After that, the dehydrant was removed from the tissue by xylene which miscible with paraffin. This process is called clearing. Finally, the clearing agent (xylene) was replaced by paraffin. The paraffin infiltration is the last step in tissue processing.

2.5.3 Embedding

After the tissue was fixed, dehydrated, cleared and filtrated with paraffin in tissue processing, it was embedded in paraffin wax. The embedding was done using embedding machine (Leica, Model EG 1160, Singapore).

The tissue was removed from the cassette and placed in a metal block half filled with hot liquid paraffin, with the inner surface of the half

ovary facing downward. Then the block was completely filled with paraffin wax. The paraffin blocks were let to be solidified by placing them on the icy section of the embedding machine. The hardened paraffin blocks containing the tissue samples were then removed from the metal block for sectioning.

2.5.4 Tissue Sectioning

For sectioning, a rotatory microtome (Leica, Model RM 2245, Singapore) was used consisting of three parts; microtome body, knife attachment and knife, and tissue holder. The paraffin block was placed on the tissue holder part of microtome, and the knife was placed in the knife attachment. The cutting operation is achieved by the rotatory action of a hand wheel that makes the block moves up and down in a vertical plane and towards the knife. Before sectioning, trimming was done. The aim of trimming is to expose a required area of the tissue sample, so it can be then sectioned. The thickness of trimming sections was 20 microns, while those for microscopy were only 5 microns thick.

2.5.5 Preparation of the Slides for Staining

The sections then were put in a water bath (Leica, Model HI 1210, Singapore), adjusted at 45°C, to make the paraffin wax and the tissue sections stretch to remove any wrinkles. Then a labelled glass slide was dipped into the water bath to pick up the sections, this is called fishing.

Then the slides were placed on a hot plate (Leica, Model HI 1210, Singapore), adjusted to 56°C, to make the section adhere onto the glass surface. When the slides became completely dry, they were put in an oven (Memmert, 100-800, Germany), adjusted to 56°C to confirm the adherence of the section onto the slide. Next day, the slides were taken out from the oven and stained.

2.5.6 Staining of the Slides with Hematoxylin and Eosin Stain

In hematoxylin and eosin stain, the sectioned tissues on the slides were passed through many jars filled with various chemicals. First chemical was xylene, where it replaces the paraffin in the tissue. Then the tissue sections were rehydrated by sequentially exposing them to decreasing concentrations of alcohol (90 %, 80 %, 70 % and 50 %).

After that, the tissue sections were flooded with hematoxylin solution followed by eosin as a counter stain. An increasing concentration of alcohol (70 %, 80 % and 90 %) were then used for dehydration. To confirm the dehydration the slides were put in an oven for 10 minutes then put again in xylene as a last step. Finally, wearing nitrile gloves and in a fume cupboard the H&E stained tissue samples were properly mounted in DPX resin (distyrene, plasticizer and xylene) and allowed to dry.

2.5.7 Microscopic Examination

The stained sections from right and left ovaries of each rat were examined using (Olympus, Model CX 31, Japan) light microscope. The antral, and atretic follicles as were counted. Any follicle having antrum, small or large, even in the pre-ovulatory stage was considered as an antral follicle. The follicles which had any of apoptotic changes in granulosa cells and the oocyte were considered as atretic follicles. Other morphological changes were also studied.

2.8 Statistical Analysis

The obtained data, number of ovarian antral and atretic follicles were presented as mean ± standard deviation (SD) for the number of animals in each group (n = 8). Statistical differences between the control and treated groups were determined by using one-way analysis of

variance (ANOVA) followed by Post Hoc (Tukey) test, and considered statistically significant when the p-value was less than 0.05.

3. Result

3.1 The Effect of Fenugreek Seed Aqueous Extract on The Quantification of Ovarian Follicles

The counting of ovarian follicles in the stained sections of ovaries was according to their morphological appearance under microscope. The following figures (7-11) show the different ovarian follicles stained with H& E stain.

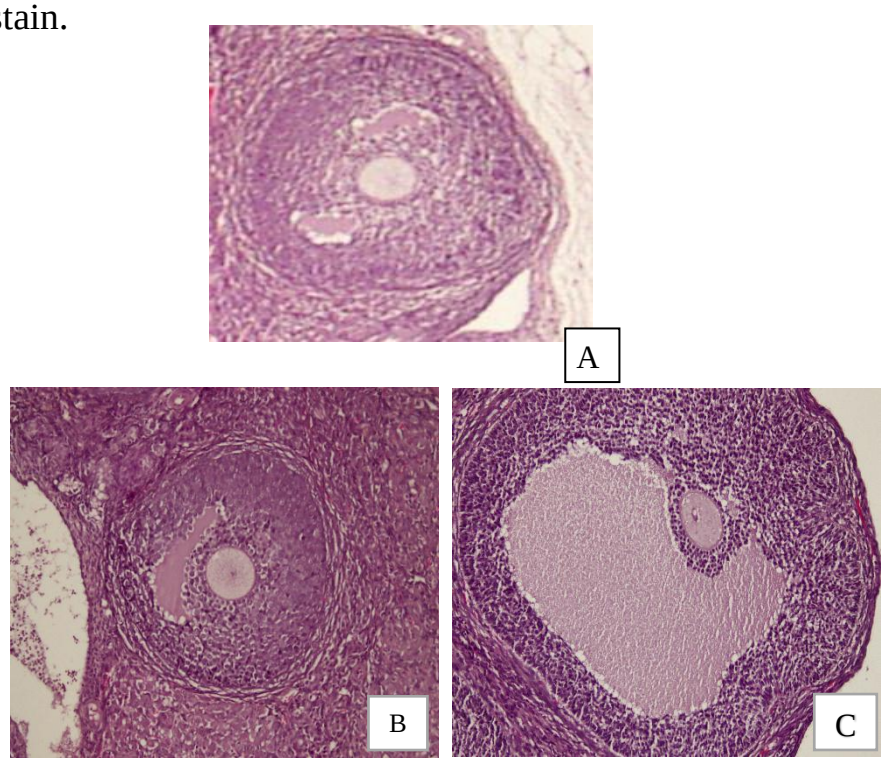

Figure 7: Photomicrograph of different morphological stages of antral follicles. A; early-antral follicle with small areas (vesicles) of antral fluid. B; antral follicle with large one antrum contains antral fluid. C; pre-ovulatory follicle, where the granulosa cells which surrounded the oocyte are almost separated from the other granulosa cells. (H & E, x200)

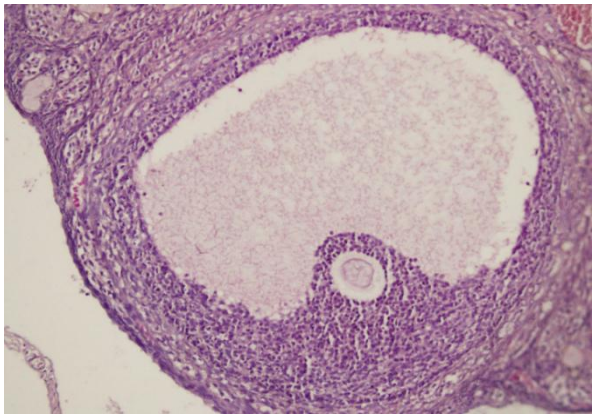

Figure 8: Photomicrograph of section of female rat's ovary shows an antral follicle undergoes atersia (atretic follicle) with apoptotic changes in granulosa cells, antral fluid and shrunk oocyte. (H&E x200).

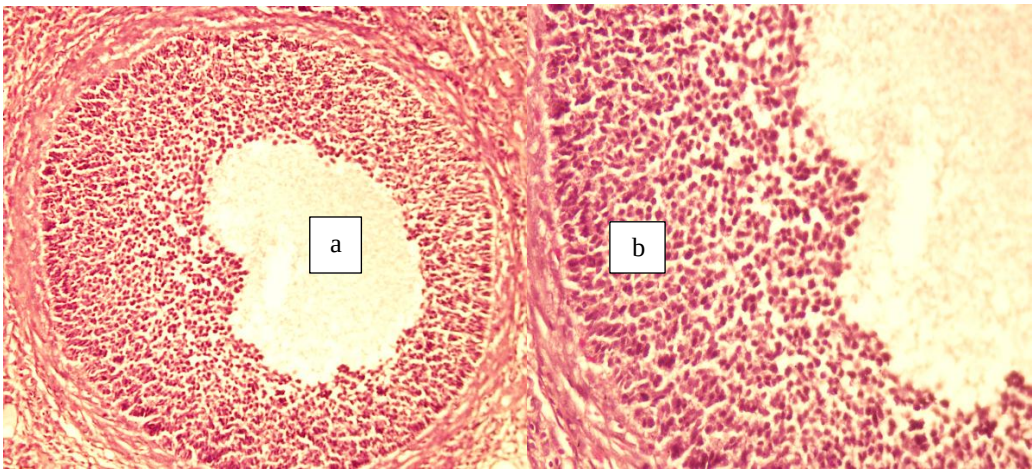

Figure 9: Photomicrograph of section of ovary of female rat from treated group B, shows an antral follicle undergoes atersia (atretic follicle) with apoptotic changes (a), x200 and (b) shows the shrinking of granulosa cells x 400. H&E.

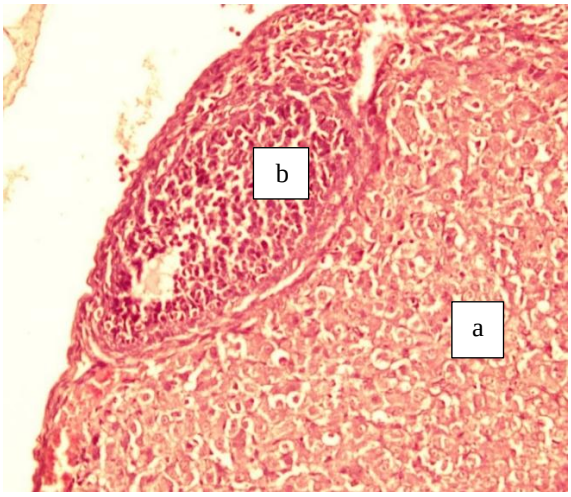

Figure 10: Photomicrograph of ovarian section of female rat from treated group B, shows the difference between the normal granulosa cells from corpus luteum (a), and shirked apoptotic granulosa cells (b) of atretic follicle. H&E, x 400.

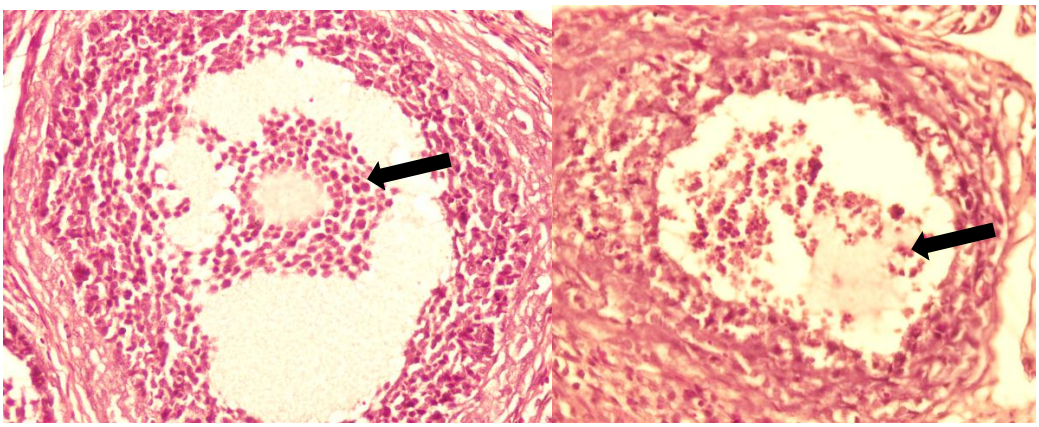

Figure 11: Photomicrograph of ovarian section of female rat from treated group C, shows degeneration of oocytes of atretic follicles, arrow indicate the oocytes. H&E, x400.

The rats in the three groups were sacrificed in the proestrous phase of estrous cycle which is the time of ovulation. Because of that some ovarian sections of the rats in control group shows oocytes in the ovarian

ducts as shown in fig. 12. However, that does not find in any ovarian sections of the two treated groups.

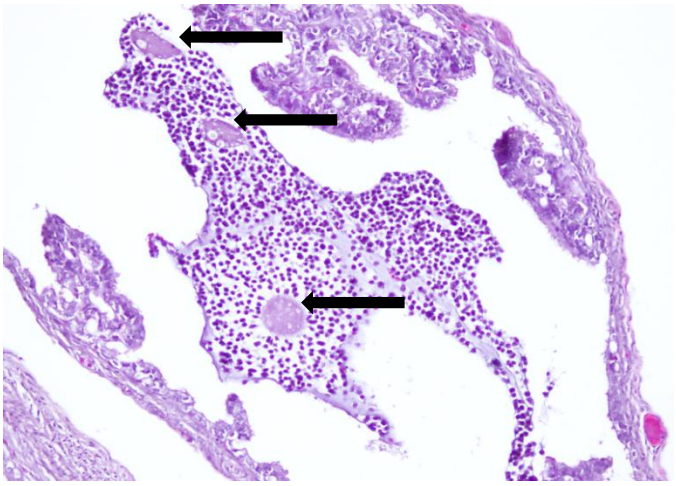

Figure 12: Photomicrograph of an ovulated oocyte in fallopian tube of female rat in control group, arrows indicate oocytes surrounded by layers of granulosa cells. (H & E x200).

3.1.1 The Effect of Fenugreek Seed Aqueous Extract on the Quantification of Antral Follicles

There was a significant (p-value = 0.005) reduction in the number of antral follicles in both treated groups in compared to control group, as shown in figure 13.

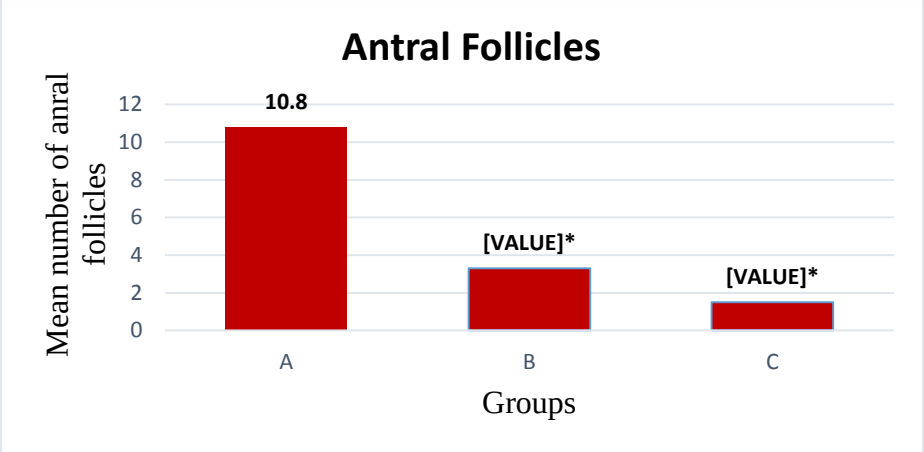

Figure 13: The effect of fenugreek aqueous extract on the number of antral follicles. Labelled values are mean for n = 8. (*) the values for the two treated groups (B and C) are statistically significant different compared to control (A)

The number of antral follicles in group C (treated with 1000mg/kg) was lower than in group B (treated with FAE 500mg/kg). However, Table 1 shows that this difference in the mean of antral follicle's number between the two treated groups B and C was not statistically significant, p value was 0.439 (>0.05). Therefore, the decrease of antral follicle's number in treated groups is dose independent as the secondary follicle's number.

Table 1: The mean difference of number of antral follicles between the three groups

Groups	Groups	Mean Difference	Std. Error	Sig.
Control	FAE 500mg/kg	7.50000*	1.40153	.000
	1000mg/kg	9.25000*	1.40153	.000
FAE 500mg/kg	Control	-7.50000*	1.40153	.000
	FAE 1000mg/kg	1.75000	1.40153	.439
FAE 1000mg/kg	Control	-9.25000*	1.40153	.000
	FAE 500mg/kg	-1.75000	1.40153	.439

. (*) the mean difference is statistically significant between the two current groups, (p<0.05).

3.1.2 The Effect of Fenugreek Seed Aqueous Extract on the Quantification of Atretic Follicles

The mean number of atretic follicles was higher in the two treated groups B and C in compared to the control group, as shown in fig. 14 This increase of atretic follicles' number was statistically significant, p-value = 0.005.

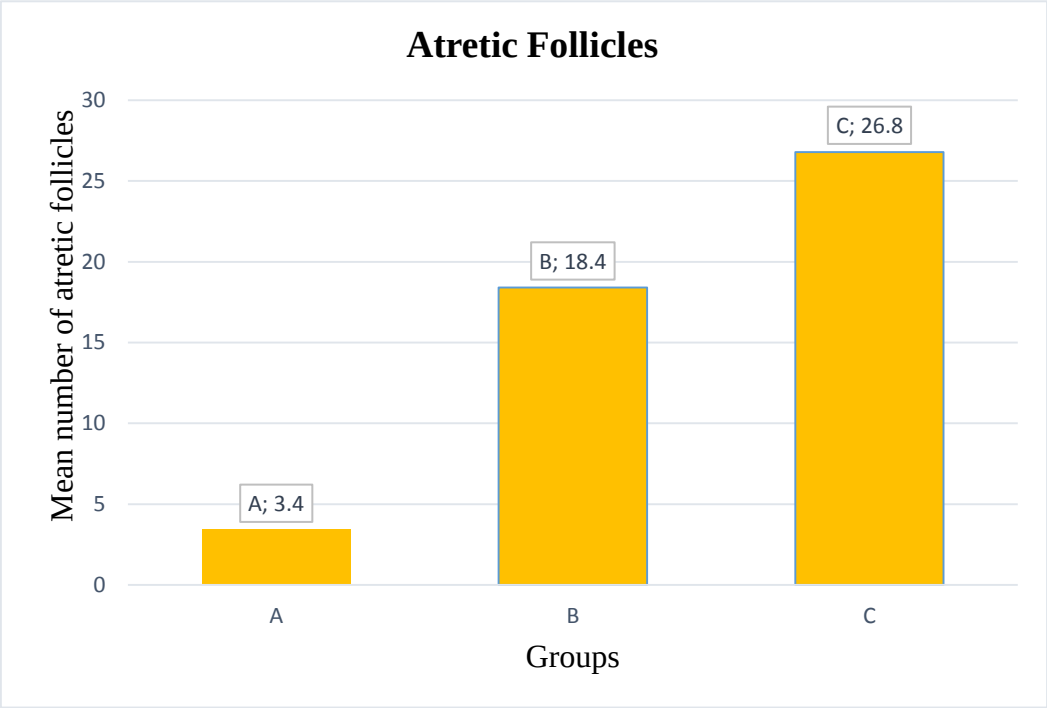

Figure 14: The effect of fenugreek seed aqueous extract on the number of atretic follicles. Values are mean for n = 8. (*) means the values of two treated groups (B and C) are statistically significant different compared to control (A), p value < 0.05.

In addition, by using Tukey test, the difference of atretic follicle's number between the two treated groups was statistically significant (p = 0.01). The atretic follicle's number in group C (treated with

1000mg/kg FAE) was significantly higher than in group B (treated with 500 mg/kg FAE), as shown in table 2.

Table 2: The difference of the mean of atretic follicles' count between the three groups.

Groups Name	Groups Name	Mean Difference	Std. Error	Sig.
Control	FAE 500mg/kg	-15.00000*	2.55243	.000
	FAE 1000mg/kg	-23.37500*	2.55243	.000
FAE 500mg/kg	Control	15.00000*	2.55243	.000
	FAE 1000mg/kg	-8.37500*	2.55243	.010
FAE 1000mg/kg	Control	23.37500*	2.55243	.000
	FAE 500mg/kg	8.37500*	2.55243	.010

(*) the mean difference between the two current groups is significant (p<0.05).

4. Discussion

This study was conducted to study the effect of fenugreek seed aqueous extract on the folliculogenesis in super-ovulated female rats. Female rodents have been used in scientific studies as a model for reproductive function in women since long time.

Ovarian follicle count gives important information on the function of ovaries, specially the folliculogenesis and its regulatory factors (14). In

this study, the count of antral and atretic ovarian follicles represents the effect of aqueous extract on folliculogenesis as well as superovulation.

In the present study, the histological study of rats' ovaries has shown a significant decrease in the number antral follicles after the administration of the fenugreek seed aqueous extract for 14 days. On the other hand, a significant increase in the number of atretic follicles was found after the administration of the extract for same period. This means the fenugreek seed aqueous extract caused disruption in folliculogenesis. The growth and differentiation of ovarian follicles from primordial follicles till pre-ovulatory follicles are stimulated and controlled by gonadotropins, mainly FSH, and interstitial ovarian factors (15). Therefore, this disruption of folliculogenesis by the fenugreek seed aqueous extract might be due to its effect on gonadotropins or on local ovarian growth factors. The fenugreek seeds have an effect on reproductive hormones, where it was found to cause decrease in FSH blood levels (13), but in the present study the exogenous rhFSH was given to induce the superovulation. Therefore, the effect of the aqueous extract on hormonal factors (FSH) that regulate the folliculogenesis can be excluded. This suggests that the fenugreek seed aqueous extract has a direct effect on ovaries, and it disrupts the folliculogenesis by affecting on the ovarian growth factors.

The increase of atretic follicles was significantly higher in the treated group with high dose of fenugreek seed aqueous extract. Steroidal saponins in fenugreek seeds have high ability to dissolve in water. Thus, the presence of steroidal saponin particularly diosgenin in fenugreek seeds aqueous extract can be considered as a cause of this significantly increase in atretic follicles. In 2010, a study also found that the aqueous extract of fenugreek seeds has teratogenic and foetotoxic effect in mice

(16). Accordingly, the present study suggests that these compounds could be responsible for the disruption of folliculogenesis and the significant increase in atretic follicles' number, and it might be considered as a reproductive toxicity of the fenugreek seeds aqueous extract on the ovaries.

5. Conclusion

The present study showed that both doses of fenugreek seed aqueous extract, 500 and 1000 mg/kg body weight, have an effect on the folliculogenesis in super-ovulated female rats.

The administration of fenugreek seed aqueous extract resulted in reduction in the number of antral follicles, and increase in the atretic follicles' number. In conclusion, fenugreek seed aqueous extract suppresses the growth of ovarian follicles in super-ovulated female rats.

Doses of 1000 mg/kg of the extract caused significant increase in atretic follicles' number compared to 500mg/kg of the extract, thus a 90-days study is recommended to study the possible reproductive toxicity effect of 1000 mg/kg of fenugreek seed aqueous extract. As well, further ultra-structural and immune-cytochemical studies are needed to explain the mechanism of action of fenugreek seed aqueous extract on the ovaries.

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