Fine structure of prolactin cell of female albino rat as affected by some antifertility drugs - A comparative electron microscopic study

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One antioestrogenic compound as well as some antifertility drugs have been administered to female albino rats over a period of six months to study their long term effects on fine structures in PRL cell. Almost in all the cases, the dynamics of hormone synthesis and secretion have been affected. Fine structure is suggestive of activation of synthetic machinery of the cell. The cell picture under the estradiol valerate regimen presents a transitional stage progressing towards involution due to accelerated cell cycle. Sparse granulation, frequent granule extrusion and misplaced exocytosis under the influence of tamoxifen citrate or levonorgestrel + ethinylestradiol are similar to those observed in adenomatous PRL cell. Fine structural correlates of stepped up synthesis are also observed following chronic progesterogenic influences of progesterone and norethisterone heptanoate, but the magnitude of the change is on a lower scale. All the fine structural changes have been discussed in the context of ultrastructural pathology.

Information is meagre about the effects of fertility regulating drugs on fine structure of anterior pituitary, which is often the principal site of action of such drugs. Even when the drugs exert their effect through a peripheral mode following a long term administration, alterations in pituitary cytology occur due to feedback mechanisms as a sequel to the disturbed endocrine milieu of the subject.

Family planning programme in India as well as in other countries implies an ever increasing exposure of more and more women to such fertility regulating drugs. Unfortunately, there is little evaluation, if any, on an individual basis, nor is there an adequate infrastructure for a subsequent follow up. Indiscriminate and long term use of contraceptive drugs is likely to increase the chances of long term endocrine disturbances including adenomatous lesions of the pituitary. Seriousness of the issue is also compounded by the fact that silent pituitary adenomas do not reflect in hormone assays and may remain undetected. Hence there is a scope for screening these drugs on the basis of their effects on the ultrastructure of the adenohypophysial cell types using a suitable animal model.

Drugs administered in this study fall under four categories. They are synthetic antioestrogenic (tamoxifen citrate), oestrogenic (oestradiol valerate), progestogenic (progesterone and norethisterone heptanoate) and a combination regimen of progestogen and oestrogen (levonorgestrel + ethinylestradiol). The targeting of prolactin cell (PRL cell) for this study can be justified because a cyclicity of morphological alterations of this cell synchronised with reproductive cycle has been demonstrated in different mammals.6 Besides, this cell is known to be very sensitive and it is possible to alter the orderly intracellular processes of prolactin synthesis by physiological changes as well as by experimental manipulations.7 Furthermore, gonadal dysfunction in physiological, pathological or experimental hyperprolactinemia is also well known8-11. However, despite the established sensitivity of this cell to a wide range of endogenous and exogenous influences, very scanty information is available, so far as its altered morphology under these influences is concerned.

Materials and Methods
Young adult female albino rats of Wistar strain (200 ± 10 g) with a history of proven fertility were procured from Haffkine Biopharma, Mumbai. The animals were acclimatized for a period of 15 days and divided into 5 sets with 8 animals in each set. These animals received the drugs as per the experimental protocol mentioned in Table 1. A control set of 8 rats was also simultaneously maintained. Pelleted rat food (Lipton India Ltd.) and water were available to the animals ad libitum. On completion of the treatment
period, the animals were anaesthetised and killed by decapitation. Their pituitary glands were removed quickly and processed for transmission electron microscopy.

Transmission electron microscopy—Pituitary glands were sliced into 1 mm pieces in a drop of glutaraldehyde fixative (3%) and immersed in ice cold fixative for 2 hr, followed by transfer to cacodylate buffer (0.1M). These were then rinsed briefly in buffer and post-osmicated in osmic acid (1%) for 2 hr. Thereafter the tissues were dehydrated in ascending series of alcohol and propylene oxide, and finally embedded in resin, polymerised at 60°C. Blocks were prepared in araldite and 1μm sections were cut with a glass knife on LKB-2000 ultramicrotome, mounted on glass slides and stained with buffered toluidine blue. Appropriate areas were selected from a light microscope and ultrathin sections were then cut with a diamond knife, picked on copper grids and stained with uranyl acetate and lead citrate for final viewing. These sections were scanned and photographed on electron microscope (JEM 100 S Joel).

Results

Ultrastructure of PRL cell of female control rat—This was a large elongated cell with large and eccentrically placed nucleus. The nucleus showed a distinct nucleolus and margined chromatin. The most conspicuous feature of this cell was its abundant, polarised but well organised RER, displayed in loose lamellar arrays in the peripheral cytoplasm. Other noticeable fine features were the juxta-nuclear golgi zone. Condensing secretion (forming secretory granules) was often seen in the core of this golgi zone. Mitochondrial inclusion in this cell was heavy, most of these being elongated with normal matricial density clear cristae. Occasionally, solitary multivesicular body was noticed distal to the golgi zone (Fig. 1).

Effect of tamoxifen citrate—Enlarged PRL cell of the female albino rats treated with tamoxifen citrate had increased density of its cytoplasm due to rich concentration of polyribosomes. The nucleus was somewhat round and indented with margined chromatin (Fig. 2). There were severely depleted granulation and frequent exocytosis (Fig. 2). RER showed noticeable augmentation. Very often the cisternal membranes became diffused and non-discernible due to engorgement of their lumen by secretion (Fig. 3). Irregular whorling of RER cisternae

| Table I—Experimental protocol (drugs administered for 24 weeks). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Dosage per 100g/week | Tamoxifen citrate | Oestradiol valerate | Progesterone | Norethisterone heptanoate | Levonorgestrel + Ethinyloestradiol |
| Vehicle | Ethanol & sesame oil | Olive oil | Arachis oil | Olive oil | Alcohol & water |
| Mode of administration | sc | im | im | im | Oral intubation |

Fig. 1—Electronmicrograph of the PRL cell of a control female rat. The large elongated cell has a large eccentrically placed nucleus. Note the abundant, polarized and well organized lamellar arrays of RER, and the core of golgi zone with prosecretory granules (PSG). Mature secretory granules are typically pleomorphic. A heavy mitochondrial inclusion is noticeable. [X 16,000]
Fig. 2—Electron micrograph of the prolactin cell of female albino rat treated with tamoxifen citrate for 24 weeks. The cell has an indented nucleus (N) with chromatin margination. Note the severely depleted granulation and frequent exocytosis (arrows). Sizeable lysosomes (L) have appeared in the cytoplasm. [X 8000]
Fig. 3—Electron micrograph highlighting a small portion of the prolactin cell of the female albino rat treated with tamoxifen citrate for 2 weeks. The cisternal membranes of the rough endoplasmic reticulum (RER) appear diffused due to engorgement of their lumen by secretion (arrow). Note the precocious granule (arrow) in the cisternae of rough endoplasmic reticulum. Note the appearance of lysosomes (L; [X 13,000])
and inclusion of secretory granules as well as deformation of mitochondria in their cores was another manifestation of the drug treatment (Fig. 4a). There seemed to be precocious formation of secretory granules within the RER cisternae (Fig. 3). Dilated and distorted golgi zones were seen, aggregating prosecretory granules (Fig. 4b).

Enlarged mitochondria lying on the trans side of the golgi zone were characterized by degenerative changes like break in wall and loss of cristae (Fig. 4b). Presence of multivesicular bodies and lysosomes (Fig. 2) is also clear in this cell following chronic tamoxifen treatment.

**Effect of oestradiol valerate**—Prolactin cell of oestradiol valerate treated female rats were characterized by hypertrophy (Fig. 5a), small nucleocytoplasmic ratio and eccentrically placed nucleonema. Characteristic sparse granulation, commonly occurring exocytosis and conspicuously augmented RER membranes were other ultrastructural changes induced by this drug (Figs 5a, 5b). RER had undergone the most dynamic change following oestrogen administration, as endorsed by the frequently encountered nebenkerns formed by 12 to 14 concentric membranes of ergastoplasm with attached ribosomes (Figs 5a, b). Some of the concentric membranes presented themselves as rounded masses, whose cores showed focal vacuolar dilatations with either sequestered cytoplasm and mitochondria (Fig. 5b). Another morphological variant of RER was the dense lamina formed by an intimate apposition of the confronting cisternae and eventual disintegration of the ribosomes trapped between them (Fig. 5a). Associated with these changes, were strong dilations and distortions in golgi sacculles, presenting vacuolated appearance to the cell.

Enlarged mitochondria showed breaks in the membranes, disintegrated cristae and matricial lucency (Figs 5a,b,c,d). Though not consistent, there were also present large lysosomes which were fused with the secretory granules as well as with light multivesicular bodies and the annular lipid inclusions (Fig. 6).

**Effect of progesterone**—Following progesterone administration, PRL cell got elongated and somewhat irregular in shape. Its euchromatin nucleus showed bordered clumps of dense chromatin. In the nucleus of some cells distinct paired nucleoli were seen (Fig. 7). Scarcity of secretory granules was pronounced. However a few, but typically large pleomorphic granules were loosely scattered. Golgi cores were occupied by prosecretory granules (Figs 7, 9). RER was increased in amount and presented as solitary, parallel strands with terminal dilations. Concentrically arranged RER cisternae formed nebenkerns, at the

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Figs 4 a, b—Following 24 weeks of tamoxifen citrate treatment—(A)—The rough endoplasmic reticulum (RER) of the prolactin cell formed an irregular whorl. The core of such whorls include degenerated mitochondria (arrow) and secretory granules (arrow head); and (B) Golgi zone of this cell is dilated and distorted and contains aggregating prosecretory granules (arrows). Degenerative changes in the enlarged mitochondria are visible. [A - X 16,000; B - X 25,000]
Figs 5 a, b, c, d—Electron micrographs of prolactin cells of female albino rats following 24 weeks of oestradiol administration. (A) Nucleus of hyperplastic cells show excentrically placed nucleolus (arrow). Note the sparse granulation and phenomenal augmentation of rough endoplasmic membranes in all the figures. Characteristic nebenkerns (NEB) as seen in the figure are common; (B)—Concentric RER membranes also appear as rounded masses with focal vacuolar dilatations (arrow) and myelination (arrowheads); (C)—Confronting cisternae (arrow) abound in the cytoplasm; and (D)—Extensive and cystically dilated cisternae of rough endoplasmic reticulum (arrow) are seen. [A—X 8,000; B—X 8,000; C—X 10,000 and D—X 10,000]
Fig. 6—Electron micrograph showing annular lipid inclusions (arrows) with lucent centres in the cytoplasm of the prolactin cell of female albino rat after 24 weeks of oestradiol valerate treatment. [X 8,000]; Figs 7 – 9—Fine structure of prolactin cells of female albino rat following 24 weeks of progesterone administration—(Fig. 7)—Nucleus (N) shows bordered chromatin clumps and paired nucleoli (arrow heads); (Fig. 8)—Mature secretory granules (arrows) and granular membranes with empty cores (small arrows) sequestered in nebenkern cores; and (Fig. 9)—Hypertrophied golgi zone (GZ) shows accentuated mitochondria (M). Lysosomal degradation of the secretory granules (arrow) is also seen in golgi zone (Fig. 7). [Fig 7,8—X 10,000; and Fig. 9—X 13,000]
Figs 10-12—Electron micrographs showing portions of the prolactin cell of the female albino rat treated with norethisterone heptanoate for 24 weeks. (Fig. 10)—Densely packed secretory granules (SG) in the cytoplasm and in the nebenkerne; (Fig. 11)—Aggregating prosecretory granules (arrows) in golgi zone; and (Fig. 12). Circular golgi zone shows dilated components and increased mitochondrial associations (M), as also seen in Fig 11,12. [Fig. 10 X 8,000 and Figs 11, 12 X 13,000; Fig. 13—Oligocitium at the end of ergastoplasmic confronting cisternal pair in prolactin cell of female albino rat treated with levonorgestrel + ethinylloestradiol for 24 weeks. [X 40,000]
Fig. 14—A high power electron micrograph of the prolactin cell of the female albino rat treated for 24 weeks with levonorgestrel + ethinyloestradiol combination. Note the Golgi zone with prosecretory granules (arrows). The cell shows misplaced exocytosis (curved arrow). [X 13,000]
center of which small portions of cytoplasm containing mature secretory granules as well as granular membranes with empty cores were sequestered (Fig. 8). Golgi underwent hypertrophy and was associated with increased number of mitochondria (Fig. 9). Lysosomal degradation was seen within and outside the golgi zone (Fig. 7). Multivesicular bodies also appeared in the cytoplasm.

Effect of norethisterone heptanoate—Following the administration of this drug, stimulation of the PRL cell was evident from the increased cytoplasmic volume of the cell with reduced nucleo-cytoplasmic ratio, presence of densely packed clusters of mature pleomorphic secretory granules (Fig. 10) and aggregation of prosecretory granules in various stages of development in the golgi zones of these cells (Figs 11, 12). Type 1 secretory granules were found either within the golgi sacules or in close association with them. The relatively large type 2 secretory granules were typically pleomorphic. The type 3 variety were not as variable as type 2, whereas the type 4 prosecretory granules were more or less rounded and ovoid. The increased size of the juxta-nuclear golgi zone was another conspicuous feature of this cell. The circular Golgi zone showed substantially dilated components (Figs 11, 12).

Augment profiles of RER existed as extensive parallel lamellar arrays or were irregularly scattered in the cytoplasm. In some cells they also occurred in the form of nebenkern with secretory granules in their cores (Fig. 10). Detectable increase occurred in the size and number of mitochondria, most of which were in the proximity of the golgi zone (Figs 11, 12). Many of these mitochondria had breaks in their walls, as well as loss of matrix and cristae. Lysosomal aggregates appeared in the cytoplasm.

Effect of levonorgestrel + ethinyl oestriadiol—PRL cell showed significantly altered picture following this treatment regimen. Golgi cores of this cell contained varieties of prosecretory granules (Fig. 13). The formed spherical granule content were found to be released between the lateral borders of the cell resulting in misplaced exocytosis (Fig. 13). RER cisternae were less pronounced and occurred in parallel arrays outside golgi zone (Fig. 13). Golgi zone underwent hypertrophy and distortion, and the number of golgi units showed an increase. Many short, spherical or sinuous, enlarged mitochondria with normal matrical density and cristae were intimately associated with golgi complex. Massive lysosomal aggregates were present in the core and in the vicinity of the golgi area. An interesting ultrastructural finding following the administration of this regimen, was the occurrence of an oligocilium outside the golgi zone at the end of an ergastoplasm confronting cisternal pair (Fig. 14).

Discussion

From our observations it was clear that all the five regimens had a profound influence on the ultrastructure of the prolactin cell. Increased electron density of the cell observed after tamoxifen citrate, oestriadiol valerate, progesterone and levonorgestrel + ethinyl oestriadiol treatment may be due to accumulation of numerous free ribosomes in the cytoplasm and may suggest hyperactivity. Similar observations have also been recorded for PRL cell stimulated by pimozide treatment. Stimulated state and hypertrophy of prolactin cell in tamoxifen and oestriadiol valerate treated rats is endorsed by the increased cell size. The increased volume of the stimulated prolactin cells under the estrogenic effect has been reported earlier.

A predominantly euchromatic nucleus is a marker of metabolically active cell. Such nuclei, as were observed in the cells of tamoxifen, progesterone and levonorgestrel + ethinyl oestriadiol treated rats, have been reported following estrone administration in female rats. Invaginated nuclei with crenated margins, observed in the cells under tamoxifen citrate and oestriadiol treated rats suggested an increase in the metabolic status of the cells since the invaginations are a design aimed at maintaining a normal nuclear surface to volume ratio. Paired nuclei seen in PRL cell of progesterone regimen are of common occurrence in the stimulated PRL cells following chronic administration of oestrogens in rats.

As revealed from our observations, the granulation dynamics is of paramount importance in assessing the cellular activity. Except for norethisterone heptanoate, in all other protocols PRL cell was sparsely granulated. The sparse granulation under the influence of tamoxifen citrate and levonorgestrel + ethinyl oestriadiol combination, the increased rate of granule extrusion and a simultaneous occurrence of forming secretory granules within the active golgi zone of the cell, as well as increased RER profiles may reflect discharge of secretion and its simultaneous packaging with a view to regranulate the cell. Extrusion of prolactin granules represents the morphological equivalent of prolactin release. Such a cell picture is suggestive of sustained stimulation o
PRL synthesis. It is relevant to note that oestrogen administration has been one of the common methods of inducing pituitary tumours that secrete PRL\textsuperscript{21-23}. Also, it is found that an overwhelming majority of prolactinomas belong to the sparsely granulated variety\textsuperscript{7,24}. The prolactin cell picture obtained in this study following the administration of tamoxifen citrate, oestradiol valerate and levonorgestrel + ethinylestradiol were identical with the ultrastructural features of a sparsely granulated PRL cell adenoma\textsuperscript{12,14,24} and also resembled those of the non-tumorous counterparts of lactating and oestrogen treated rats\textsuperscript{7}.

Rapid discharge of immature secretory granules through misplaced exocytosis from prolactin cell of the female rat treated with combination regimen of levonorgestrel + ethinylestradiol indicated that these PRL cells were in hyperactive secretory phase and also indicated a morphological manifestation of an abnormally high rate of hormone release\textsuperscript{7,25}.

Presence of discrete secretory granules within RER cisternae in prolactin cell of tamoxifen treated female rats may be a result of an intense secretory activity of the cell causing a precocious formation of visible secretory product before it reached the Golgi region\textsuperscript{20}. In this context it may be mentioned that PRL can be directly secreted from RER without going through golgi or the secretory granule, as a matter of constitutive pathway\textsuperscript{36,27}.

Increased rate of PRL synthesis was endorsed by accentuated RER in the form of nebenkerns that are of common occurrence in PRL cells of pregnancy and lactation\textsuperscript{14,20,28}, as well as in the hyperplastic and adenomatous PRL cells formed under oestrogenic stimuli\textsuperscript{7,12,28,30}. The papilliferous and vesicular profiles consequent of intracisternal sequestration in norethisterone schedule is possibly a mechanism of disposing off the excess RER by cell. The short solitary and long winding cisternae in PRL cells of tamoxifen citrate and oestradiol valerate regimens have been reported from the rapidly dividing cells progressing towards neoplasia\textsuperscript{13}. Bizarre RER formations noticed in PRL cells of oestradiol valerate treated rats indicate a transitional stage progressing towards involution\textsuperscript{13}. Such a cell picture could be interpreted as being suggestive of accelerated cell cycle\textsuperscript{32}.

Occurrence of an oligocilium in PRL cell of the combination regimen was an interesting find of this study. Despite the scattered reports on the presence of cilia in the adenohypophysis by light\textsuperscript{33} and electron microscopy\textsuperscript{34-40}, no unifying hypothesis regarding their functional significance has been proposed\textsuperscript{41}.

Proliferative changes that occur in golgi zone following sustained stimulation of PRL cell through administration of tamoxifen citrate, oestradiol valerate and levonorgestrel + ethinylestradiol in particular are indicative of endocrine hyperactivity. Gross enlargement of mitochondria in close association with golgi, attested the hyperactive status of the latter.

Heightened lysosomal activity was a common feature of all the drug regimens, being more pronounced in tamoxifen, oestradiol and the combination protocol of levonorgestrel + ethinyl oestradiol. This may be viewed as a built-in check to prevent hyperprolactinemia, since the lysosomes have been reported to control the rate of hormone production by digesting and diminishing the secretory granules through crinophagy\textsuperscript{42,43}.

From the above discussion it clearly stands out that in most of the cases, the dynamics of the hormone synthesis and secretion was affected. Overwhelming response of PRL cell to oestradiol valerate may have culminated into prolactin cell tumour. A word of caution here is that most often distinction between hyperplasia, nodules and adenoma is not possible\textsuperscript{13}.

Such a significant increase in the number of PRL cells and cytological criteria of increased secretory activity have been seen following a high dose of oestradiol administration to female rats\textsuperscript{44}. Striking hyperprolactinemia and premature death following oestrogen treatment of female mice has also been reported\textsuperscript{45}. In this context it has been noted that a direct stimulating effect of oestradiol on prolactin gene transcription and accumulation of mRNA in the cytoplasm, has been documented in cultured rat pituitary cells\textsuperscript{46,47}. It has also been established that stimulation of PRL cell is mostly due to the effect of decreased dopamine inhibition and that oestradiol has an integrated effect to prevent dopaminergic inhibition of PRL mRNA levels, PRL synthesis and PRL secretion\textsuperscript{48}.

The cell picture suggestive of enhanced synthesis and secretion under the influence of tamoxifen seems to be confusing considering the antioestrogenic activity of this compound. However, pharmacology of tamoxifen is complex, as well as species and target specific\textsuperscript{49}. The compound can also behave as an oestrogen agonist\textsuperscript{50,51}. Evidence of elevated PRL levels following tamoxifen administration may be ascribable to partial estrogen agonistic potency of this compound in rats\textsuperscript{32,55}. 
Ultrastructure implying enhanced synthetic function of PRL cell of progesterone regimen is concordant with the demonstration of progesterone induced hyperprolactinemia\(^{56,57}\). It is also known that progesterone suppresses the pituitary gonadotrophins and promotes breast development\(^{58}\). Increased risk and induction of mammary carcinomas as a sequel to progesterone induced hyperprolactinoma has also been documented\(^{56,57}\). It is possible that some of the progesterone is metabolised to oestrogen in vivo before it acts on pituitary. Besides, progesterone is metabolised to oestrogen which is concerned, reports are available of men and women with high doses of such hormone and promotes breast development\(^{58}\). Increased risk and induction of mammary carcinomas as a sequel to progesterone administration are suggestive of increased PRL cell tumours\(^{62}\). Probably the oestrogenic response of PRL cell in such a case is evoked by the oestrogenic fraction of the combination oral pills. Some investigators have reported higher levels of PRL in women using combination oral pills\(^{63}\).

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