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A Morphometric Study on the Endometrium of Rat Uterus in Hypothyroid and Thyroxine Treated Hypothyroid Rats

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ABSTRACT

Hypothyroidism increases the rate of pregnancy loss. Other manifestations include menstrual disorder, and infertility. Serum levels of gonadotropins are low in hypothyroid patients. Though studies of uterine ultrastructure are well established as approaches to investigating the pathophysiology of infertility, they have scarcely been extended to the study of hypothyroid related infertility. The present study investigates the effect of hypothyroidism on the ultrastructure of uterine epithelium.

Three groups of Wistar rats were studied. Two groups were initially made hypothyroid using methimazole, and the third group was an untreated control. One hypothyroid group was given daily injections of thyroxine for six weeks. The uteri were removed in all three groups, and processed for transmission electron microscopy and morphometry. It was found that absolute epithelial cell volume was decreased in hypothyroidism. The volume of the nucleus had decreased though its relative volume in the cell had increased. The height of the luminal epithelium in hypothyroid rats also decreased by (33.8%) as compared with controls. Basement membrane thickness was significantly increased in hypothyroidism. The changes were all substantially abrogated by the administration of thyroxine.

This study suggests that thyroid hormones might be importantly concerned in the maintenance of the normal structure of uterine epithelial cells.

INTRODUCTION

It is well known that human hypothyroidism is almost always associated with sub fertility (1). Hypothyroid women are known to have difficulty in getting pregnant (2-3). Furthermore, should a hypothyroid individual get pregnant, successful conclusion of pregnancy is difficult there being a high risk of both abortion, and still birth in such mothers (4-5). Gonadotropin profiles in hypothyroid women have been extensively

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investigated (6-7). Most findings have demonstrated a decrease in the serum level of FSH and LH (8-9). Based on this observation. It has generally been believed that the reason for infertility in hypothyroid women was anovulation, as there is poor ovarian stimulation by the pituitary gland (10). However, until recently the structure of the hypothyroid uterus had been little studied. The study of uterine epithelial morphology has importance since for implantation to occur, there has to be a process whereby the fertilised ovum and the surface epithelium interact. This necessitates the epithelium being adequately prepared for pregnancy. Indeed, surface epithelial structure had been shown to determine the success of implantation (11). IT has been reported previously that methimazole-induced hypothyroidism caused gross changes to the histology of the uterus (12). These alterations could functionally translate into an inability of the uterus to maintain pregnancy, and hence explain why abortion is common in hypothyroidism.

The aims of this study were to establish via morphometric methods whether Hypothyroidism had any effect on the ultrastructure of rat uterine luminal epithelium.

MATERIAL AND METHODS

Animal model

Hypothyroid Wistar rat was used as a model for the study. Hypothyroidism was created by oral administration of 0.02% Methimazole in the drinking water (12-13) Two groups (A and B) were initially made hypothyroid, with one group, (B), receiving exogenous thyroxine intraperitoneally after hypothyroidism was established. A third control group. (C), of normal rats was maintained on tap water. Daily vaginal smears were obtained from rats in all groups to determine the stage of the oestrus cycle. The animals were six weeks old at the start of treatment and were killed after a further six weeks. Each rat was killed by an intraperitoneal overdose of anaesthetic (xylazine and ketamine hydrochloride in ratio of 1:2 v / v) before removing the uterine horns.

Tissue processing

One horn from each rat was randomly selected and cut transversely into three equal portion. Each portion was further chopped into pieces of about 1 mm^3 and then fixed in 2.5% phosphate buffered glutaraldehyde (pH 7.24) for 1 8 hours at 4°C. The fixed pieces were briefly washed in phosphate buffer (pH 7.2) dehydrated in ascending concentrations of ethanol up to and including dried absolute ethanol. The dehydrated tissues were then immersed in propylene oxide for 20 minutes with one change followed by infiltration in a mixture of Araldite and propylene oxide (ratio 1:1) for 30 minutes. The infiltrated tissues were then dropped into pots containing fresh 100% Araldite resin and left to polymerise at 60° for 48 hours (14). The tissues were embedded in this way so as to ensure randomness in orientation of the tissue when sections were obtained. This was achieved by letting the tissue sink to the bottom of the embedding moulds unaided. Blocks were selected, and trimmed, and

semi-thin sections (t = 0.5µm) obtained on a Reichert-Jung ultramicrotome (JX 301). These were stained with Toluidine Blue and examined under a light microscope (Leitz Laborlux K). A ribbon of ultra-thin sections was then cut using a diamond knife (Diatome MX 3776). The sections were expanded with chloroform vapour, and those producing silver-grey interference colour were picked up on 200µm mesh copper grids (Gilder), stained with uranyl acetate, followed by lead citrate (15).Electron micrographs were taken on cut film with a Philips 301 transmission electron microscope operated at an accelerating voltage of 60 kv. Micrographs used for morphometric estimates were taken at a nominal magnification of x1300. A micrograph of a cross-grating replica (2160 /mm) was included in each negative series as a magnification standard.

Sampling protocol

A systematic random sampling procedure was carried out in order to obtain a representative sample of tissue from each group of animals (16). Six animals were used in each group. In an earlier pilot experiment, using a two-way ANOVA we had established that there was no significant difference in structure between the two horns of the same rat, and between the various regions of the same horn (12). One horn from each rat was randomly selected and cut transversely into three equal portion. Each portion was further chopped into pieces of about 1 mm side and processed into Araldite blocks for TEM. From each animal, three blocks were selected by lottery (one block per portion). From each block a ribbon of silver sections was obtained and examined under a transmission electron microscope. A raster of systematic random fields of view was obtained on the microscope and six micrographs per block were taken. From each rat six micrographs were selected by lottery (two from each block), and measurements carried out.

Morphometry

Morphometric analysis was performed on electron micrograph negatives viewed under a projecting microscope at *x*13 magnification. An overlay screen bearing an array of points was randomly applied onto the projected image, and the number of points hitting various components of the cell were counted and used to estimate volume densities (Vv). Epithelial cell height was measured on profiles showing the whole epithelium from basement membrane to the luminal surface taking into consideration the micrograph magnification. The volume-weighted mean volume of epithelial cell nucleus was estimated b\ the point-sampled intercept method (17-18). A random array of test points was superimposed on the electron micrograph negatives. Where a point hit a nuclear profile, a line which crossed the profile was drawn through the point at a randomly selected angle. This was repeated for all nuclear profiles hit by the test points. The part of the line which intercepted the nuclear membrane was measured (*l*).Each intercept was then raised to the power of 3 (l^3) and the mean (l^3) was calculated. This mean nuclear intercept length was used to estimate the nuclear volume thus;

$$\overline{V_V} = \frac{\pi}{3}\overline{l^3}$$

The major *(a)* and minor *(b)* axes of the epithelial nuclear profiles were also measured, and the mean diameter *(d)* obtained from

$$d = \frac{a+b}{2}$$

The axial ratio $\frac{a}{b}$ of the nucleus was also calculated (19). Absolute volume V, of the average epithelial cell was estimated using the volume density, Vv, and volume weighted mean volume of the nucleus $\overline{v_v}$ using the formula;

$$V = \overline{Vv}/Vv$$

Harmonic and arithmetic mean thickness of epithelial basement membrane as measured using the orthogonal intercept method (20-21). Micrograph negatives containing slices of the luminal epithelium were projected at a final magnification of x12744. This image was overlaid with random test lines that made chance intersections with the basement membrane surface. Taking the plasmalemmal side of the basement membrane as the horizontal, a line was drawn at 90° (orthogonal) through the intersection point towards the outer edge of the basement membrane. The lengths of these orthogonal intercepts were measured using a logarithmic ruler. These, together with the number of observations, were used to estimate 'harmonic mean' thicknesses of the basement membrane, t_h (20-21). All variances were computed and expressed on a 'between animal' basis. Analysis of differences in absolute cell and organelle volumes, epithelial height, and basement membrane thickness between animal groups was performed using the non-parametric Mann-Whitney-U test. In each case, the null hypothesis was rejected if the probability of no difference was found to be less than 5% (i.e. $P < 0.05$). All statistical computations were made using the Instat statistical package, version 1.15 (Graphpad Softwares, 1990) run on a personal computer.

RESULTS

Ultrastructure of normal uterine horn luminal epithelium in the rat

Luminal epithelial cells in uterine horn of control rats were more or less uniform. They were columnar shape sitting on a thin basement membrane, and having clear cut cell borders as shown in Figure 1A. The nucleus, placed one third of the way up the cell, was ovoid and mainly euchromatic with a prominent nucleolus. It was about one fourth

of the length of the cell (axial ratio 3.1). Cytoplasm was rich in rough endoplasmic reticulum, with a few lipid droplets situated mainly in the infranuclear region. The luminal surface had numerous slender microvilli with a few electronlucent vesicles just beneath them. Giant autolysosomes were present occasionally in the infranuclear regions of the cells.

Ultrastructure of hypothyroid rat uterine horn luminal epithelium

As shown in Figures 1B and 2B epithelia from uterine horn in hypothyroid rats were also columnar and lay on a thicker basement membrane than that below normal epithelia (Fig 2A). Nuclei were also ovoid, mainly euchromatic and with a prominent nucleolus. The nuclei were about one half of the cell height, and were situated in the bottom halves of the cells. Lipid droplets were abundant in the cytoplasm and were situated both in the supranuclear and infranuclear regions. Rough endoplasmic reticulum was scanty. Microvilli appeared shorter than in euthyroid epithelia. All the epithelial structural changes observed were less pronounced in hypothyroid rats given thyroxine.

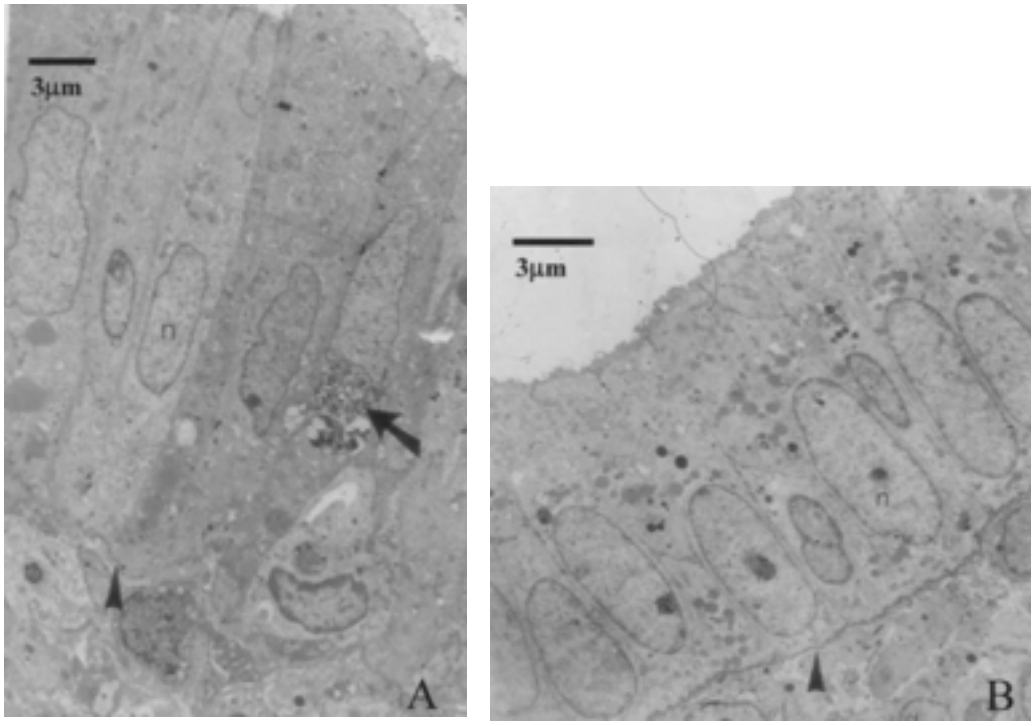

Figure 1. (A) Uterine horn luminal epithelium in control rat. Notice the giant autolysosomes (long arrow) typical of oestrus stage, and a thin basement membrane (arrow head). Nucleus (n).
(B) Uterine horn luminal epithelium in hypothyroid rat. The cells were shorter, with a much thicker basement membrane (arrow head) as compared to euthyroid epithelia. Autolysosomes are absent

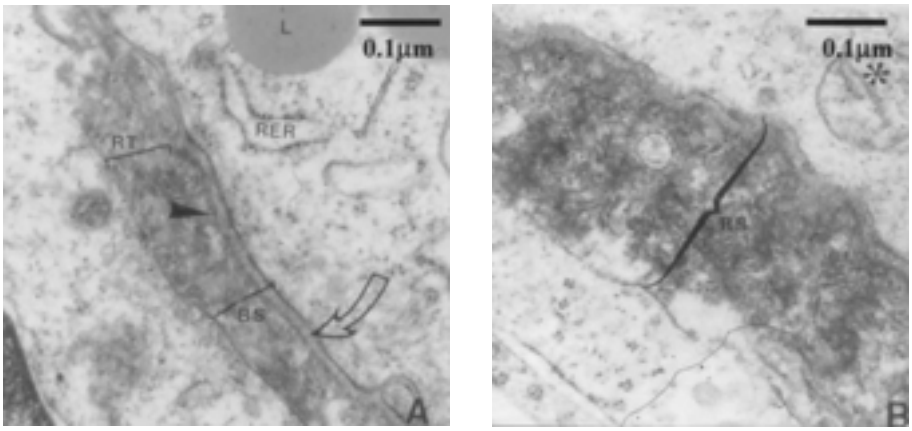

Figure 2. (A) High power view of the basement membrane (BS) in euthyroid rat luminal epithelium. Notice the lamina lucida (open arrow) and the reticular lamina (RT) Lipid droplet (L).
(B) High power view of the basement membrane (BS) in hypothyroid rat luminal epithelium. Notice the increase in thickness.

Estimated parameters of normal rat uterine horn luminal epithelium

The mean absolute volume of a luminal epithelial cell (cv % in parentheses) was 1235.50 (17.5) μm^3 with a mean height of 35.14 μm (18.2). Volume weighted mean volume of the nucleus was 222.39 (21.6) μm^3 and the nuclear/cytoplasmic ratio was

Variable	Control	MMI	MMI+T4
Epithelial Height (μm)	35.14* (18.2)	23.42 (12.4)	27.81* (18.5)
Nuclear/Cytoplasmic ratio	0.22* (19.9)	0.35 (15.7)	0.26* (19.5)
Nuclear profile diameter (μm)	7.18 (16.7)*	6.27 (20.9)	6.47 (14.3)
Nuclear profile Axial ratio	3.1 (14.8)	2.9 (27.9)	2.8 (18.5)
'Harmonic mean' basement membrane thickness (nm)	143.86* (25.5)	252.93 (18.1)	184.52* (16.9)
Arithmetic mean basement membrane thickness (nm)	187.53* (3.9)	272.07 (9)	232.96* (8.6)

*p<0.05 vs hypothyroid rats

Tabell 1. Mean and coefficient of variation (CV%) of volumetric parameters of different compartments of the uterine horn luminal epithelium in euthyroid (control), hypothyroid (MMI) and thyroxine treated hypothyroid rats (MMI+T4).

0.22 (19.9). Mean volume fraction of nucleus in the cell was 0.18 (21.6) whilst that of mitochondria in cytoplasm was 0.061 (19.5), and lipid droplets 0.03 (33.8). Harmonic basement membrane thickness was 143.86 (25.5) nm. Mean nuclear diameter was 7.18 (16.7) μm with an axial ratio of 3.1 (14.8) (see Tables 1 and 2)

Compartment	Volume densities Vv			Absolute volumes V (μm^3)		
	Control	MMI	MMI+T 4	Control	MM!	MMI+T4
Nucleus	0.18* (21.6)	0.26 (18)	0.21* (23)	222.39* (21.6)	88.55 (17.2)	134.90* (23.3)
Mitochondria	0.061 (19.5)	0.060 (15.1)	0.060 (12.5)	75.36* (19.5)	20.43 (15.1)	39.18* (12.5)
Lipid droplets	0.029* (33.8)	0.11 (13)	0.053* (18.6)	35.83* (33.8)	37.46 (13)	34.04* (18.6)
Whole cell				1235.50* (17.5)	340.57 (14.4)	642.38* (15.1)

* = P<0.05 vs hypothyroid rats.

Tabell 2. Mean and coefficient of variation (CV%) of morphometric parameters for uterine horn luminal epithelium in euthyroid (control) hypothyroid (MMI) and thyroxine treated hypothyroid rats (MMI+T4).

Changes in parameters of the luminal epithelium in hypothyroid rats

Changes in volumetric and other parameters in hypothyroid rat uterine horn luminal epithelia are illustrated in Tables 1 and 2. The absolute volume of the average epithelium was reduced by 72.4% in hypothyroid (MMI) animals as compared to euthyroid (control) animals, whilst in hypothyroid rats given thyroxine the decrease was by 48%. Nuclear volume decreased by 60.1% and 39.3% in hypothyroid, and hypothyroid rats given thyroxine respectively. However, the volume density of nucleus in the cell was increased significantly in hypothyroid rats as compared with euthyroid ones. Total volume of lipid droplets, was increased by 4.5% in hypothyroid as compared with euthyroid rats. There was no increase in lipid volume in hypothyroid rats given thyroxine. Mitochondrial volume density was unchanged in all three groups, though its absolute volume had decreased by 72.8%. in hypothyroid (MMI), and by 48% in hypothyroid rats given thyroxine. The difference in parameters between hypothyroid rats given thyroxine (MMI+T4) and those not given (MMI), was significant at $p<0.05$ (see Table 1). Nuclear-cytoplasmic ratio was significantly higher in hypothyroid rats as compared with euthyroid ones. Basement membrane thickness was increased by 75.8% in hypothyroid, as compared with euthyroid rats. This increase however, was by 28.3% in hypothyroid rats given thyroxine when compared with euthyroid animals. Height of luminal epithelium had decreased by 33.3% in hypothyroid (MM1) as compared with euthyroid (control) rats. in hypothyroid rats given thyroxine (MMI+T4) the decrease

was by 20.8%. The diameter of epithelial nuclei was greater in euthyroid than in hypothyroid rats ($p<0.05$) though the mean nuclear axial ratio was not significantly different in all three groups (see Table 2).

DISCUSSION

This study has demonstrated the occurrence of significant structural change in the uterine horn luminal epithelium in hypothyroidism. Such change was decreased by thyroxine administration. The improvement in epithelial morphometric parameters effected by exogenous thyroxine administration to hypothyroid rats in this study. suggests that in healthy animals the presence of the hormone helps maintain normal epithelial structure. This correlates with the clinical experience which demonstrated that thyroxine administration helped correct menstrual irregularities in hypothyroid women (22-23) Similarly, in pregnant hypothyroid women thyroxine administration improves the outcome. The decrease in cell size, nuclear volume. and mitochondrial volume in hypothyroid animals could be thus a direct effect of thyroxine deprivation, particularly since thyroid hormone is believed to exert its influence on cells by facilitating the transcription of DNA and hence new protein synthesis. Indeed, the rat uterus has been shown by Evans et al. (25) and Mukku et al. (26) to contain thyroid hormone receptors suggesting that it is a specific site for thyroid hormone action. Absence of the hormone could thus slow down the epithelial metabolic processes resulting in a decrease in cell and nuclear volumes as well as a decrease in mitochondrial volume. The fact that this study demonstrates in hypothyroid uterine epithelia a decrease in nuclear size apparently without alteration of shape further suggests that there may be a decrease in nucleic acid transcription in the nuclei of these cells. An increase in nuclear-cytoplasmic ratio coupled with a decrease in total cell volume in hypothyroid animals suggests that the cytoplasmic volume was far more reduced than was the nuclear volume. As the basement membrane is composed of collagen (including type IV), and a number of glycosaminoglycans, an increase in its thickness suggests that hypothyroidism influences the turnover of these components. Harvey et al. (27) have shown that hypothyroidism is associated with a significant decreased collagen breakdown. Others (28-30) have reported increased deposition of collagen type IV in tissues from hypothyroid individuals. The apparent increase in collagen and glvcosaminoglycan deposition in hypothyroidism is thus probably not due to an increase in synthesis, but to a decreased breakdown of these substances (31). Since the thickness of basement membrane is known to be inversely proportional to the rate of diffusion of substances across that membrane (32), the increase would be expected to impair diffusion to the basal aspect of the cells. This would be of particular relevance with respect to large molecular weight substances such as hormones, enzymes etc. Impaired diffusion of such control substances is likely to result in altered metabolism and probably impaired morphology, which might explain the generalised decrease in cell size observed

here. Similarly, an increase in basement membrane thickness might well adversely affect specific epithelial function as in the case of medical conditions such as glomerulonephritis. in fact, there are also reports in the literature of hypothyroidism being associated with basement membrane damaging glomerulonephritis (33-34). Hypothyroidism is known to increase the serum levels of lipids and cholesterol in humans. It also causes excessive deposition of fat under the skin (35). The present study shows that extra lipid accumulation occurs within the uterine epithelium suggesting that hypothyroidism may influence generally cellular lipid metabolism. The fact that the observed changes in epithelial structure in this study were similar to those found during dioestrus stage of the oestrus cycle in rats by Spornitz et al. (36) further suggest that hypothyroidism affects ovarian steroid hormone metabolism. Therefore, alteration in steroid hormone levels during hypothyroidism is especially likely since the dioestrus stage in rats is characterised by low steroid hormone levels, and the hypothyroid rats in this study had a preponderance of dioestrus vaginal smears (12). However, it could also be claimed that the changes we have seen might have been due to another indirect effect of hypothyroidism on gonadotropins, since it has been shown that hypothyroidism leads to a decrease in the serum levels of the pituitary gonadotropins FSH and LH (8, 37-38). This could in turn lead to a decrease in ovarian stimulation and hence a decrease in ovarian steroid hormone levels, causing a decrease in uterine epithelial stimulation resulting in the structural changes observed in this study.

In conclusion, it seems reasonable to suggest that though the rarity of pregnancy in hypothyroid women has generally been passed off as due to a high incidence of anovulation (39), the observations made here suggest, that in addition epithelial structural change might be present contributing an adverse affect by making interaction with fertilised ovum less successful. Similarly, altered steroid hormone metabolism in hypothyroidism could disturb an existing pregnancy. Study of ovarian histochemistry in hypothyroidism, particularly investigation of the activity of steroid synthesising enzymes are indicated and these may prove important since progesterone deficiency is well known to result in infertility, or during pregnancy to abortion.

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