ORIGINAL ARTICLE

Effects of Simvastatin 20mg on the Histology of Albino Rat Ovary

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ABSTRACT

Background: Simvastatin is an effective hypocholesterolemic drug that inhibits cholesterol synthesis selectively in the liver but at the same time could have potential side effects on the adrenal glands, ovary and testis as these three glands use cholesterol for their hormonal biosynthesis.
Design: In this study 30 female albino rats were taken and randomly divided into three groups A, and B. Group A was designated as the control group whereas group B was the experimental group in which simvastatin was given in dosage of 20mg a total of 42 days.
Results: In the cortex the number of primordial follicles were decreased in the experimental group B (P-value <0.001), whereas, the secondary follicles showed a decrease in diameter in the same group. In the same way the Graafian follicles also showed a decrease in diameter which was 839µm in group A and 750µm group B. The corpus luteum showed an increased in size in the experimental group B. Congestion and dilatation was seen in group B. The lipid profile showed a decrease of more than 50% of total cholesterol and triglycerides in both the experimental groups (P-value <0.001). However LDL was decreased by approximately 40% but HDL was increased by 10%.
Conclusion: This drug as a hypocholesterolemic agent (statin) decreased the total serum cholesterol, triglycerides and LDL but it seems has an adverse effect on the morphology and histology of ovaries even when given for a short time in a dose of 20-mg/day.
Keywords: Ovary, albino rat, simvastatin

INTRODUCTION

Cholesterol is an apparently indispensable lipid for numerous process required for cell proliferation[1]. Cholesterol formed in cells called endogenous cholesterol is produced in greater quantity as compared to exogenous cholesterol. Essentially all the endogenous cholesterol that circulates in the lipoproteins of the plasma is formed by the liver, but all other cells of the body form at least some cholesterol[2]. The first two reactions in the cholesterol synthesis path way lead to the production of 3-hydroxy-3-methylglutaryl CoA (HMG CoA). The next step, the reduction of HMG CoA to mevalonic acid is catalyzed by HMG CoA reductase, and is the rate limiting step in cholesterol synthesis[3]. Simvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key enzyme in the synthesis of cholesterol[4]. Endogenous cholesterol acts as a precursor of testosterone and other steroid hormones. In a recent study variable doses of Simvastatin were given to normal male rats for 30 to 60 days. It was concluded that inhibition of denovo pathway of cholesterol biosynthesisss negatively affects testosterone levels[5].

As listed by various researches cholesterol is the precursor of glucocorticoids, mineralocorticoids and sex steroids, besides being a structural component of cell membrane. Adrenal, testicular and ovarian glands are composed of steroid secreting cells which secrete steroid hormones. These steroid secreting cells synthesize these hormones by utilizing cholesterol either in the form of LDL circulating in the blood or by denovo cholesterol synthesize which is controlled by HMG-CoA reductase enzyme. Since Simvastatin both decrease circulatory LDL and inhibit denovo cholesterol synthesis, they are likely to affect the synthesis of steroid hormones[6].

MATERIAL AND METHODS

In this experimental study 20 female albino rats of Wistar strain weighing between 200 and 250grams were used. All of these animals were kept in cages for 14 days in the animal house of Zoology Department, University of Punjab Lahore for the purpose of acclimatization. A twelve hour light and dark cycle was maintained at room temperature between 22ºC-25ºC. The food and water was provided to these animals ad libitum. The food given was in the form of chick feed No.1[7].

Experimental design: After 14 days the animals were randomly divided into two groups labeled as A,

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and B. Each group comprised of 10 animals. These rats were then marked with a permanent marker for identification and placed in their respective cages labeled with allocated tags. Blood was taken from each rat through cardiac puncture in which the needle of a 5cc syringe was introduced just behind the xiphoid cartilage of the sternum slightly left of the midline for lipid profile before administering the drug to obtain baseline values. Simvastatin (Simplacor, Novartis) 20mg was used in this study which was given orally to the rats through a 1cc insulin syringe on a daily basis.

Group A (Control): This was designated as the control group and comprised of 10 albino rats. These rats were not given any medication except for 1ml of distilled water via 1cc insulin syringe.

Group B (Experimental): This group consisted of 10 albino rats and were given Simvastatin 20mg/Kg(one 20mg tablet) dissolved in 1ml of distilled water on a daily basis for six weeks (42 doses) via insulin 1cc syringe.

Simvastatin tablets were removed from the blister pack and were placed in a small mortis. These tablets were then crushed to powder form using a small wooden crusher. The powder form of medicine was then transferred to a beaker and 1 ml of distilled water was added. Using a glass mixer the contents were mixed together until a homogenous mixture was made. This was then given to each of the experimental rat 1cc insulin syringe. The feed was given ad libitum for a total of 42 days.

RESULTS

Ovaries were removed and immediately fixed in 10% formalin solution. Tissue processing was carried out and paraffin tissue blocks of each ovary were made. Serial 5 micrometer tissue sections were cut by a rotary microtome and stained with haematoxyline & Eosin (H&E) stain. Stained sections were studied under the light microscope. The following histological parameters were studied and compared with the normal histological parameters of ovaries of the control group. The lining epithelium of the control group A was seen covering the outer aspect of the ovary. It was made up of cuboidal epithelium with central rounded nuclei (Fig 1).

The lining epithelium of experimental group B was also lined by cuboidal epithelium. Only places where the corpus luteum or large follicles reached the periphery, the epithelium appeared in segments of flat cells (Fig 2). Chi-square analysis between the control group A and group B was significant (P-value < 0.001) (Table 1). The capsule of the control group A was made up of a thin layer of dense connective tissue immediately underlying the lining epithelium (Fig-1). In the experimental group B 07 out of 10 animals had a thinner capsule as compared to the control group A. The histological examination of the capsule was statistically significant when the control group A was compared with the experimental group B (P-value < 0.001, Table 2).

Table 1: Association of lining epithelium of albino rats with drug dosages control & experimental groups

Groups	Lining epithelium		Total
	Normal	Abnormal	
A	10	0	10
B	3	7	10

Chi-Square 15.20 P-Value = 0.001

Pairwise comparison	Groups	Chi-sq	P-value
A	B	13.68	<0.001*

*Statistically significant difference (P<0.001).
†Statistically insignificant difference (P>0.001).

Table 2: Association of capsule of albino rats with drug dosages-control & experimental groups

Groups	Capsule		Total
	Normal	Abnormal	
A	10	0	10
B	3	7	10

Chi-Square 28.84 P-Value = <0.001

Pairwise comparison	Groups	Chi-Sq	P-value
A	B	13.68	<0.001*

*Statistically significant difference (P<0.001).
†Statistically insignificant difference (P>0.001).

The primordial follicles in group A were seen occupying the peripheral part of the cortex. Spherical oocytes were seen surrounded by flattened follicular cells embedded in stromal connective tissue (Fig-1). The primordial follicles in the experimental group B were decreased in number and somewhat smaller in appearance. The oocytes were also surrounded by flattened follicular cells as seen in the control group A. Statistical analysis between the control group A and experimental group B was significant (P-value < 0.001).

The secondary follicles of the control group A were occupying a deeper segment of the cortical stroma. The oocytes were spherical in shape, surrounded by polyhedral shaped granulosa cells with rounded to oval shaped centrally placed nuclei. These cells were lying on a thin basement membrane which separated them from the underlying theca interna cells. The cells of theca interna were variable, composed of spindle shaped to polygonal cells with oval central nuclei. In between these cells small blood vessels were observed. Irregular spaces were noted among cells of granulosa layers. These spaces were filled with follicular fluid (Fig-1).

The follicles of group B were centered by a rounded oocyte and occupied a deeper portion of the ovarian cortex. The granulosa cells surrounding the

oocyte were polygonal in shape with central rounded to oval shaped nuclei. As compared to the control group A more intercellular spaces were seen among the granulosa cells and the number of cell layers was also increased. A thin basement membrane separated granulosa cells from theca interna cells. The theca interna cells showed variation between polyhedral and squamous cells with rounded to plate like nuclei respectively. Small blood vessels were seen in between these cells. Theca interna blended with the surrounding theca externa (Fig-2). By using Chi-square the pair wise comparison of the secondary follicle in this group with the control group A showed atrophy (P-value <0.001, Table-3). The mean diameter of the secondary follicle in the control group A was 81.50 µm (±8.87) in group B it was 69.00 µm (±16.63). Pair wise comparison of control group A and experimental group B by using Tukey was significant (P-value <0.001, Table 4). These findings showed a decrease in size of the secondary follicle in group B when treated with simvastatin.

Table 3: Association of secondary follicle (primary oocyte & surrounding cells) of albino rats with drug dosages-control & experimental groups

Groups	Secondary Follicle (Primary Oocyte & Surrounding Cells)		Total
	Normal	Atrophy	
A	10	0	10
B	0	10	10

Chi-Square 38.19 P-Value = <0.001

Pairwise comparison	Group	Chi-sq	P-value
A	B	16.20	<0.001*

Statistically significant difference (P<0.001).
†Statistically insignificant difference (P>0.001).

Table 4: Diameter of secondary oocyte in mature follicle (µm) of albino rats-control & experimental groups

Group	Mean	Sd	Min	Max
A	81.50	8.87	70.00	100.00
B	69.00	16.63	50.00	100.00

ANOVA

Source	Sum of squares	Df	Mean square	F	Sig.
Between Groups	3251.667	2	1625.833	9.890	0.001
Within Group	4438.500	27	164.389		
Total	7690.167	29			

Pair-wise comparison between groups by using Tukey HSD

Group (I)	Group (J)	Mean difference(I-J)	Std. Error	Sig.
A	B	12.50000	5.73391	0.000*

SD:*Standard Deviation.,* ***F:****f-test (Ratio of variances),* ***DF:*** *Degree of Freedom*
*******Statistically significant difference (P<0.001).*
†*Statistically insignificant difference (P>0.001).*

In group A, the Graafian follicles were observed occupying a large part of the ovarian cortex and were near the surface of the ovary. The oocyte was eccentrically placed in the follicle. It was surrounded by 2-3 layers of granulosa cells forming corona radiata. The granulosa cells around the oocyte were multilayered and were thick as compared to the opposite wall. The basement membrane between granulosa and theca interna was not well defined. Theca interna showed more polygonal cells with rounded to oval shaped nuclei. The number of blood vessels increased between the theca interna cells. The theca interna blended with theca externa which was made up of flattened cells with plate like nuclei (Fig 1).

The mature follicle in the experimental group B showed the oocyte eccentrically placed surrounded by granulosa cells. The granulosa cells were multilayered around the oocyte which were slightly more as compared to the control group A. Towards the opposite pole, the granulosa cells were thicker which was again slightly more than the control group A. The demarcation between granulosa cell layers and theca interna was not clearly defined. The number of follicles was often seen increased with more infolding of cellular walls along with an increase in the number of cell layers. Theca interna cells were variable between polygonal and flattened cells with central oval to flat nuclei. Blood vessels were slightly more prominent in between theca interna cells as compared to the control. Flat cells of theca externa were seen surrounding the Graafian follicles (Fig-2&3). The Graafian follicles in this experimental group showed atrophy as compared to the control group A by Chi-square analysis (P-value <0.001) (Table 5). The mean diameter of the Graafian follicle in group A was 839.20µm (±68.51) whereas the mean diameter of Graafian follicles in group B was decreased as compared to the control group A 750.00 µm (±31.89).

Table 5: Association of Graafian follicle (secondary oocyte & surrounding cells) of albino rats with drug dosages-control & experimental groups

Groups	Graafian follicle (secondary oocyte & surrounding cells)		Total
	Normal	Atrophy	
A	10	0	10
B	0	10	10

Chi-Square 38.19 P-Value = <0.001

Pairwise Comparison	Groups	Chi-Sq	P-value
A	B	16.20	<0.001*

*****Statistically significant difference (P<0.001).
†Statistically insignificant difference (P>0.001).

Table 6: Diameter of graafian follicle (µm) of albino rats-control & experimental groups

Group	Mean	Sd	Min	Max
A	839.20	68.51	650.00	900.00
B	750.00	31.89	700.00	820.00

ANOVA

Sources	Sum of squares	DF	Mean square	F	Sig.
Between Groups	67175.25	2	33587.633	9.131	0.001
Within Group	99320.10	27	3678.522		
Total	166495.37	29			

Pair-wise comparison between groups by using Tukey HSD

Groups (I)	Groups (J)	Mean difference(I-J)	Std. Error	Sig.
A	B	89.20	27.12	0.001*

SD: Standard Deviation, **F:** f-test (Ratio of variances, **DF:** Degree of Freedom

*****Statistically significant difference (P<0.001).

†Statistically insignificant difference (P>0.001).

Percentage change in total cholesterol of albino rats-control & experimental groups

Group	Mean	Sd	Min	Max
A	40.13	52.34	-31.50	129.20
B	26.34	21.15	-51.90	19.70

Pair-wise comparison between groups by using Tukey HSD

Groups (i)	Groups (j)	Mean difference (I-J)	Std. Error	Sig.
A	B	66.47000	15.52045	0.001*

SD: Standard Deviation.

Statistically significant difference (P<0.001).

Percentage change in ldl cholesterol of albino rats-control & experimental groups

Group	Mean	Sd	Min	Max
A	56.40	64.37	2.70	170.30
B	32.17	15.30	-56.40	-13.50

ANOVA

Sources	Sum of squares	Df	Mean square	F	Sig.
Between Groups	48731.193	2	24365.596	16.355	<0.001

Pair-wise comparison between groups by using Tukey Hsd

Groups (i)	Groups (j)	Mean difference (I-J)	Std. Error	Sig.
A	B	88.57000(*)	17.26124	<0.00*

SD: Standard Deviation.

*****Statistically significant difference (P<0.001).

Percentage change in HDL cholesterol of albino rats-control & experimental groups

Group	Mean	Sd	Min	Max
A	25.06	45.59	-26.30	100.00
B	28.34	23.22	-12.50	73.70

ANOVA

Sources	Sum of squares	Df	Mean square	F	Sig.
Between Groups	124.209	2	62.104	0.061	0.941

Pair-wise comparison between groups by using tukey hsd

Groups (i)	Groups (j)	Mean difference (I-J)	Std. Error	Sig.
A	B	-3.28000	14.27517	0.971

SD: Standard Deviation.

Statistically significant difference (P<0.001).

The corpus luteum of group A was seen to occupy various spots in the cortex of the ovaries. Majority were located in the peripheral zone of the ovarian cortex. The granulosa luteal cells were large and polyhedral with large central rounded to oval shaped nuclei. These nuclei exhibited a single darkly staining nucleolus. A copious amount of pink staining cytoplasm surrounded the nuclei giving a more vacuolated appearance or showing empty irregular spaces within the cytoplasm. Small capillaries and venules were seen scattered throughout the corpus luteum. Towards the periphery of the luteal gland smaller polygonal theca luteal cells with relatively darkly staining rounded nuclei were observed (Fig 1 & 4).

The corpus luteum in the experimental group B was seen occupying the outer as well as inner zones of the ovarian cortex. The luteal glands were increased as compared to the control group A. The granulosa cells appeared to be smaller in size as compared to the control group A. The cells were polygonal in shape with a single central, lightly staining rounded nucleus. A single spherical nucleolus was also seen in majority of the granulosa cells. Smaller irregular shaped cells noted infiltrating the granulosa luteal cells. Intercellular spaces were appreciable among the theca luteal cells. The peripheral segment of the luteal gland showed smaller polygonal cells of theca interna with central rounded, darkly staining nuclei. The blood vessels in the form of capillaries and venules were congested as compared to the control group A (Fig-2). Comparison of these findings was statistically insignificant when compared with the control group A (P-value > 0.001, Table-7).

Table 7: Association of corpus luteum of albino rats with drug dosages-control & experimental groups

Groups	Corpus Luteum		Total
	Normal	Hypertrophy	
A	10	0	10
B	4	6	10
Total	14	6	20

Chi-Square 27.99 P-Value = <0.001

Pairwise comparison	Groups	Chi-sq	P-value
A	B	5.95	0.010†

*****Statistically significant difference (P<0.001).

†Statistically insignificant difference (P>0.001).

The ovarian medulla of the control group A showed blood vessels of different diameter occupying the central portion of the ovary. These arteries, arterioles, capillaries, veins, venules and lymphatic vessels were embedded in a bed of loose connective tissue (Fig 4). The blood vessels in the experimental group B revealed relative moderate dilatation as compared to the control group A. The lumen of these blood vessels was irregular with intact endothelial lining surrounded by a loose connective tissue network (Fig 2 &3).

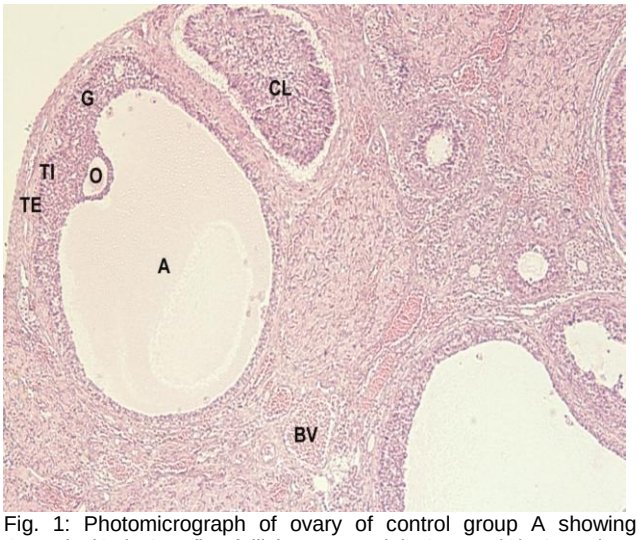

Fig. 1: Photomicrograph of ovary of control group A showing Capsule (CP), Graafian follicle, Antrum (A), Oocyte (O), Granulosa cells (G), Corona radiate (CR),Theca Interna (TI), Theca Externa (TE), Secondary oocyte (SO), Corpus Luteum (CL)and Blood vessel (BV) in cortex (H&E, x5)

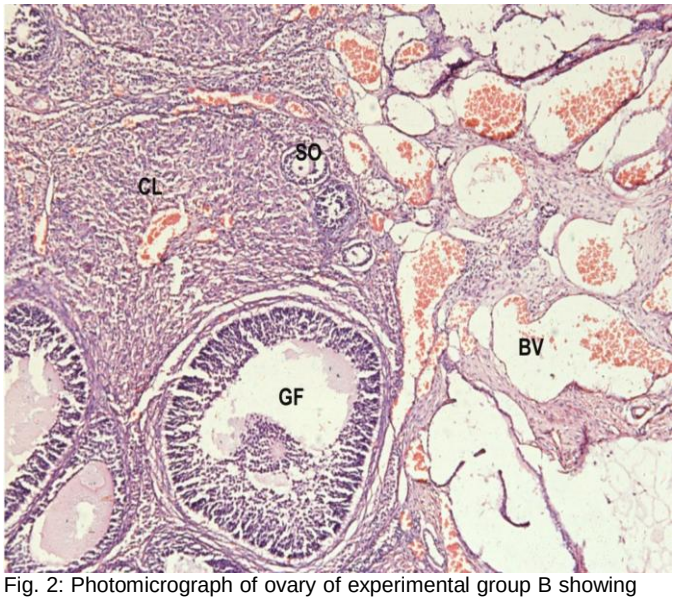

Fig. 2: Photomicrograph of ovary of experimental group B showing Graafian follicle (GF), Secondary Oocyte (SO), Corpus Luteum (CL) and Blood Vessel (BV) in medulla (H&E, x5)

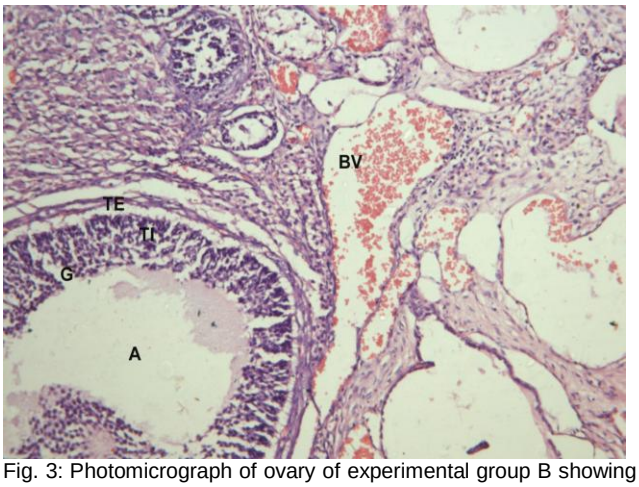

Fig. 3: Photomicrograph of ovary of experimental group B showing Graafian follicle with Antrum (A), Granulosa cells (G), Theca interna (TI), Theca externa (TE) and Blood Vessel (BV) in medulla (H&E, x10)

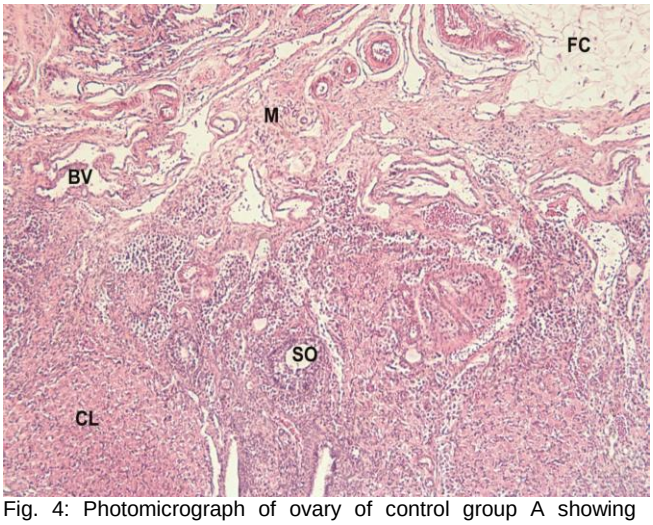

Fig. 4: Photomicrograph of ovary of control group A showing Secondary oocyte (SO), Corpus Luteum (CL) and Blood vessel (BV) in medulla (H&E, x5)

DISCUSSION

In the present study the detail histological study of the ovaries of the control group A revealed the ovulatory cycle in different stages of folliculogenesis, progressing from primordial follicle to Graafian follicle. In addition the differentiation of both granulosa cells and theca interna cells, formation of antrum, cumulus oophorus, corona radiata and corpus luteum were also observed and depicted the normal histological findings and coincide with the findings reported by Amina Boubekri (2007) in her study of rat histology. These follicles were seen embedded in an internal supporting or connective tissue called stroma[9]. The lining epithelium of the ovary was made up of low cuboidal to squamous epithelium[10]. This observation was seen in both the groups in the same section or in different sections

especially where the graafian follicles or corpus luteum reached the peripheral segment of the ovary. The underlying connective tissue capsule termed as tunica albuginea was variable in thickness being thin in most of the experimental animals of group B. The number of primordial follicles in the experimental group B was reduced as compared to the control group A. The size of primordial follicles in both the experimental group B seemed to show a relative smaller size as compared to the control group A. This decrease in size seems to be in agreement with the work done by Sokalska et al in which he states that statins induce an inhibition of DNA synthesis[11]. These findings suggest that Simvastatin plays a negative role in the development of primordial follicle especially the growing oocyte.

The secondary or preantral follicles were occupying a more central segment of the cortex in all the groups. The analysis of the results revealed that there was a relative and gradual decrease in the diameter of the secondary follicles in the experimental group B when compared with the control group A. The atrophy of the secondary follicles in the experimental group B as compared to the control was highly marked despite the fact that proliferation and differentiation of granulosa cells and theca cells is in progress at this stage. In addition to this the cells of theca interna are acquiring the characteristics of steroid secreting cells which include abundant profile of smooth endoplasmic reticulum, mitochondria with tubular cristae and numerous lipid droplets. These cells are known to synthesize steroid hormone androstenedion which is later transported to granulosa cells[12]. In vitro studies and a randomized, crossover study conducted by Banaszewska et al has shown that statins may reduce ovarian androgen production by inhibiting the proliferation of theca cells[13]. This means that statins decrease the proliferation and steroidogenesis of ovarian theca cells.

The Graafian follicle in the experimental group B showed a pair wise decrease in the mean diameter which was deemed insignificant statistically (P-value >0.001,Table 11) was compared with the control group A. These results showed a gradual decrease in the size of the Graafian follicle in the experimental group. The granulosa cell layers were increased in the experimental group B as compared to the control. The cellular walls of the experimental groups also exhibited infoldings (Fig 7). Sources of cholesterol for steroid synthesis in ovarian granulosa cells and theca cells include plasma lipoproteins (LDL and HDL), stored cholesterol esters and de novo- synthesized cholesterol. Most of the cholesterol is provided by the circulating lipoproteins and the preference for different classes varies between species. Stimulation of LH receptor has been reported to stimulate incorporation of acetate into sterols and steroids in granulosa cells. Interestingly the cholesterol synthesis inhibitor Simvastatin decreased the incorporation of acetate into cholesterol in periovulatory granulosa cells as seen by Emilia Rung et al[14]. This study is in agreement with the findings of present study that Simvastatin inhibits steroidogenesis at other sites in addition to HMG-CoA reductase and hence leads to decrease cell proliferation and growth in theca and granulosa cells. This explains the decrease in diameter of the secondary and graafian follicles. However the increase in the number of cell layers in the experimental groups can be explained by the hypothesis that granulosa and theca cells acquire cholesterol required for proliferation through a pathway other than via HMG-CoA reductase. One such pathway may be through formation of free cholesterol from cholesterol esters as occurs for steroidogenesis[15]. Another being that rats predominantly use HDL as their source of cholesterol and Simvastatin increases HDL as a result of its mechanism of action[16]. The hypertrophy seen in the experimental group B as compared to the control group A can be explained on the basis of steroidogenesis involving cholesterol transport. Under normal conditions the majority of cholesterol is synthesized in the liver and transported to steroidogenic tissues such as the adrenal cortex, follicles, corpus luteum and testis in the form of lipoproteins. LDL and HDL are the most common sources of cholesterol for the production of steroid hormones by the corpus luteum. There appears to be some species related difference in their preference for LDL or HDL, but either source can be utilized by luteal cells of most species[17]. It has been seen that rats, mice and ruminants utilize HDL whereas human, rhesus macaques and porcrine use primarily LDL for luteal steroidogenesis[16]. Circulating HDL contributes cholesterol to luteal steroid synthesis and is the principal cholesterol supply in murine rodents[18]. However under condition of lipid deprivation i.e., reduced synthesis of lipoproteins luteal cells are capable of synthesizing cholesterol from acetate[19]. Since Simvastatin increases the availability of HDL in the blood it seems logical that increased amount of HDL is utilized by the luteal cells leading to hypertrophy as compared with present study.

The medulla of the ovaries in the experimental groups exhibited changes in the blood vessels as compared to the control group. The blood vessels were seen to be dilated and congested in the experimental group B. This change can be explained by the fact that statins have effects on angiogenesis as shown by research. It was seen that statins have a

biphasic dose dependent effect on angiogenesis characterized by a proangiogenic affect at low dose and antiangiogenic and proapoptotic effect at high doses. This dual theory was introduced in 2002 and is widely accepted[20]. In addition to this in vascular endothelium statins increase the concentration of nitric oxide (NO) which has vasodilator and antithrombotic properties. This was also reported by Tandon V et al in his study on the mice in which Simvastatin enhanced the production of NO in vascular endothelium[21]. This explains increased dilatation of vascular endothelium in group B.

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