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Evaluating the effects of Levonorgestrel on female gonads of *Rattus albicans*

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Abstract

Though the repeated or long term use of progesterone only birth control measures are found to be disruptors of reproductive cycle, it is often reported that the effects of an exogenous progesterone analog on the histology of reproductive organs cannot be predicted easily, which is utmost important for the users. Therefore, the present work is designed to enumerate whether a commonly used synthetic progestogen, Levonorgestrel (LNG) has disruptive effects on the ovarian histology at a dose of 1.5mg/kg b.w/day for 03 consecutive days at 24 h time interval in cyclic ovary-intact female rats.Present investigation reveals that administration of LNG reduces ovary sizes, causes detachment of the cumulus oophorous cells from the oocyte, changes the shape of the oocyte, increased follicular atresia along with increase in number of secondary follicles and decrease in numbers of primordial and grafiaan follicles. Number of corpus luteum was also decreases. From these results, it can be concluded that prolonged or repeated use of LNG may disrupt the ovarian activity by affecting folliculogenesis, rate of ovulation thereby the rate of fertility in females. Information from this study might help researchers to develop certain contraceptives from natural resources with little side effects rather than synthetic contraceptives.

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Introduction

Although natural products have been used to cure diseases since time immemorial, people of the recent time are using synthetic drugs to get quick relief from any disease or to suppress some physiological anomalies of the body instantly. Synthetic steroids are one such group of chemicals which are used as birth control measures. However, the steroids, like synthetic progestins or progestogens alone at contraceptive dose were reported as disruptors of reproductive cycle (Benagiano *et al.,* 2000). It was also reported that in comparison to combined estrogen-progesterone contraceptive pills, the progesterone-only pills (POPs) exhibit higher incidence of menstrual cycle irregularity (The ESHRE Capri Workshop Group, 2001). POPs inhibit release of ova and also affect the endometrium to prevent unwanted birth. But the actual effects of these POPs into the endometrium is unpredictable (Rawal, 2016), because progestogens exhibits a number of dose dependent actions (Larsson *et al.,* 1970). Menstrual disturbances as a result of POPs include spotting, breakthrough bleeding, cycles with abnormal duration etc. (Hatcher *et al.,* 1988; Speroff and Darney, 1992).Among several commonly used synthetic progestins, Levonorgestrel (LNG) is one which acts an active ingredient of a number of birth control measures like oral contraceptive pills (OCPs), intra-uterine devices (IUDs) etc. LNG prevents pregnancy either by suppressing ovulation or by thickening the cervical mucus. However, literatures reported LNG to have severe effects such asclosed uterine lumen and suppression of the epithelial cell division in mice (Martin *et al.,* 1970), decidualization of the uterine stroma as well as atrophy of the endometrium in human (Martinez and Aznar, 1975), prolonged estrous cycle along with suppressed levels of serum estrogen (E_2) in monkeys (Heikinheimo *et al.,* 1996), disruption of normal reproductive cycle and impairs oocyte maturation in human (Alvarez *et al.,* 1986), decreased levels of progesterone (P_4) and E_2 in human females (Pritis *et al.,* 2005), small indistinct endometrial glands; low columnar epithelium (Mazur and Kurman, 2005), decreased levels of serum E_2 and P_4, leading to disrupted ovulation and luteal activity in *Mongolian gerbils* (Hui and Zhao, 2011), prolongation of the estrous cycle length and significant reduction of mRNA and expression of follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor(LHR) in ICR mice (Chen and Shi, 2016), delayed menstruation, metrorrhagia, dysmenorrhea, menorrhagia, premenstrual syndrome, breast tenderness, nipple disorder, breast enlargement, ectopic pregnancy in human female (Kurian *et al.,* 2018) etc. Besides these, the earlier research work of Hazarika and Choudhury, 2021 states that LNG causes disruption in both cyclicity as well as the level of E_2 and P_4 hormone when administered repeatedly at a dose of 1.5mg/kg body weight/day for six consecutive estrous cycle in adult female *Rattus albicans*. However, literature considering the effect of LNG on ovarian folliculogenesis, rate of ovulation and the rate of fertility is limited. Therefore the present study aims to find out whether the synthetic contraceptive LNG exhibits any adverse effects on female reproductive system especially on the folliculogenesis which may lead to infertility.

Materials and methods

Experimental animals

Female cyclic Wistar rats, *Rattus albicans* weighing 130g to 150g, were procured for the present work. All experiments were done following approved protocols of the Institutional Animal Ethics Committee (IAEC) and guidelines were followed for the care of the laboratory animals. Rats were fed with standard diet (containing maize-powder, corn-husk, oilcake and salt) with Vitamin supplement and water *ad libitum* and housed in paddy husk bedded polypropylene cages. Animals were maintained under natural light/dark cycle and at ambient temperature (Max.32^{0}C. and Min. 28^{0}C). Estrous cycle was observed by vaginal smear technique prior to the experiments for confirmation of regular cyclicity. Six numbers of healthy female rats were taken in each group. Animals were anesthetized with a combination of ketamine (50mg/kg body weight) and xylazine (10mg/kg body weight) intra-peritoneally at 2:1 ratio (Margarete *et al.,* 2001).

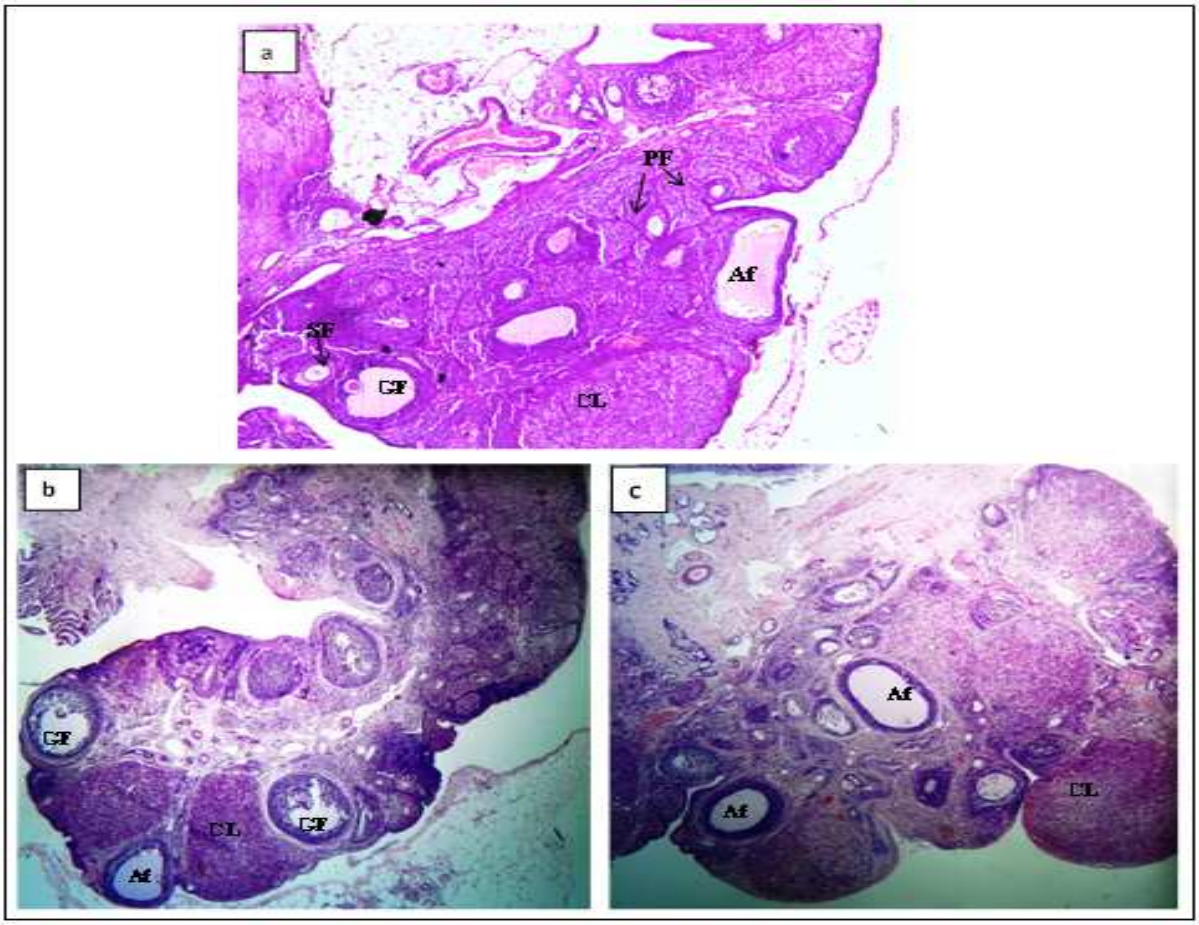

Fig. 1. Photomicrographs of ovarian histology of: **(a).** Group 1 (Control), **(b).** Group 2 (1.5mg/kg b.w/day LNG treated), **(c).** Group 3(after discontinuation of 1.5mg/kg b.w/day LNG treatment) showing different follicles 50x magnification. PF: Primary Follicle; SF: Secondary follicle, GF: Grafiaan follicle; CL: Corpus luteum, Af: Atretic follicle.

Chemicals

LNG was purchased in powdered form from TCI Chemicals, Tokyo. It was selected for the present study since it is used as an active ingredient in some hormonal contraceptives. To prepare the stock solution of LNG, 0.0125g LNG was dissolved in 2ml of sesame oil (solvent). LNG was routinely administered by diluting LNG to the required concentration of doses in sesame oil and was administered at a dose of 1.5mg/kg b.w/day sub-cutaneously at a volume of 500µl for 3 consecutive days at 24 h time interval. The injectable volume was prepared as per OECD guidelines. Sesame oil is used commonly as a vehicle in scientific experiments since it has been proven to possess no side effects. In the present study it was used as a vehicle for the administration of LNG. It was administered at a volume of 500µl for 03 consecutive days at 24 h time interval. A combination of ketamine (Aneket, Ketamine hydrochloride, Neon Laboratories Limited) and xylaxine (Xylaxine hydrochloride; Indian Immunologicals Limited, Hyderabad) at a ratio of 2:1 was used as anesthesia during experimentation.

Experimental design

Experiments were carried out in 18 cyclic ovary-intact female rats and were divided into three groups (n=6) according to OECD guidelines (OECD 407, 2008). Group 1 served as the control group and received sesame oil at a volume of 500µl for 3 consecutive days at an interval of 24 h. Group 2 received 500µl of LNG at a dose of 1.5 mg/kg b.w/day sub-cutaneously for 3 consecutive days at an interval of 24 h. Group 3 were administered LNG (1.5mg/kg b.w/day) for 3 consecutive days and then left untreated for two consecutive estrous cycles (8 days) to examine

whether the effects of LNG remains even after discontinuation of LNG administration.

Histology

Histology of ovarian tissues was done by means of routine haematoxylin-eosin (H and E) method described by Scudamore, 2014 with some minor modifications. Counting of ovarian follicles was done by cutting approximately 150 serial sections of 4µ thick ovarian tissue which were cut from each ovary. To obtain an overall view of the follicular populations per ovary, every 12th and 20th section was studied for counting smaller and larger follicles respectively (Pedersen and Peters, 1968; Butcher and Keller, 1984).

Quantification of follicles

The follicles were classified according to the method of Meyers *et al.,* 2004 and criteria described in 7th edition of Essential Reproduction,2013 by Johnson, into: (i). Primordial follicles: having an oocyte enclosed by a layer of flattened squamous granulosa cells; (ii). Primary follicles: consist of a cuboidal granulosa cells layer; (iii).Secondary follicles: having 2-4 granulosa cells layers without antrum and (iv). Graafian follicles/antral follicles/tertiary follicles: mature follicle having areas of follicular fluid called antrum. The averages of differential follicular counts for each class of follicles in all the treatment groups were expressed as mean ± SEM.

Quantification of corpus luteum

The methods used by Jefferson *et al.,* 2005 and Brasil *et al.,* 2009 with some minor modifications were used to quantify the corpus luteum of ovary of each rat. About 4µ thick H and E stained ovarian sections were taken for quantification of corpus luteum. During counting, a gap of 50µm was maintained between the sections to avoid the same being considered twice. Three tissue sections of ovary per animal per treatment group (3 tissue sections × 6 animals per group) were taken for counting the number of corpus luteum (Banks *et al.,* 2001). Percentage of rats showing corpus luteum in ovarian histology was determined using the following formula.

$$\text{Percentage of rats showing corpus luteum} = \frac{\text{Number of rats showing corpus luteum}}{\text{Total number of rats per group}} \times 100$$

Statistical analysis

Results of animal experimentations were tested for statistical significance with one way analysis of variance (ANOVA) using window's Statistical Package of Social Sciences (SPSS version 21) followed by Tukey's Post hoc test. Student's t-test using Microsoft Office Excel 2007 was also performed. Data were expressed as mean ± SEM. $p < 0.05$ was considered to be statistically significant.

Results

Histoarchitecture of ovaries

The histoarchitecture of ovaries of both control and treated rats of different groups was examined considering the counting of primordial, primary, secondary, Grafiaan and atretic follicles and the corpora lutea (Figure 1). It was found that after LNG administration, the size of ovaries decreased in comparison to that of control group. Besides, histological observation revealed detachment of cumulus oophorous cells from the oocyte along with detachment of granulosa cells. There was also a change in the shape of the oocytes from round or oval shaped to disc-shaped (Figure 1b and 1c).

Ovarian follicular count

In control rats, the ovaries were found to consist of healthy pre antral and antral follicles of various stages i.e., primary, secondary and grafiaan follicles along with a number of corpus luteum. Change in the number of follicles of various stages was observed in the histology of ovaries after the administration of LNG at a dose of 1.5mg/kg b w/day. As shown in table 1, the number of secondary follicles (268.14 ± 4.36) increased in the ovary of LNG treated females when compared with the control counterparts (193.29 ± 3.59). However, the number of primordial follicles (120.26 ± 5.96) and grafiaan follicles (8.45 ± 3.04) decreased significantly in the LNG treated ovaries.

These alterations in follicle counting remained almost similar even after discontinuation of LNG

administration. Variation in the number of atretic follicle was clearly observed in the histological sections of both control and treated individuals (Table 1). The number of such follicles was very few in control rats (9.61 ± 1.86) whereas, it showed significant ($p<0.01$) increase immediately after the three day long administration of LNG (32.84 ± 3.21) as well as after 8 days of discontinuation of LNG administration (31.33 ± 2.93).

Quantification of Corpus luteum

Histology of ovaries of cyclic females was observed both in control and treated rats for the quantification of corpus luteum (Figure 1). As shown in table 2, the ovaries of rats receiving LNG at a dose of 1.5mg/kg b.w/day, showed a few number of corpus luteum (13.7 ± 0.3) which was significantly ($p<0.05$) less than the control counterparts (27.6 ± 0.3). Number of corpus luteum remained similar to group 2 (14.4 ±0.1).

Table 1. Ovarian follicular count in different experimental groups of rats (n=6).

Types of Follicles	Experimental Groups		
	Group 1	Group 2	Group 3
Primordial follicle	198.22 ± 4.34	120.26 ± 5.96[a]	121.74 ± 4.18[a]
Primary follicle	160.46 ± 4.86	218.71 ± 7.22[a]	220.52 ± 7.27[a]
Secondary follicle	193.29 ± 3.59	268.14 ± 4.36[a]	265.04 ± 5.34[a]
Grafiaan follicle	15.17 ± 4.04	8.45 ± 3.04[a]	8.61 ± 2.08[a]
Atretic follicle	9.61 ± 1.86	32.84 ± 3.21[a]	31.33 ± 2.93[a]

Data are expressed as mean ± SEM. [a]denotes significant difference from Group1 at $p < 0.01$.

Discussion

Use of chemical contraceptives is a common practice for those women throughout the world who want to avoid pregnancy. Although synthetic hormone analogues are components of most widely used contraceptives of the present time, repeated use of such synthetic components may have deleterious effects in the physiological processes of their users, especially to the reproductive system causing permanent damage. Considering the relevance of this issue, an attempt was made to see the effects of a synthetic progestin, LNG on some reproductive parameters of female rats. Reduction in the number of primordial and mature follicles and increase in the number of secondary, immature and atretic follicles in LNG treated rats was a noteworthy finding of this study. Recovery from these alterations was not seen even after withdrawal of LNG treatment for two estrous cycles. These alterations may be considered as the side effects of the studied synthetic contraceptive. Because, LNG was found to disrupt the reproductive cycle as well as the level of E_2 and P_4 hormone when administered in repeatedly to adult *Rattaus albican* sat a dose of 1.5mg/kg body weight/day (Hazarika *et al.,* 2021).The results of the present study are in agreement with the work of Chen and Shi, 2016 who evaluated the side effects of Mifpristone and LNG on the ovarian function of mice.

Table 2. Corpus luteum count in the ovaries of different experimental groups of rats (n = 6).

Experimental Groups	Percentage of rats showing corpus luteum	Average numbers of corpus luteum in both ovaries
Group-1	6/6 (100%)	27.6 ± 0.3
Group-2	4/6 (66.7%)	13.7 ± 0.3[a]
Group-3	4/6 (66.7%)	14.4 ±0.1[a]

Data are expressed as mean ± SEM.

[a] denotes significant difference from Group 1 at $p < 0.05$.

They reported that the change was due to inhibition of FSHR and LHR expression in the ovary which may be repairable or irreparable. Such inhibition affects the level of E_2 and P_4 hormones in blood causing ovarian malfunction. As stated by Manikkam and Rajamahendran, 1997 large scale follicular atresia in

the present observation may be due to the synthetic progestin administration.

The present observations have similarities with the work of Bhowmik and Mukharjee, 1988 and Girija and Kamakshi, 2003 who described the atretic ovarian follicles after prolonged exposure of rat ovaries to injectable progesterone. Follicular atresia in turn results in insufficient production of endogenous E_2 and P_4. A number of abnormalities ranging from anovulation to insufficient luteal phase may occur due to the disruption in the hormone profile (Segal *et al.,* 1991). As a result of LNG administration, a decrease in the number of corpus luteum was also observed in the present investigation which might be indicative of low ovulation rate, because corpora lutea are formed only after ovulation.

Conclusion

Continuous use of LNG (3 days in this study) at a dose of 1.5mg/kg b.w/day was found to cause malfunction in the histoarchitecture of the ovaries. The number of mature follicles and corpora lutea were also found to be decreased. Also, the number of atretic follicles was found to be increased after LNG administration. From these findings, it can be concluded that, continuous use of this commonly used progestin might affect the rate of fertility in females by affecting the primary reproductive organs. However, further investigations in the gene level may help to confirm the observed effects.

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