Effects of Repeated Dose of Levonorgestrel on Uterine Histomorphology of Adult Female Albino Rats

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ABSTRACT: *The present study was done to observe the effects of Levonorgestrel (LNG), a progestin, on the uterine histoarchitecture of female rat. Adult cyclic female albino rats were divided into three groups: control, LNG treated (1.5mg/kg body weight/day) and sesame oil treated. Treatment was done sub-cutaneously for 06 consecutive cycles (one injection/cycle). Half of the animals were sacrificed after 24 hours of end of the treatment and half were left untreated for another two estrous cycles to observe sustainable effects, if any. Uterine histology together with their wet weight was studied in all the groups. Decreased wet weight of the uterus, narrow and elongated uterine lumen, decreased numbers of endometrial glands, thin luminal epithelial linning, decidualization of the stroma were observed both immediately after the treatment and even after two cycles of discontinuation of the treatment. The present study revealed that repeated or long term use of LNG doses may cause some permanent changes leading to malfunctioning of the female reproductive system which may be the cause of female infertility.*

Key words: Progestin, endometrial glands, anti-estogenicity, infertility.

1. INTRODUCTION

Levonorgestrel (LNG), a synthetic 13β-ethyl substituted 19-nor steroid progestin with a half life of 10-24 hours, is one of the active components used in different types of birth control measures like oral contraceptive pills (OCPs), Intra uterine devices (IUDs) etc. Currently, due to the effectiveness and ease of use, LNG is the most commonly used clinical drug for emergency contraception in females of reproductive age (Apter, 2003). Convenience of emergency contraceptives has led some women to use it repeatedly for long due to which women are facing problems related to infertility. Alvarez *et al*., 1986 and Segal *et al*., 1991 reported that, LNG suppresses maturation of ovarian follicles along with ovulation by lowering the secretion of follicle stimulating hormone (FSH) and inhibiting the preovulatory luteinizing hormone (LH) surge. The mechanism of LNG also includes actions affecting corpous luteum formation, fertilization, blastocyst implantation, endometrial function etc **(**Croxatto *et al.*, 2001; Marions *et al.*, 2004). Besides these, LNG also induces changes in endometrial secretions that modulate sperm functionality (Hernandez *et al.*, 2018). Earlier studies revealed that LNG can affect menstrual cycle and menstrual flow (Koyama *et al.*, 2013). The most common reason for discontinuation of progestin only contraception is irregular bleeding (Findlay, 1996; Hickey *et al.*, 1999) which is related to the vasculature of the uterus. IUS containing LNG are known to alter the morphology and function of endometrium (Critchley *et al.*, 1998a) which include pseudo-decidualization of the stromal compartment with leukocytes infiltration, atrophy of glandular and surface epithelium, and alterations in the vasculature (Jones and Critchley, 2000). But according to some literature these actions of LNG are reversible. Now the question is what would happen if a female uses it repeatedly for long. So the aim of the present investigation is to study the effect of repeated use of high dose of LNG as emergency contraceptive on certain parameters of the uterus.

2. MATERIALS AND METHODS

2.1. Experimental Animals: Adult cyclic female albino rats weighing 130 to 150 gm, showing regular estrous cycle were used for the present study. Rats were allowed free access to water and food and housed in standard poly cages bedded with paddy husk under natural light/dark cycle at room temperature. Cyclicity

was observed prior to and during treatment. All the experimentation was done in accordance with the institutional ethical guidelines.

2.2. Work design: Experimental animals were divided into three groups each having six rats. The first group was the control, second group received 1ml Levonorgestrel solution at the dose of 1.5mg/kg body weight/day where sesame oil was used as vehicle and the third group received sesame oil only. Each animal received one subcutaneous injection/cycle at a dose of 1.5mg/kg body weight/day for six consecutive cycles during follicular phase. Half of the animals were sacrificed 24 hours after end of the treatment and half were kept for another two cycles (8days) without treatment. Uterine wet weight of all the animals was measured with the help of Sartorius Electronic Balance (DJ602A, Germany) and then the uterine horns were fixed in 10% formaldehyde for histological observation. Histology was done according to a Haematoxyline-eosin (H&E) protocol (Scudamore, 2014) with some laboratory modifications.

2.3. Uterine morphology & Measurement of uterine wet weight: The uteri were photographed using Nikon Digital Camera (COOLPIX S3500) for morphological examination. Immediately after dissection, wet weight of uterus was measured to avoid desiccation of the tissues, using Sartorius Electronic Balance (Germany).

2.4. Histological observation: Histological slides of uterus, were observed in various magnifications (50x, 100x and 400x) using Labored L x 400 and Radical R x L r -3 Bright field microscope system.

2.5. Measurement of different parts of uterine tissue: Diameter of the lumen and height of luminal epithelium was measured from the 4µm thick uterine tissue sections using 100µm scale bar in Leica DM5000B Micro system and Zen software in the ZEISS AXIO observer Z1. Measurement was done in three areas of the same section and three sections per animal per treatment group (3 tissue sections × 06 animals per group).

2.6. Quantification of Endometrial Glands: Number of endometrial glands was counted in the image transfer system (model no.F629) at Biotechnology Hub, B. Borooah College, Guwahati. Gland number was counted in serial cross sections of uterine tissues obtained from the middle region of the uterus. Counting was done in three sections per animal per treatment group (3 tissue sections x 06 animals per group).

2.7. Statistical analysis: Calculated data from all results were expressed as mean $\pm$ S.E.M. Statistical analyses were performed using Microsoft Office Excel 2007. Values are significant at $p< 0.05$. The significance of differences between groups and within groups was ascertained by the student's t test.

3. RESULTS

Uteri collected on 24 hours after end of the treatment and uteri collected after two untreated cycles exhibited similar results. In the present study, uterine morphology showed visible differences between the control and treated groups. The uteri of LNG treated groups appeared to be thinner in its morphological structure when compared to the control and vehicle treated groups. This thinning remains even after two cycles of withdrawal of the LNG treatment. The ovaries also found in contracted appearances in both of these two treatment stages (Plate-1). Wet weight of the uteri was significantly decreased in both group-2 (0.33g) and group-3 (0.35g) in comparison to group-1 (0.48g). Group-4 exhibited almost similar result (0.46g) to that of group-1 as shown in the table 1.

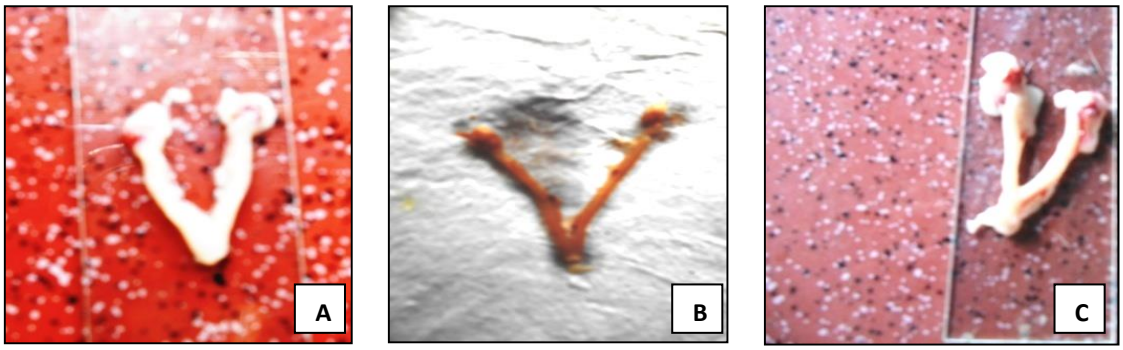


Plate.1: Photomicrographs showing morphology of uterus of different groups of adult female albino rats. (A).Normal control; (B).After 24 hours of LNG treatment; (C).After two estrous cycle of discontinuation of LNG treatment.

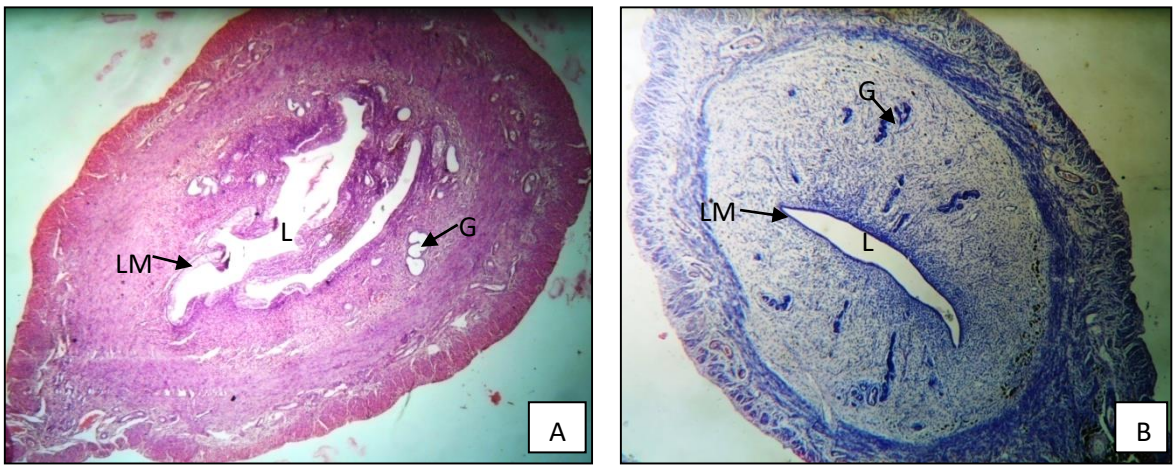
Table 1: Effects of repeated use of LNG on the wet weight of uteri in comparison to normal control and vehicle treated uteri of adult female albino rats. (Data are expressed as Mean ± S.E.M; *p< 0.05):

Groups of rats	Wet weight of the uteri (in g) (mean ± S.E.M)
Group-1 (Control)	0.48 ± 0.027
Group-2 (LNG treated)	0.35 ± 0.030*
Group-3 (LNG treated)	0.33 ± 0.031*
Group-4 (Sesame oil)	0.46 ± 0.014

Histology of uterus of different groups is shown below in Fig.2. Endometrial linning possess low cuboidal epithelial cells whereas the vehicle treated and normal control groups possess endometrial linning with columnar epithelial cells.

Lumen of the LNG treated uteri were narrow, almost closed, elongated and less vascular with a diameter of (83.901 ± 4.7694*) µm and (81.879 ± 3.4192*) µm in comparison to those of control (298.293 ± 1.7348) µm and vehicle treated (302.293 ± 1.7348) µm as shown in the Fig.3. Height of the luminal epithelial linning was also decreased i.e., (9.287 ± 0.3867*) µm and (8.134 ± 0.247*) whereas it is (36.668 ± 1.0763) µm in control and (35.966 ± 0.6327) µm in vehicle treated groups (Fig.4).

Uterine glands were found very narrow and less in number when compared to the other groups. Crowding of the endometrial gland was seen which may result in glandular hyperplasia. Whereas glands of the control and vehicle treated were distributed throughout the stroma and were very prominent.



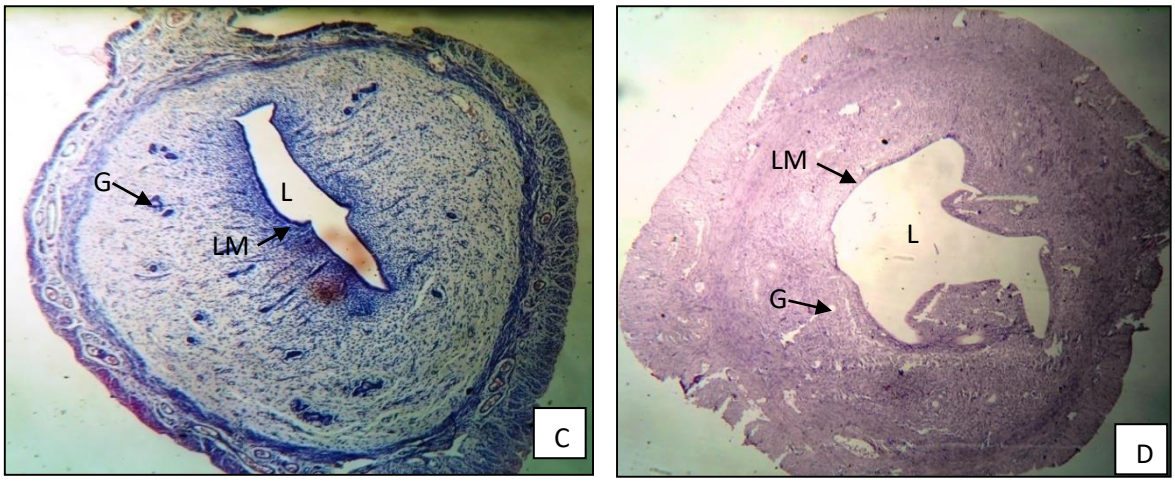


Plate 2: Photomicrographs showing changes in the histology of uterus: (A). normal control; (B). LNG treated (tissue collected after 24 hrs of treatment) (C). LNG treated (tissue collected after two cycles of withdrawal of treatment) (D). Vehicle treated

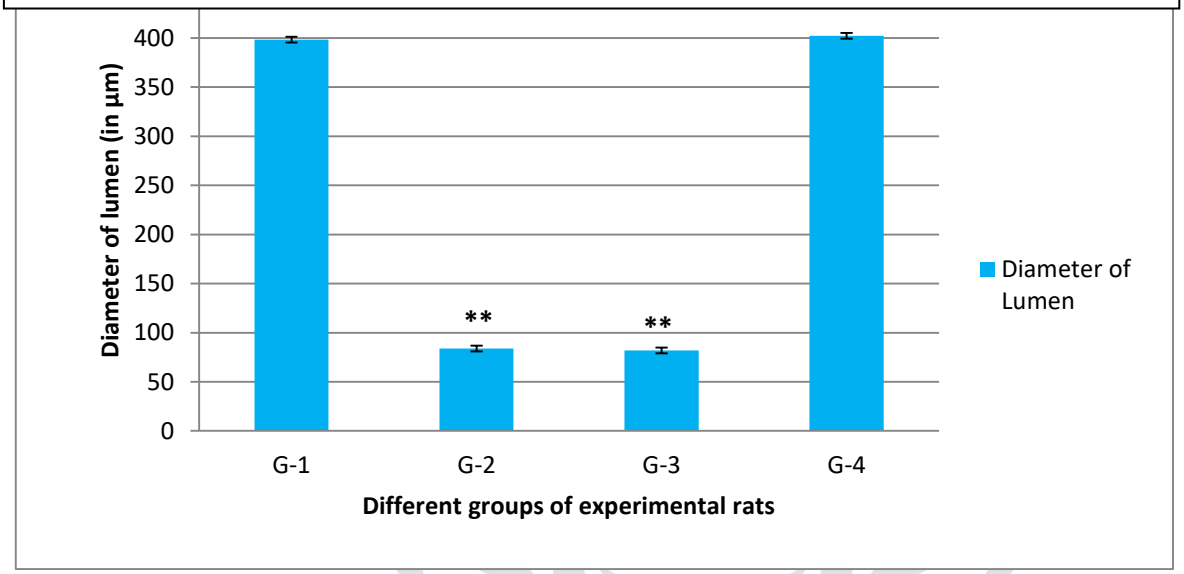


Fig.3: Bar diagram showing diameter of uterine lumen in different treatment groups. G-1 (control); G-2 (LNG treated, tissue collected after 24 hrs of treatment); G-3 (LNG treated, tissue collected after two cycles of withdrawal of treatment) G-4 (Vehicle treated). *p<0.05

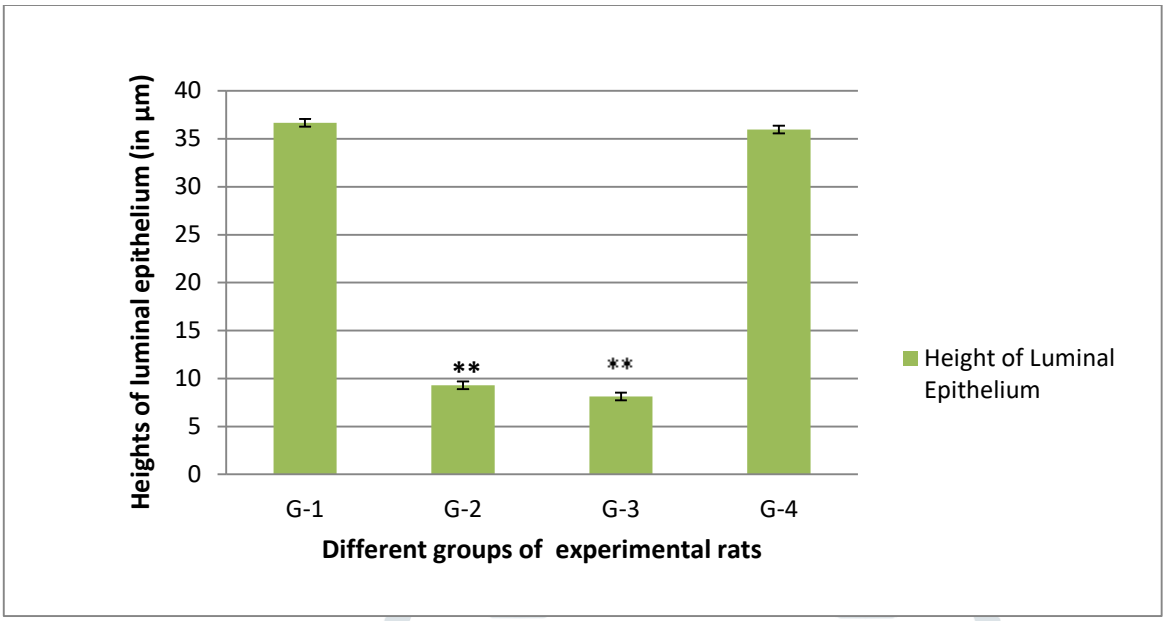


Fig.4: Bar diagram showing heights of luminal epithelium in different treatment groups. G-1 (control); G-2 (LNG treated, tissue collected after 24 hrs of treatment); G-3 (LNG treated, tissue collected after two cycles of withdrawal of treatment) G-4 (Vehicle treated). *p<0.05

4. DISCUSSION

Exogenous progestins used as contraceptives induce changes in the histology of endometrium and uterine glands that differ from those occur during the normal menstrual cycle (Dinh, 2015). But whether these changes if remain for long even after discontinuation of a progesterone only contraceptive cause permanent damage to the reproductive system is often questioned. Therefore the present study was aimed to evaluate the effect of repeated use of Levonorgestrel on the uterus of adult female albino rat and to analyze its possible effects after discontinuation.

Weight of uteri with luminal fluid (wet weight) or without luminal fluid (Blotted) can be considered as the most reliable data of an uterotrophic bioassay to identify estrogenecity or anti-estrogenecity of a substance. Increased uterine weight indicates estrogenic activity of a test compound, while anti-estrogenic compounds diminish the estrogen dependent uterine weight increase in intact immature or ovariectomized adult females (Debnath, 2013). Differences in the morphology and wet weight of the uteri were observed in group-2 and group-3 when compared to group-1. Such decrease in size and weight of the uteri might be due to the anti-estrogenic activity of a substance (Sreenivasulu, 1992).

It is difficult to interpret the effects of a progestin on the uterine histology because the changes vary by the type of progestin used, concentration of the progestin, duration of use, the degree of inhibition of follicular activity and may also vary depending on whether or not estrogen is used (Smith, 2005; Dinh, 2015). Use of progestin-only contraceptives for long can create an atrophic pattern of the uterine endometrium that consists of small and indistinct glands and are lined by low columnar epithelium (Mazur, 2005). Manautou *et al.*, in 1975 described that the morphological changes of the endometrium caused by LNG are related to atrophy or a suppression of the superficial layers of the endometrium with decidualization of the stroma. These literatures can be correlated to the findings of the present work where decidualized endometrium with atrophied uterine glands and diminished luminal epithelial linning was seen in the uteri treated with Levonorgestrel. Narrow slit- like lumen was exhibited by the LNG treated groups which may be indicative of inhospitable uterus for implantation. Similar results were also observed by Martin *et al.*, 1970 in their work on effects of progesterone and estradiol-17β on the luminal epithelium of the mouse uterus. They found that daily dose of progesterone changes the morphology of the uterine epithelium including closure of the uterine lumen, suppression of epithelial cell division etc. These results

are also in consistent with the results of Alvarez *et al.*, 2009, who found that after LNG treatment the uterus of mice exhibited decidualization of stromal cells; atrophy of glandular epithelium and down regulation of ERα and PR in all cellular components of the endometrium.

A number of workers have described the causes of endometrial changes in different ways. These include the long term use of LTPOC (Hickey, 2000), inhibitory effects of LNG (Hui & Zhao, 2011) and down regulation of expression of ER & PR due to LNG (Salmi, *et al.*, 1998; Alvaez *et al.*, 2009 & Meng *et al.*, 2010). Causes of the endometrial alterations in the present investigation may be attributed to the findings of the above workers.

5. CONCLUSION

From these observations it can concluded that LNG causes some permanent changes in the endometrium which may cause malfunctioning of the reproductive system in future. Further study on this topic including hormone assay or in terms of gene expression might help to find out the appropriate reason and solution.

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