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Progestogenic effect of norethisterone on uterine chemistry and uterine endometrium ultrastructure of albino rat (wistar strain)

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Abstract
Rat endometrium stimulates by progestogen. Synthetic progestogen like Norethisterone heptanoate (Nor-Hn) is used therapeutically. Study of norethisterone to establish a structural, functional properties concerning its contraceptive efficacy. Effects of progesterone on endometrium are dose dependent. Under treatment with norethesterone heptanoate endometrial epithelium showed certain variations. This study is supported with ulatrastructural changes with uterine biochemistry.

Keywords: Nor-Hn, Norethisterone enanthate, therapeutic, contraceptive efficacy, ultrastruture, uterine biochemistry

1. Introduction

Progestogen brings about the changes in the endometrium of the uterus. The changes are such as to favour the implantation of a fertilized ovum and its subsequent gestation. The only naturally occurring progestogen is progesterone but a number of synthetic progestogens are used therapeutically and possess some of the biological actions. The pharmacokinetics of synthetic progestins can vary depending on the route of administration and whether the progestin is given alone or in combination with an estrogen. Animal studies are of little relevance to humans because of differences in absorption and metabolic clearance among various species.

Progestogen exhibits a profound influence on both normal and abnormal endometrial tissue (Robert *et al*. 1974) [17]. This investigation was designed to examine the subcellular alterations in the endometrial glands. The objective was to study the subcellular structural response to norethisterone to establish a structural, functional properties concerning its contraceptive efficacy. Electron microscopy was employed to provide close intracellular scrutiny and it was not possible with light microscopy. The hormonal contraceptive suppresses ovulation and at the same time, affects the endometrium. The net effect of these steroids into the endometrium cannot be predicted.

In the present study the fine structure of the rat endometrium under Norethisterone as a progestogen stimulation was performed to help us acquire an insight into the fine structure of the endometrium and provide us with some knowledge regarding its efficacy as a contraceptive at the endometrial level.

Progestogens were found to have a range of actions which were dose dependent (Larsson-Cohn *et al*. 1970). The contraceptive effectiveness of a number of these progestogens had been described (Larsson-Cohn *et al*. 1970b [11] and Moghissi and Marks, 1971) [15].

An intramuscular injection of 200mg of a synthetic progestogen (Norethisterone oenanthate) compound produced antifertility effect. Its efficacy appeared to be similar to that of DMPA (Geraldine *et al*. 1975) [6]. The uterus as at target organs seemed to react independently to norethisterone reaction (Bjorklund *et al*. 1991) [1]. Progesterone had a relaxant effect on rabbit, rat and human uterus (Journal article, Review. Ginecol obstet, 1977) [8].

There is some evidence that the effects of NOR-HN on endometrium are dose dependent. Blood plasma concentrations are also recorded in this study. Progestogen exhibits a profound influence on both normal and abnormal endometrial tissue (Robert *et al*. 1974) [17]. This investigation was designed to examine the subcellular alterations in the endometrial glands. NOR-HN was administered and the various changes were studied.

2. Materials and Methods

Animals

Young, healthy, sexually mature female albino rats of Wistar strain (120-150 gms body weight) with normal reproductive history were procurred from Haffkine Biofarmaceuticals. The animals were kept under uncontrolled room ambient temperature and photoperiod. Food pellets marketed by Lipton India Limited and water provided ad libitum. The rats were acclimatized for a month to the laboratory conditions prior to the commencement of any experiment. Animals were divided into two sets for control and drug treatment, both the set of an experiment a population of female rats belonging closely to a certain weight group were selected, the reason for which all the groups of rats at the commencement of the treatment did not weigh the same.

The animals were divided into control and experimental groups. The treatment lasted for 24 weeks duration i.e 24 injection of i.m.injectable Norethisterone heptanoate. Drug was of 100% purity which is available in the market with same trade name.

On the completion of the treatment period, the animals were weighed and sacrificed under light ether anaesthesia. Blood was drawn from the ventricles period to the sacrifice. Oxalated and non-oxalated glass bulbs were used for the separation of whole blood, plasma and serum which were used for the biochemical parameters. Care was taken to avoid any hemolysis of the whole blood. Reproductive tract was quickly excised cleared off the adhering fat blotted and weighed after which processed for the various light and ultrastructral and biochemical studies. Simultaneously uterus was separated

from the reproductive tract and processed to extract the uterine tissue for the biochemical analysis.

Drug Chemistry

Norethisterone (norethindrone) C20 H26 O2

Norethisterone is a white or creamy white odourless, crystalline powder with a slightly bitter taste. It is insoluble in fixed oils but soluble in dioxane, most alcohols and slightly soluble in ether. It is prepared by chemical synthesis, usually from the precursors obtained from the Mexican yam (Diascorea).

3. Electron Microscopy

Uterus from the control, and Norethisterone heptanoate treated animals. The organs were sliced into 1mm pieces in a drop of 3 % Glutarldehyde and immersed in fresh ice cold fixative for 2 hours in 0.1 Cacodylate buffer. Tissues were then postosmicated for 2 hours, dehydrated in ascending series of alcohol and finally embedded in araldite ultrathin sections were cut with a glass knife on a LKB-2000s ultramicrotome. The sections were scanned and photographed on a JemJeol 100s electron microscope after staining with Uranyl acetate and Lead Citrate.

4. Result and Discussion

Uterus

Light microscopy

Histologically the uterus is divided into three layers. The outer serosa is perimetrium, the middle muscularis is myometrium and the inner layer as endometrium.

The perimetrium is composed of specialised squamous epithelium. Myometrium is a very thick layer of interwoven bundles of large smooth muscle cells, which are separated by connective tissue.

The endometrium with simple columnar epithelium contains endomerial stroma and uterine glands (fig.1).

The epithelial cells of the rat endometrium are glandular epithelial cells localised in the uterine glands and as luminal epithelial cells cover the uterine surface(fig.1).The surface epithelium is lined by a single layer of columnar epithelial cells having centrally located oval nuclei occuping most of the cells volume surrounded by eosinophilic cytoplasm.

The patches of ciliated cells are seen on the surface of the epithelium facing the lumen of the uterus (fig.1).The major portion of endometrium consists of numerous tubular uterine glands and endometrial stroma which is loosely arranged and often has darkly stained oval nuclei. The stroma is highly cellular with moderate network of blood vessels (fig.1).

NOR- HN Treated Uterus

The effect of norethisterone treatment on endometrium revealed that norehisterone decreased the number of glandular mitosis and induced subnuclear vacuolation. The treatment does not affect glandular epithelial height, stromal mitosis and induced edema, thereby stromal edema is moderate and marked (fig.2).

Luminal epithelial cells changed from cuboidal to tall columnar, in which nuclei back at the basal region and oval in shape. These nuclei are surrounded by moderate eosinophilic cytoplasm (fig.2).

The endometrial glands show suppressed proliferative activity atrophy. The endometrial stroma is made up of loose network of collagenous fibers interspersed with amorphous, non-stained ground substances (fig.2).

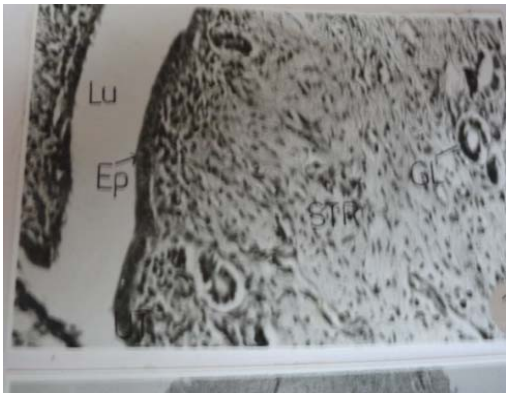

Fig 1: Control Uterus- Lumen (Lu), epithelial (Ep), endometrial stroma (STR), &uterine Gland. (X-75)

Fig 2: Norethisterone treated uterus- Eosinophilic cytoplasm (CY), edematous stroma (STR), Epithelial cell (Ep) contained nuclei (N) towards basal region, uterine lumen (Lu). (X-75)

Uterus: Electon microscopy

The uterus of the control animal shows following ultrastructrural features. Fig. 3 and 4 shows the salient features of the supranuclear cytoplasm of the younger population of gland cells including some strands of granular endoplasmic reticulum with cisternal distension, free ribosomes, several pleomorphic (round, oval and elongated) mitochondria, prominent Golgi membrane, occasional lipid droplets and a multivesicular body. The apical surface bored microvilli, some of them had a slender shape.

The nuclei of the glandular cells of the control specimen were oval and did not exhibit a degree of irregularity in their contour. Nuclei were placed mostly in the middle to mid basal portion of the cell with nucleoli. Nuclear membrane was evenly approximated and regular. Nucleoli were placed peripherally (fig.3) and this inclusion assumed a central position with an ovoid, regular nucleus.

The mitochondria seen in the glandular cells of the control specimen were pleomorphic, predomonantly situated in the apical portion of the cell and their cristae were infrequent, only a few extending completely across the organelle. The matrix of the mitochondria was homogeneous, some mitochondria showed loss of cristae may be because of an artifact (fig. 4).

Granular endoplasmic reticulum as described by many investigators, was common in the glandular cells of the control

specimen. The rough endoplasmic reticulum appeared chiefly as randomly scattered profile of irregular vesicles and dilated short tubular structures (fig.3). Moderate number of ribosomes was present in the cytoplasm (fig.3 and 4).
The Golgi apparatus of the glandular cells was prominantly positioned apically (fig 52 and4). The cisternae were well organised and closely approximated but occasional cisternal distension was noted (fig.4).
Multivesicular bodies were commonly found in the apical cytoplasm and within these bodies were found variable numbers of small vesicles which were fairly uniform in size. The background matrix of the multivesicular bodies was generally of lighter density than the cytoplasm (fig. 3, 4). Lysosomes like bodies were also present.

Control Uterus

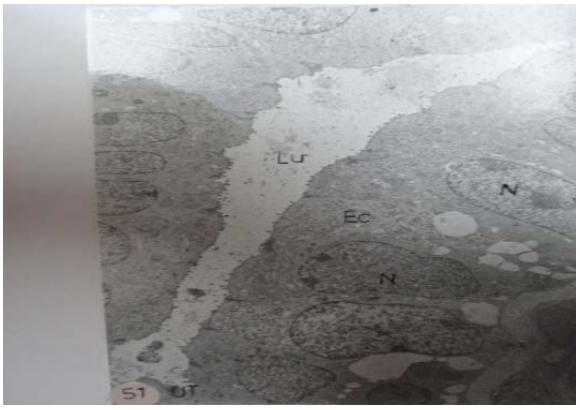

Fig 3: Low power electron micrograph of Endometrial epithelium of control uterus surrounding the lumen(Lu), Elongated epithelial cells(Ec), Nucleus(N) more toward the basal end, sparse chromatin network with irregular contour. (X- 2000)

Fig 4: High power electron micrograph showing scanty microvilli (MV) Mitochondria(M), Golgi apparatus(G), few profile of endoplasmic reticulum(ER), ribosomes(R), dilated inter cellular membrane. X- 10,000)

Noretisterone Treated Uterus

Under treatment with norethesterone heptanoate endometrial epithelium showed certain variations. The microvilli decreased in size, frequency and regularity after treatment and became low clubbed and inactive as they appeared shorter when a cross section was observed. (fig.5&6).
The nuclear characteristics varied after treatment with the development of nuclear irregularity and invaginations. There were two types of nuclei present in the glandular cells after norethesterone administration. The first type was similar to that seen in the controls. The second type of nucleus contained an inclusion that was granular in nature. Nucleoli were placed peripherally in an irregular nucleus and was placed centrally with an ovoid regular nucleus (fig.5).
The cytoplasm was dense with an abundant group of ribosomes i.e. polysomes (fig.6) but the frequency of ribosomes did not alter.
The mitochondrial morphology was similar to the controls but mitochondrial enlargement was noticed with no change in the cristae pattern (fig.6). The mitochondrial matrix was homogenous and mitochondria was more frequent in the apical portion of the cell (fig.6).
The granular endoplasmic reticulum was present to a less degree and exhibited a random distribution in the cytoplasm. The cisternae became progressively shortened (fig.6).
The Golgi apparatus exhibited more cisternal distention. Certain cisternae were well organised and closely approximated and less distended. Multiple Golgi apparatus were frequently noted. They were again well organised with an electron dense matrix (fig.6).
Lysosome bodies were less frequent in treated specimens than control specimens (Fig. 6)

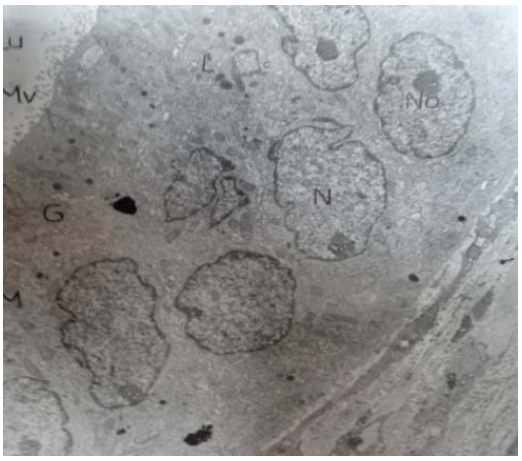

Fig 5: NOR-HN treated Uterus shows the cells are oval and cuboidal, Nucleus (N) Mostly spherical and irregular in shape with centrally placed nucleioli (No), & microvilli (Mv). (X-2000)

Fig 6: Microvilli are accumulated in the lumen (Lu), Mitochondria (M) with unchanged cristae, the bodies exhibited more cisternal distention (G), and increased in number. (X – 10000)

Total Uterine Profile
Biochemical Assay

Table 1: X ± SEM Values

Parameter	Control values X1 (6)	Norethisterone Treated values X1 (6)
Alkaline Phosphatase	60.25 ± 17.2	238* ± 58.1
Acid Phosphatase	16.3 ± 3.12	22.75 ± 2.54

(P > 0.05 significantly different)

Table 2: X ± SEM Values

Parameter	Control values X1 (6)	Norethisterone Treated values X1 (6)
Sodium	156.5 ± 2.04	130.25* ± 6.33
Potassium	1.4 ± 0.315	2.3 ± 0.61
Calcium	1.1 ± 0.115	1.43 ± 0.31
Chloride	165 ± 0.865	149.25 ± 9.335
Triglyceride	10 ± 0	8.5 ± 2.3
Cholesterol	13 + 0.5	7.25 ± 3.04

(P > 0.05 significantly different)

Alkaline Phosphatase
Alkaline phosphatase activity was demonstrable in the uterus of both experimental and control animals. Significant increase was registered after the treatment of Norethisterone (Table No.1).

Acid Phosphatase
Acid phosphatase activity increased under the influence of Norethisterone but it is not significant. (Table No.1).

Sodium
Volumes of uterine extract collected after the contraceptive norethisterone treatments. Sodium levels of the animal significant decrease observed in Norethisterone treated animals. (Table No.2).

Potassium
The mean potassium concentration was unchanged after the NOR-Hn (Table No.2)

Calcium
Serum calcium no significant change was observed in norethisterone treated animals (Table No. 2).

Chloride
The concentration of chloride in animals treated with norethisterone decreased non-significantly. (Table No. 2).

Triglycerides
The uterine epithelial triglycerides varied quantitatively with the reproductive state of the females. The levels of triglyceride present after the treatment of norethisterone, were low. (Table No. 2).

Cholesterol
Concentration of cholesterol no significant decrease was observed after norethisterone treatment. (Table No. 2).

5. Discussion
The results of present study demonstrate the effect of Norethisterone heptanoate (NOR-HN), on alkaline phosphatase, acid phosphatase, sodium potassium, calcium, cholesterol and triglycerides in rat uterus. The alkaline phosphatase levels significantly increased after norethisterone.
Alkaline phosphatase is associated with the apical plasma membrane of the uterine glands in the uterine cavity. It is related with the process of growth and differentiation of new cells and also associated with membrane transport (Charles Flower, 1974) [4], According to McAlpine, (1955) [14], alkaline phophatase is a histochemical marker for primordial cells of rat.
Histochemical investigations of the endometrial alkaline phosphatase activity levels vary with the stage of the estrous cycle and this estrous cycle is under the influence of hormones as revealed by Moss *et al*. (1954) [16], Skjerven (1956) [18]. The present findings mean that NOR-HN, may alter the transport mechanism across the cell surface. Acid phosphatase (ACP) level registered no significant increase after NOR-HN treated rat uterus. Acid phosphatase is a hydrolytic enzyme associated with lysosomes and Golgi apparatus (Dott and Dingle, 1968) [5]. Function of this enzyme includes degradation and digestion of the secretory products and tissue debris, Charles Flower, (1974) [4].
Increased level of acid phosphatase is supported by ultrastructural observation of uterus which indicate the presence of lysosomal bodies and Golgi apparatus in the cytoplasm in the norethisterone treated rat uterus. The relationship of both acid and alkaline phosphatase activity to the stage of the estrous cycle compares closely with histochemical determinations of the same enzyme in the endometrium found by Kenney (1964) [10].
The uterine sodium levels after norethisterone treatment decreased. Level of potassium Nor-HN, treated rat, potassium level increased non-significantly.
Howard and Defeo (1959) [7] found that sodium level increased at the expense of potassium level in the uterine fluid. This was not the case in the present study. It is difficult to make a direct comparison between these results, however we can say that the contraceptive efficacy of hormones on these elctrolytes is independent.
Calcium concentration in uterus in norethisterone treated animals calcium level was unchanged. The comparison of the uterus in the rat is under the control of hormones was demonstrated by the fact that the concentration of potassium, chloride, calcium, sodium alkaline phosphatase and acid phosphatase, the greatest variations occured due to progestogenic effect.
In progestogenic groups (Norethisterone) no significant alteration was observed in uterine triglyceride levels which may be due to a lack of significant effect on uterine triglyceridogenesis.
Triglycerol metabolism in the uterine epithelium reflects the reproductive state of the female which itself is under control of the ovarian hormones (Boshier *et al*.; 1981) [3]. Although functional correlations of these changes in uterine triglycerol content must be conjectural (Boshier *et al*.; 1981) [3]. Boshier (1976) [2], Kennedy (1977) [9] have suggested that the epithelial triglycerides would be a suitable and readily available energy and metabolites source for the use by the uterus.
In case of uterine cholesterol, significant changes are manifested by the component of norethisterone administration significantly decreased the uterine cholesterol concentration,

Changes of level of cholesterol studied by Moss *et al*. (1954) [16], Skjerven (1956) [18] by progestogenic compound. Our data suggest that progestogen alone with low potency counts for major alteration in cholesterol and triglyceride profile.

The current study is designed to give answer to, whether the activity of cholesterol is sensitive to the presence of sex hormones. (Lichenstein *et al.* 1983 [12] and Martinez *et al.* 1990) [13]. Administration of these hormones modulates cholesterol activity.

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