

EFFECT OF AQUEOUS GARLIC EXTRACT ON ANDROGEN INDUCED CHANGES IN OVARIES OF PREPUBERTAL FEMALE ALBINO RATS

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ABSTRACT

Background and Objectives: Administration of exogenous androgens to immature female rats had been reported to produce polycystic ovaries. The current study is designed to evaluate these effects and to assess the protection provided by aqueous garlic extract (AGE).

Methods: Fifty prepubertal female rats of age 21 days were divided into five groups A, B, C, D and E. Group A served as control and received 5 ml/kg/day of propylene glycol subcutaneous for 14 days. Group B received testosterone propionate (TP) 10 mg/kg/day dissolved in 5 ml/kg of propylene glycol subcutaneous for 14 days. Group C received TP 10 mg/kg/day dissolved in 5 ml/kg of propylene glycol subcutaneous and concomitantly AGE 200 mg/kg/day orally for 14 days. Group D received TP 10 mg/kg/day dissolved in 5 ml/kg of propylene glycol subcutaneous for 14 days and, aqueous garlic extract 200 mg/kg orally from day 14 – 21. Group E received TP 10 mg/kg/day dissolved in 5 ml/kg of propylene glycol subcutaneous for 14 days with no intervention till day 21. Animals of group A, B and C were sacrificed at day 15 and of group D and E at day 22, ovaries were removed and examined.

Results: There was no significant difference in gross parameters of the ovaries between control and experimental groups; however weight and volume of ovaries were significantly increased in group B. Histological sections stained with H&E, showed significant increase in number of large cystic and antral follicles in groups B and C respectively; however the number and size of cystic follicles were reduced after treatment with AGE.

Conclusion: The results showed that AGE prevents and decreased the number and size of cystic follicles in androgen treated ovaries of immature rats.

Key words: Androgens, immature rats, aqueous garlic extract, ovaries, follicle.

INTRODUCTION

Recent studies in human and non human primates suggest that elevated androgens play an important role in the development of conditions resembling polycystic ovarian syndrome (PCOS).¹ Early exposure of androgens either due to environmental or genetic factors in young females can cause polycystic ovarian syndrome (PCOS) like symptoms, which exhibits an increase in the number of growing preantral and antral follicles and an arrest of growth of follicle in mid-antral follicle stage, leading to antrum expansion, increased granulosa cell degeneration and, development of cystic follicles with thin granulosa cell walls.^{2,3} Regulation of androgen concentration in the ovaries is necessary for the normal ovarian function, when they are in excess they interfere in the process of maturation of follicles, preventing the recruitment of late antral follicle, leading to follicular atresia.⁴

Rodent models of polycystic ovaries have shown many characteristics similar to that in the human polycystic ovarian syndrome (PCOS) which includes hyper-

androgenism, disrupted cyclicality, presence of follicular cysts/polycystic ovaries.⁵ Different androgens like Dihydrotestosterone (DHT), estradiol-valerate, dehydroepiandrosterone (DHEA), and testosterone were used to develop polycystic ovaries in rodents.^{5,6} In this study we used Testosterone propionate (TP) to develop a rodent model of the condition.

The effect of testosterone propionate during fetal and neonatal life on ovaries and also in prepubertal and adult female rats have been studied and reported that it resulted in a significant increase in body weight; the total number of follicle was unchanged, but there was significant increase in activating transitory follicles and a decrease in small antral follicles.⁷ Testosterone propionate also induced granulosa cell atresia in advanced follicles; it also resulted in underdeveloped streak ovaries in both prepubertal and adult rats.⁷

In another study histological observations of folliculogenesis and follicular atresia in immature female rats (0-35 days old) were studied; after day 30 numerous primary follicles and atretic follicles were obser-

ved in the ovaries, antrum grew larger in the follicles where oocyte disappeared and granulosa cell layer became thin.⁸

Oxidative stress (OS) plays an important role in the pathophysiology of polycystic ovarian syndrome (PCOS). Antioxidants in the body help to modulate the effect of reactive oxygen species (ROS) by preventing the oxidative stress. *Allium Sativum* (Garlic) is one of the herbs which are used in daily food in Asian countries, whether in raw or cooked form. Raw garlic homogenate is the major preparation of garlic that is used for intensive scientific studies, as it is the usual way of garlic consumption. Garlic has been shown to have many of the medicinal properties including anti-thrombolytic,⁹ cancer preventive¹⁰, cardioprotective effect,¹¹ and its antioxidant. It is very effective in treating many conditions regarding male reproductive system. The effect of garlic produced in male reproductive system on administration of androgen had been extensively studied¹² but reports on its manifestations upon female reproductive system treated with androgens are yet to be established. This study is, therefore, designed to evaluate the effect of aqueous garlic extract on Testosterone propionate (TP) induced changes in ovaries of prepubertal female rats.

ANIMALS AND METHODS

50 Female prepubertal albino rats, 21 day of age and weighing 40-50gms were obtained from the colony raised in the Animal House, University of Health Sciences, Lahore and were housed under controlled temperature of $25^{\circ} \pm 2^{\circ}\text{C}$, humidity 55 ± 5 and light and dark cycles of 12 hours each. The animals were fed on

standard rat diet and tap water *ad libitum*. The experiment was carried out in accordance with the instructions and guidelines of Ethical Committee of UHS. The animals were randomly divided to five groups, A, B, C, D and E using balloting method containing 10 animals each.

Groups of Experimental Animals

Group A was control and was given 5ml/kg body weight of propylene glycol subcutaneously daily for 14 days.

Group B was given TP 10mg/kg/day subcutaneously for 14 days, dissolved in 5ml/kg of propylene glycol.

Group C was given TP 10mg/kg/day subcutaneously for 14 days, dissolved in 5ml/kg of propylene glycol and concomitantly AGE 200mg/kg/day orally for 14 days.

Animals of group A, B, and C were sacrificed on day 15 of the experiment.

Group D was given TP 10mg/kg/day subcutaneously for 14 days, dissolved in 5ml/kg of propylene glycol and AGE 200mg/kg was given orally from day 14 to 21; animals were sacrificed on day 22.

Group E was given TP 10mg/kg/day subcutaneously for 14 days, dissolved in 5ml/kg of propylene glycol, no intervention from day 14-21; animals were sacrificed on day 22.

Chemical

Testosterone propionate was purchased from Ipca Laboratories Ltd., Batch No.4002 TH1RN, India.

The dose and method of preparation of aqueous garlic extract was adopted from the earlier work.¹²

Table 1: Showing Intervention and Dosage Schedule.

Groups N = 10	Intervention/Treatment	Route of Administration	Duration	Sacrificed
Group A	Propylene glycol 5ml/kg	Subcutaneous	Day 1 to 14	On day 15 th of experiment
Group B	Testosterone propionate 10mg/kg dissolved in 5ml/kg propylene glycol	Subcutaneous	Day 1 to 14	On day 15 th of experiment
Group C	Testosterone propionate 10mg/kg dissolved in 5ml /kg propylene glycol	Subcutaneous	Day 1 to 14	On day 15 th of experiment
	Aqueous garlic extract 200mg/kg	Oral	Day 1 to 14	
Group D	Testosterone propionate 10mg/kg dissolved in 5ml/kg propylene glycol	Subcutaneous	Day 1 to 14	On day 22 st of experiment
	Aqueous garlic extract 200mg/kg	Oral	Day 14 to 21	
Group E	Testosterone propionate 10mg/kg dissolved in 5ml/kg propylene glycol	Subcutaneous	Day 1 to 14 (Animals were sacrificed a week later on day 21.	On day 22 st of experiment

Similarly the dose of TP was derived from the work reported earlier.¹³ Doses were adjusted according to the weight of the animals.

On day 15 of the experiment, animals of group A, B and C were weighed and then sacrificed under anesthesia. After assuring all the aseptic measures, abdomen was opened and ovaries were identified, exposed, removed and weighed, using digital balance (Sartorius, model TE-214-S). Volume of the ovaries was determined by using indirect water displacement method, and these were washed with normal saline, and fixed in 10% formalin for 48 hours. The tissue was then processed in the automatic tissue processor and paraffin blocks were prepared. Sections of 4µm thickness were obtained using rotary microtome (Leica RM 2125). Animals of group D and E were sacrificed on day 22 of the study and their ovaries were processed similarly.

Histological Examination

The slides, after staining with Haematoxylin & Eosin, were examined under a light microscope (Lieca DM 1000) using objective of X10. Sections were examined by using calibrating ocular graticule for the number and diameter of primary, antral and atretic follicles and number and size of the ovarian cysts, by the method described earlier.¹⁴ Follicles were counted and classified according to a previously published method.¹⁵

Statistical Analysis

The data was entered and analyzed by using SPSS 20.0. Mean \pm SD were given for the quantitative variables. One way ANOVA was applied to compare means of variables among the control and experimental groups. Post hoc Tuckey test was applied to compare the means of groups. Chi-square test was applied to categorical variables. P-value \leq 0.05 is considered statistically significant.

RESULTS

Gross Parameters: The mean weight of both ovaries of groups A, B, C, D and E was 0.035 ± 0.008 , 0.072 ± 0.018 , 0.037 ± 0.008 , 0.039 ± 0.015 and 0.033 ± 0.006 gm respectively. One way ANOVA showed the difference was statistically significant ($< 0.001^*$) when groups A, B, C, D and E were compared among one another (Table 2). Post hoc Tukey's test showed that mean value of combined weight of both ovaries in group B was statistically significant (p-value $< 0.001^*$), when compared with the mean values of groups A, C, D and E.

The mean value of volume of combined ovaries of groups A, B, C, D, and E was 0.045 ± 0.014 , 0.082 ± 0.033 , 0.047 ± 0.016 , 0.051 ± 0.025 and 0.039 ± 0.010 respectively. One way ANOVA test showed the difference was statistically significant when mean value of combined ovaries ($< 0.001^*$) of groups A, B, C, D and E were compared among the groups (Table 3). Post hoc Tukey test showed that the difference in the mean value of combined volume of both ovaries in group B was statistically significant, when compared with the mean values of groups A, C, D and E.

Histological Parameters

Primary Follicles

One way ANOVA test showed the difference was statistically significant when mean value of diameter of primary follicles ($< 0.001^*$) of group A, B, C, D and E were compared among one another. However, mean of number of primary follicles, when compared among one another showed no significant difference ($= 0.180$) (Table 4, Fig. 1, 2, 3, and 4). Post hoc Tukey's test showed that the difference in the mean value of diameter of primary follicles in group C and E was statistically significant, when compared with the mean value of groups A and B respectively.

Table 2: Showing comparison of mean weight of combined ovaries between groups A, B, C, D, and E.

Parameter	Group A Mean \pm SD	Group B Mean \pm SD	Group C Mean \pm SD	Group D Mean \pm SD	Group E Mean \pm SD	p-value
Combined weight of ovaries (gm)	0.035 ± 0.008	0.072 ± 0.018	0.037 ± 0.008	0.039 ± 0.015	0.033 ± 0.006	$< 0.001^*$

*p \leq 0.05 is considered statistically significant.

Table 3: Showing comparison of mean values of volume of combined ovaries between groups A, B, C, D and E.

Parameter	Group A Mean \pm SD	Group B Mean \pm SD	Group C Mean \pm SD	Group D Mean \pm SD	Group E Mean \pm SD	p-value
Volume of Ovaries (ml)	0.045 ± 0.014	0.082 ± 0.033	0.047 ± 0.016	0.051 ± 0.025	0.039 ± 0.01	$< 0.001^*$

*p \leq 0.05 is considered statistically significant.

Table 4: Experimental strategy on the effect of Testosterone propionate and Aqueous Garlic Extract on the ovaries of Immature (21 days old) rats. Values given are mean \pm SD.

Parameters		Group A n = 10	Group B n = 10	Group C n = 10	Group D n = 10	Group E n = 10	p-value**
Primary Follicles	Number of Primary Follicles	9.800 \pm 5.07	12.100 \pm 6.78	15.200 \pm 6.08	10.070 \pm 6.53	8.500 \pm 6.94	0.180
	Diameter of Primary Follicles	17.085 \pm 2.52	16.349 \pm 3.36	14.120 \pm 1.58	15.093 \pm 1.72	12.953 \pm 1.78	< 0.001*
Antral Follicles	Number of Antral Follicles	8.500 \pm 3.13	9.501 \pm 1.77	9.700 \pm 1.88	7.400 \pm 2.31	4.400 \pm 1.89	< 0.001*
	Diameter of Antral Follicles	172.432 \pm 34.65	257.321 \pm 72.83	203.471 \pm 33.62	229.380 \pm 44.75	181.810 \pm 60.70	0.004*
Atretic Follicles	Number of Atretic Follicles	4.000 \pm 1.49	4.000 \pm 1.33	4.200 \pm 1.22	4.300 \pm 1.56	4.400 \pm 1.64	0.962
	Diameter of Atretic Follicles	219.401 \pm 30.36	231.720 \pm 55.63	198.801 \pm 27.84	185.201 \pm 32.70	148.140 \pm 42.75	< 0.001*
Cysts	Number of Cyst	0.000 \pm 0.00	3.600 \pm 1.07	3.100 \pm 0.99	2.600 \pm 0.51	0.900 \pm 0.73	< 0.001*
	Diameter of Cyst	0.000 \pm 0.00	299.261 \pm 70.97	128.920 \pm 25.17	140.072 \pm 50.70	117.350 \pm 91.16	< 0.001*

**Statistics according to single factor ANOVA.

Antral Follicles

One way ANOVA test showed the difference was statistically significant when mean value of number of antral follicle (<0.001*) and diameter of antral follicles (0.004*) of groups A, B, C, D and E were compared among one another (Table 4, Fig. 1, 2, 3, 4 and 5). Post hoc Tukey's test showed that mean value of number of antral follicles in group E was statistically significant, when compared with the mean value of groups A, B and C respectively, and the mean value of diameter of antral follicles in group B was statistically significant (p-value 0.005*) and when compared with the groups A and E the p-value is 0.017*.

Atretic Follicle

One way ANOVA test showed the difference was statistically significant when mean value of diameter of atretic follicles of groups A, B, C, D and E was compared among one another, the p-value was < 0.001*. However, mean value of number of atretic follicles, when compared with one another showed insignificant difference (p-value 0.962) (Table 4, Fig. 1, 2, 3, 4 and 5). Post hoc Tukey's test showed that mean value of diameter of atretic follicles in group E was statistically significant, when compared with the groups A, B

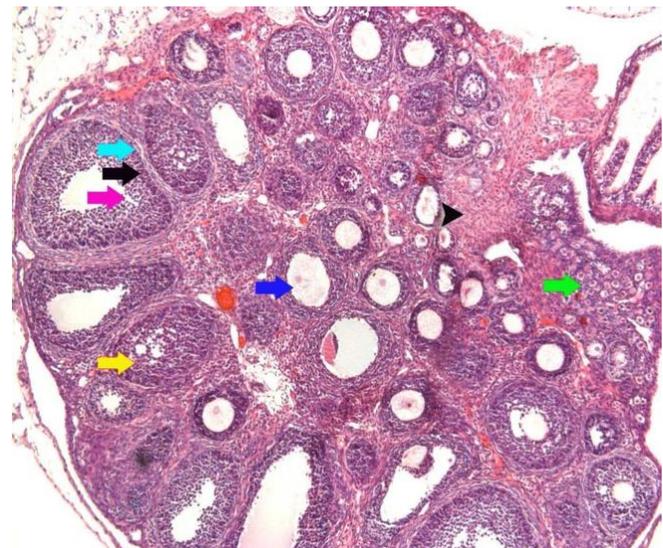


Fig. 1: Photomicrograph of ovary from group A (Control), showing follicles at various stages of development. Primordial follicle (light green arrow), Antral follicle (blue arrow), Atretic follicle (yellow arrow), Stroma (black arrow head), Granulosa cells (pink arrow), Theca interna (black arrow) and Theca externa (light blue arrow). H&E, 100X.

and C.

Cyst

One way ANOVA test showed the difference was statistically significant when mean values of number of cysts (< 0.001*) and diameter of cysts (< 0.001*) of groups A, B, C, D and E were compared among one another (Table 4, Fig. 2, 3, 4, and 5). Post hoc Tukey’s test showed that mean value of number of cysts in group E was statistically significant (p-value < 0.001*), when compared with the mean value of groups B, C and D. The mean value of size of cysts in group B (p-value < 0.001*) was statistically significant, when compared with the groups C, D and E.

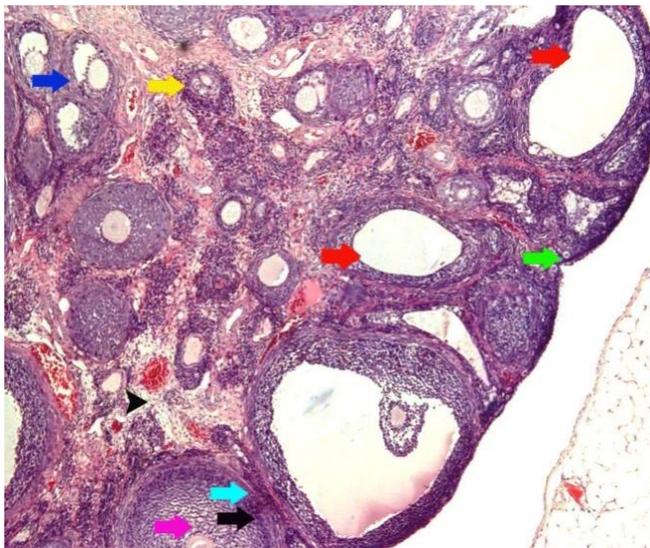


Fig. 2: Photomicrograph of ovary from group B (treated with TP for 14 days), showing large cystic follicles and other type of follicles at various stages of development. Primordial follicle (light green arrow), Antral follicle (blue arrow), Atretic follicle (yellow arrow), Stroma (black arrow head), Granulosa cells (pink arrow), Theca interna (black arrow), Theca externa (light blue arrow) and Cystic follicle (red arrow). H&E, 100X.

DISCUSSION

In the present study, the difference in the mean weight of ovaries single or in pair was statistically significant among various groups treated with TP and AGE for different durations. The mean weight of ovaries from group B which was given testosterone propionate was significantly higher when compared with groups A, C, D and E (p value = 0.001). These findings were in accord with those reported earlier.¹⁶ The reason for this increase in weight is insulin resistance and an increase plasma insulin concentration which were associated with hyperandrogenism, causing increase in weight and deposition of fat.¹⁷ Likewise, the volume of ovaries individually and both increased which was statistically

significant when compared among various groups. The mean volume of ovaries in group B was significantly

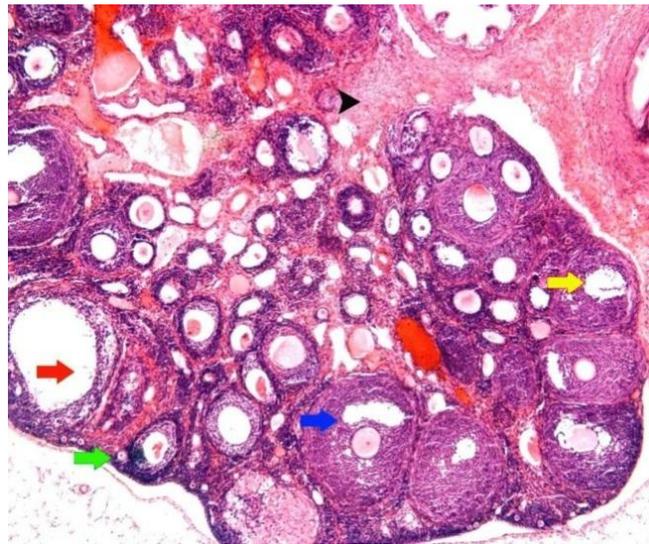


Fig. 3: Photomicrograph of ovary from group C (treated with TP 10mg/kg and AGE 200ml/kg daily for 14 days), showing large number of antral follicles with decrease in diameter as compared with other groups. Primordial follicle (light green arrow), Antral follicle (blue arrow), Atretic follicle (yellow arrow), Stroma (black arrow head), and Cystic follicle (red arrow). H&E, 100X.

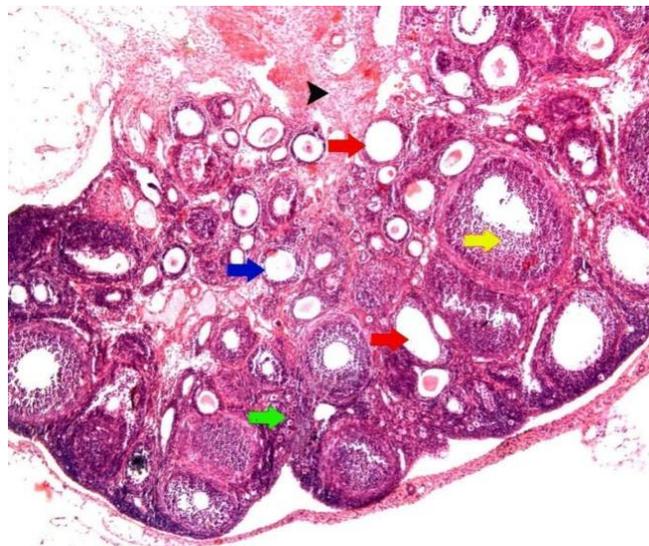


Fig. 4: Photomicrograph of ovary from group D (treated with TP 10mg/kg for 14 days, and AGE 200mg/kg from day 14-21), showing all types of follicles at various stages of development. Primordial follicle (light green arrow), Antral follicle (blue arrow), Atretic follicle (yellow arrow), Stroma (black arrow head), and Cystic follicle (red arrow), size of cyst is reduced as compared with those from group B. H&E, 100X.

higher when compared with groups A, C, D and E (p value = 0.001).

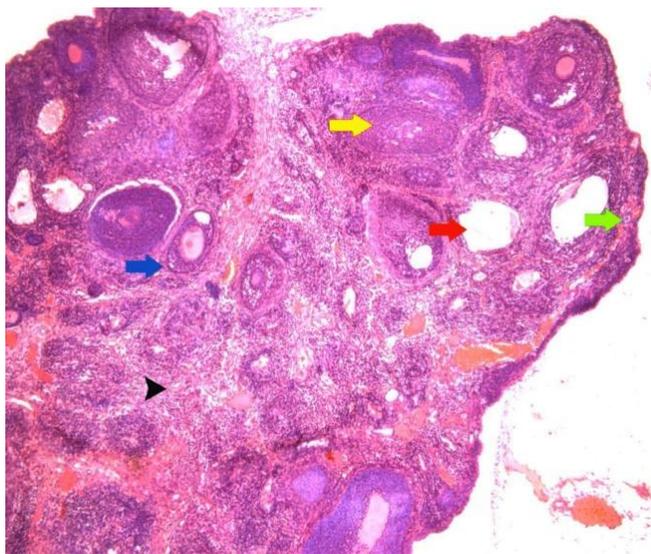


Fig. 5: Photomicrograph of ovary from group E (treated with TP 10mg/kg for 14 days, animals were sacrificed on day 22). Number of all types of follicles is reduced with increase in stroma, and number and size of cyst is decreased as compared to other groups. Primordial follicle (light green arrow), Antral follicle (blue arrow), Atretic follicle (yellow arrow), Stroma (black arrow head) and Cystic follicle (red arrow). H&E stain, 100X.

In this study, histological examination of the ovaries for the number of primary follicles showed statistically insignificant difference when the mean of number of primary follicles were compared among various groups. Androgens predominantly targeted granulosa cells, which showed that TP had no direct effect on the number of primary follicles, which neither increased nor decreased after the administration of TP. However, the mean diameter of primary follicles in group A (control) was significantly higher when compared with those from groups B, C, D and E (p value = 0.001).

The effect of testosterone and other androgens on the primate ovaries was studied and 10 days after treatment of animals showed that testosterone resulted in 4.5-fold increase in the number of follicles; cystic follicles were also seen both micro and macroscopically along with an increase in size of the ovaries.¹⁸ In the present study, after fourteen days of administration of TP in group B the number of antral follicles increased significantly (p value = 0.001), the mean diameter of antral follicles in group B was also significantly higher when compared among various groups (p value = 0.004). Group B also showed large cystic follicles along with large number of small and medium size dantral follicles. Many atretic follicles were also seen. This was presumably because androgens targeted gra-

nulosa cells where they initiated follicular growth and maturation and enhanced the number of follicles at various other stages of development. However, the mean number and diameter of antral follicles in group E significantly decreased when compared with other groups.

Comparative to this was group C, which was given TP and AGE concomitantly; it showed significantly increase in number of antral follicles when compared with other groups but their diameter was decreased; cystic follicles were also seen but their number and diameter was decreased as compared to the group B. This showed that the treatment with aqueous garlic extract had reduced the deleterious effects of TP.

The effect of testosterone propionate in prepubertal rats could be divided into two phases, in first phase (day 21 – day 28) there was formation of large cystic follicle and moderate atresia in preantral follicles; whereas in the second phase (day 28 onwards) progressive increase in atresia both in preantral and large cystic follicle were observed,¹⁵ results of current study were in accord with the same, where marked atresia and decrease in number and diameter of antral follicles was observed, the difference was statistically significant when groups D and E were compared with other group. The number of antral follicles in group D was comparatively more than those in group E, group D was treated with TP from day 1-day 14 (aged 21-35 days) and then AGE from day 15-day 21 (i.e animal aged 35-42 days). This showed that at this smaller dose (200 mg/kg), aqueous garlic extract can reverse the changes induced by TP. Present study, therefore **concludes** AGE administration showed beneficial effects on the histopathological changes induced upon administration of TP in immature female rats; it reduced the number and size of cysts significantly in the groups treated with AGE. It also reduced the atretic changes in the ovaries.

Authors' Contribution

YB: Concept, design of the work, data collection, data analysis and interpretation, drafting the article. MT: Concept and design of the work, critical revision of the article and final approval before submission for publication. KPL: Data interpretation and critical revision of the article.

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Conflict of Interest: None.

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