ARE THE EFFECTS OF STEROIDOGENESIS INHIBITOR DRUGS (KETOCONAZOLES) REVERSIBLE OR IRREVERSIBLE ON ADRENAL CORTEX OF ADULT MALE ALBINO RATS?
HISTOLOGICAL AND ULTRASTRUCTURAL STUDY

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Abstract: This study was carried out to investigate effect of Ketoconazole (KTZ) on the cortex of adrenal gland of adult male albino rat.KTZ is an antifungal drug used as steroidogenesis inhibitor. Animals were randomly divided into 3 groups of fifteen animals in each group. In (group I), animals were injected with saline, (group II) animals were injected with KTZ with 10mg/100g.b. wt in daily dose for 15 days where (group III) animals were injected with KTZ in the same manner and period then left for recovery for another 15 days. All animals were sacrificed at the end and adrenal glands were separated and processed. Light microscopic studies demonstrated accumulation of variable sized lipid droplets. Ultra structural studies exhibited hypertrophied mitochondria and some of them showed vacuolar degenerations, while others were with destroyed cristae. SER was scanty, sometimes absent. The nuclei were electron dens surrounded by irregular nuclear membrane, showing shrinkage and signs of pyknosis.Lipofuscin pigments were scattered. Recovery group showed improved pictures similar to control group. Therefore, it is noticed that the destructive impacts of ketoconazole on the adrenocortical cells reflected on their functions leading to much deficiency in their performance. But these effects fortunately were reversible.

Key words, Adrenal cortex, Ketoconazole, Rats.

INTRODUCTION

The adrenal gland is an important organ yet it is neglectable in relation to effects of endocrine disruptors [1]. Adrenocortical zones synthesize and secrete steroid hormones, which fall into three major categories; mineralcorticoid, (aldosterone) which is secreted by zona glomerulosa. Aldosterone is an important regulator of salt homeostasis and fluid balance and it is a major control unit of acid/base balance [2]. While, zona fasciculata secretes glucocorticoid, (cortisol) which is essential for life since it has a major role in responding to environmental stimuli; it decreases protein synthesis, thereby increasing the circulating level of amino acids; it elevates blood glucose by stimulating the enzymes involved in gluconeogenesis in the liver and it mobilizes fatty acids and glycerol from adipose cells. It has also anti-inflammatory effects [3]. By zona reticularis, small amounts of androgens are secreted. The two principal adrenal androgens are; androstenedione and dehydroepi-androsterone, which is far less potent than testosterone and has little physiological significance. Both hormones can serve
as substrates for the conversion into testosterone and estradiol [4]. The hormone secretions of the adrenal cortex are dependent on steroidogenic cytochrome P450 (CYP) enzymes which possibly can be the target of endocrine-disrupting drugs affecting them [5]. Ketoconazole (KTZ) is an imidazole derivative, initially developed as an orally active antifungal agent. It was introduced in clinical practice for the treatment of systemic fungal infections in the early 1980s. In fungi, it inhibits the synthesis of ergosterol, a cell membrane sterol. Shortly after its introduction as an antifungal agent, reports linked long-term KTZ administration with the development of gynaecomastia in men and low serum testosterone levels. Subsequently, glucocorticoid suppression with KTZ both in vitro and in vivo, confirming its inhibitory effect on adrenal steroidogenesis [6].

In the adrenal cortex, KTZ blocks multiple steps of steroid biosynthesis through the inhibition of cytochrome p450 enzymes such as 17α-hydroxylase, 20,22-desmolase, 11β-hydroxylase, 17,20-desmolase and 18-hydroxylase. Similarly, it blocks various steps of steroidogenesis in the gonads, leading to the potential side effect of hypogonadism [6]. Ketoconazole has also been investigated as an anticancer therapy in both clinical and pre-clinical settings [7]. Ketoconazole has been shown to interfere with steroidogenesis in patients and rat in vitro systems. Further studies raise the possibility that this drug action may be beneficially used in situations where inhibition of steroidogenesis is a therapeutic goal [8].

Also, KTZ has anti-inflammatory activity, it may prevent the development of acute respiratory distress syndrome and acute lung injury in critically ill patients. In the field of medicine, the great ability of ketoconazole to inhibit mineralcorticoid synthesis is used for palliative treatment of primary hyperaldosteronism [9]. Also, in a treatment strategy of resistant and hyper-cortisolemic depressive patients as an inhibitor of glucocorticoid synthesis [10]. As well as, in treatment of ACTH-secreting adenomas, palliative treatment of cushing disease, adrenal tumors, adrenocortical carcinoma and ectopic corticotrophin production by small-cell lung carcinoma or carcinoid tumors [9]. Ketoconazole as inhibitor of androgen has been used for treatment of prostate cancer with promising results [11].

Also, physicians use high-dose ketoconazole in women with advanced breast cancer resistant to conventional chemotherapy and for ovarian hyperandrogenism syndrome, including polycystic ovarian syndrome and hyperthecosis with considerable improvement in acne, hirsutism, and amenorrhea [9].

It is clearly noticed from the previous studies that ketoconazole has been widely utilized in the medical fields for the treatment of different types of diseases especially those needed steroidogenesis inhibition. But, unfortunately there is no attention if the influence of its administration on the adrenal cortex is reversible or not.

Thus, the present study aimed to throw light on the influence of ketoconazole on adrenal cortical tissues from the histological and ultrastructural point of view.

**MATERIALS AND METHODS**

45 adult male albino rats of average weight 150-250 gm were used in this study. The animals obtained from breeding animal house, Faculty of Medicine, Menoufia University. Animals were housed in the animal laboratory of Menoufia faculty of medicine.

Figures 1,2,3 are histological preparations stained with haematoxylin and Eosin. magnification 200x.

Fig. 1a: A section of control adrenal gland (group I) showing the capsule (C) and underlying closely packed cells of the zona glomerulosa (G) with spherical densely stained nuclei (star) being separated by trabeculae (T) extended from the capsule and large polyhedral fasciculata cells (F) with central vesicular nuclei (N) and separated by narrowed blood capillaries (arrow). Notice: suprarenal fat (L).Fig. 1b: A section of recovery adrenal gland (group III) nearly similar to control.

Fig. 2a: A section of (group I) Zona fasciculata cells (F) with central vesicular nuclei (N) arranged in long radial cords, separated by narrowed blood capillaries (arrow) Zona reticularis cells (R) small, closely packed cells arranged in irregular network of intermingled cords, separated by numerous blood sinusoids (S). Fig. 2b: A section of (group III)nearly similar to control.

Fig. 3a,b: A section of KTZ adrenal gland (group II) showing thick fibrous capsule (C) and Hypertrophied glomerulosa cells (G) and fasciculata cells (F) containing vacuoles (*) and some of their nuclei showing signs of pyknosis (P). Notice: suprarenal fat (L)& cellular infiltration (I).
Strict care and cleaning measures were utilized to keep the animal in a normal healthy condition; the animals were put in animal cages under the prevailing atmospheric conditions and also were fed standard diet and liberal supply of tap water. They were kept under a photoperiod of 12 h light: 12 h darkness, in controlled conditions of temperature (20-24 ºC). All ethical protocols for animal treatment were followed. The experimental protocol was accepted by the Ethical Committee of Menoufia faculty of medicine.

**Drug:** Ketoconazole is a synthetic imidazole of oral broad-spectrum antifungal agent [12]. It is sold under trade name; Nizoral® as a tablet of 200 mg Ketoconazole which is manufactured by JANSSEN-CILAG Pharmaceutical N.V., Turnhoutseweg 30, B-2340 Beerse, Belgium.

**Experimental procedure:** Rats were divided into three groups, included 15 rats for each as follows:

- **Group I:** (Control group): They were injected daily intra-peritoneally with 1 ml of physiological saline solution for 15 days.
- **Group II:** Animals of this group were injected with daily dose (10mg/100g. b.wt.) of ketoconazole dissolved in 1 ml of physiological saline solution for 15 days. [13].
- **Group III:** These animals were treated like group II for 15 days and then left for another 15 days for recovery.

**Histological study:** After experimental period, the animals were sacrificed and adrenal glands were removed, cleaned and fixed in Bouin’s fluid for 24 hours, then subjected to the normal procedures for paraffin sectioning. Sections, of 5 μm were dehydrated, cleared in xylene and mounted in DPX, stained with haematoxylin and Eosin, (H&E) to show the histological details [14]. The stained sections were examined and photographed by light microscopy.

**Ultrastructure study:** Freshly excised adrenal glands were cut into small blocks (1mm3), fixed directly in cold 4F1G (i.e. 4% formalin + 1% glutaraldehyde adjusted at pH 2.2) for 24 hours, then were post fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.3), dehydrated in an ethanolic series culminating in 100% acetone, and infiltrated with epoxide resin. After polymerization over night at 60oC, semithin sections (0.5 μm) were stained with 1% toluidine blue in 1% sodium borate and examined with light microscope. Areas of cortical cells were selected and the blocks trimmed accordingly. Ultrathin sections (80-90 nm) were cut, mounted on 200 mesh copper grids, and stained with uranyl acetate and lead citrate [15]. The girds were then examined with the transmission electron microscope (Seo-Russia) in Tanta E.M Center at faculty of medicine Tanta University.

**RESULTS**

**Histological studies:**

**Control group (Group I):** Control sections showing the three zones of adrenal cortex; zona glomerulosa, fasciculata and reticularis, respectively. The adrenal gland is surrounded by a fibrous connective tissue capsule. Zona glomerulosa is formed of columnar cells arranged in glomeruli-like structure, which are separated by delicate trabeculae extending from the capsule. Its cells contain acidophilic cytoplasm with fairly large rounded to oval basophilic nuclei. Zona fasciculata is composed of polyhedral cells arranged in one or two cell thick in long radial cords or fasciculae and they are separated by narrowed blood capillaries. The cells have eosinophilic cytoplasm.

**Figures 4,5,6 are electron microscopic preparations:**

**Fig. (a) & (b):** An electron micrograph of a section of a rat adrenal cortex from control (group I) and recovery (group III) groups showing cells of zona glomerulosa having numerous mitochondria (M) and some lipid droplets (Li) and oval or round shaped nuclei (N) ensheathed by a double nuclear envelope involving nucleolus (Nu), dense clumps of heterochromatin(Ht) and homogenous euchromatin(Eu), smooth endoplasmic reticulum (SER), small Golgi vesicles (G) and lysosomes (Ly).

**Fig (5a) & (b):** An electron micrograph of control (group I) and recovery (group III) groups showing cells of zona fasciculata having numerous mitochondria (M) and many lipid droplets (Li) and nuclei (N), smooth endoplasmic reticulum (SER), small Golgi vesicles (G), lysosome (Ly) and macrophage(Mg), RER and blood sinusoid(S).

**Fig. (6a)&(6b):** An electron micrograph of control (group I) and recovery (group III) groups showing cells of zona reticularis having numerous mitochondria (M) and many lipid droplets (Li) and nucleus (N), smooth endoplasmic reticulum (SER), blood capillary (cap) a macrophage (Mg), lysosome(Ly) and cell surface with some microvilli (v) are noticed.
and spherical basophilic nuclei. Zona reticularis is characterized by an irregular anastomosing network of intermingled cords separated by numerous wide blood sinusoids. The cells of these cords are columnar cells having eosinophilic cytoplasm, rounded basophilic nuclei (Figs. 1a, 2a).

**Ketoconazole group (Group II):** The fibrous connective tissue capsule being thickened with increased fibrous elements. Glomerulosa, fasciculata and reticularis cells exhibiting hypertrophy with accumulated variable sized lipid droplets which appeared as vacuoles in the cytoplasm. Some of these lipid droplets fused together and occupied almost the entire cytoplasm, thus displacing the nuclei eccentrically which showed signs of pyknosis (Fig. 3).

**Recovery group (Group III):** Study shows results more or less similar to histological structure of control group (Figs. 1b, 2b).

**Ultrastructural results**

**Control group (Group I):** Ultrastructure of zona glomerulosa cells reveal different mitochondrial configuration varying from oval to spherical shapes (Fig 4a). In addition, a fair amount of smooth endoplasmic reticulum, small Golgi vesicles and abundant number of lipid droplets are evident. The nuclei of these cells are rounded or oval in shape; sometimes wavy in appearance ensheathed by double nuclear envelopes and possessing nucleoli, peripheral dense heterochromatin and homogenous euchromatin material. Zona fasciculata (Fig 5a) exhibits the fine characteristic features including; abundance of rounded mitochondria, smooth endoplasmic reticulum, scanty rough endoplasmic reticulum, fair number of lysosomes and richness of lipid droplets. The nuclei are large, rounded, possessing prominent nucleoli, dense peripheral heterochromatin, lightly stained euchromatin and surrounded by nuclear membranes. Blood capillaries are noticed between these fasciculata cells.

Zona reticularis cells are distinguished by their richness of rounded mitochondria, smooth endoplasmic reticulum, lysosomes and lipid droplets with varying sizes. Their nuclei are spherical or ovoid in shape contained condensed heterochromatin, euchromatin and prominent nucleoli. Widened blood sinusoids are manifested (Fig 6a).

**Ketoconazole group (Group II):** Marked ultrastructural changes of zona glomerulosa (Figs 7a,b), zona fasciculata (Figs 8a,b) and zona reticularis (Figs 9a,b) cells respectively are illustrated. It is evident that their cytoplasm contain hypertrophied mitochondria and some of them possessing destroyed cristae and other have small vacuolar degenerations. In addition, lysosomes and lipid droplets of variable sizes are seen; some of lipid become so large thus occupying almost the entire cytoplasm, masking the organelles and distending the cells. The nuclei are electron dens surrounded by irregular nuclear membrane, showing shrinkage and signs of pyknosis. lipofuscin pigments are seen scattered in the cytoplasm. Blood sinusoids are observed. It is worthy to mention that smooth endoplasmic reticulum, was scanty, sometimes almost absent in all examined cells of the adrenocortical zones, suggesting that it may be disintegrated.

**Recovery group (Group III):** More or less similar to ultrastructure of control group (Figs 4b, 5b, 6b).

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_Figures 7, 8, 9 are_ electron microscopic preparations:

**Fig. 7(a) & (b):** An electron micrograph of KTZ group (group II) showing cells of zona glomerulosa having numerous hypertrophied mitochondria (M) some of them having vacuolar degeneration (*), and some lipid droplets (Li) and pyknotic nuclei (N), being shrunken ensheathed by irregular nuclear membrane (Nm), lysosome (Ly) & cell surface with some microvilli (v) are noticed.

**Fig. 8(a) & (b):** An electron micrograph of KTZ group (group II) showing cells of zona fasciculata having numerous hypertrophied mitochondria (M) some of them having vacuolar degeneration (*), swollen mitochondria with destroyed cristae (arrow), some lipid droplets (Li), RER and pyknotic nuclei (N), with irregular nuclear membrane (Nm) are seen. Notice: lysosome (Ly) & lipofuscin pigments (l) are scattered in the cytoplasm.

**Fig. 9(a) & (b):** An electron micrograph of KTZ group (group II) showing cells of zona reticularis having numerous hypertrophied mitochondria (M) some of them having vacuolar degeneration (*), some lipid droplets (Li) and pyknotic nuclei (N), irregular nuclear membrane (Nm), Irregular cell surface with some microvilli (v) are seen. Notice: lysosome (Ly) & lipofuscin pigments (l) are seen.
DISCUSSION

The adrenal gland is the most important steroidogenic tissue in the human body and essential for survival. All steroidogenic processes take place in the adrenal cortex including steroid hormones (glucocorticoids, mineralcorticoids, androgens, and estrogens) [2].

Ketoconazole is an imidazole derivative used as an antimycotic agent with a broad spectrum of activity and low toxicity that is approved by the FDA for the treatment of a number of superficial and systemic fungal infections [16]. It is the most commonly used steroidogenesis inhibitor in the USA because of its availability and relatively rapid onset of action. It inhibits steroid synthesis through inhibition of cytochrome P450 enzymes [17].

Despite KTZ effectiveness as antifungal, new researches for other uses of KTZ are processed. KTZ is an effective inhibitor of adrenal and gonadal steroidogenesis [18]. For this property, it has been used for the treatment of different types of diseases including; cushing disease, hyper-cortisolemic depressive patients, adrenal tumors, adrenocortical carcinoma, adrenal adenomas, prostate cancer, advanced breast cancer and various cancer cell lines such as hepatic metastasis and pulmonary metastasis [9].

The most striking change in KTZ group is the extensive accumulation of lipid droplets and appearance of cytoplasmic vacuolation of the three zones cells. This was consistent with the results of previous investigators [19]. Also, SER were scanty and almost absent in cells of all zones suggesting it may be disintegrated. This is coincided with Elshennawy and Aboelwafa [13].

In particular, zona fasciculateis most common site of lesions in the endocrine system. This may be explained by that zona fasciculata has disproportionately large blood supply per unit mass, its high content of lipids, the susceptibility of its unsaturated fatty acids to peroxidation damage and its high levels of cytochrome P450 which metabolize xenobiotics to reactive intermediates [20].

In the present work, the ultrastructure of adrenal cortical cells of KTZ group revealed numerous swollen mitochondria with degeneration of the cristae resulting in cavitation of their matrix. These findings could be responsible for the increased vacuolation seen by LM. This was in agreement with El-Drieny et al. [21] who explained that the impaired activities of mitochondrial enzymes lead to decreased energy levels. These changes lead eventually to cell degeneration with appearance of cytoplasmic vacuolation. This hypertrophy and cavitations in mitochondria probably resulted from inhibition of the conversion of cholesterol to pregnenolone, cholesterol may accumulate within the mitochondria. Consequently, it undergoes considerable hypertrophy and vacuolation [13]. Also, similar results were observed by Ishihara et al. [22] in human adrenal cortex. They explained that the intra mitochondrial vacuoles might be associated with the deposition of steroids or their related compounds in the cristae.

The marked lesions observed in mitochondria and smooth endoplasmic reticulum might be sufficient to cause impairment of steroid synthesis in accordance with Guerrero et al. [23], who reported that these organelles play a great role in steroidogenesis within the cortex, through cytochrome P450 and the enzyme 3β-hydroxysteroid dehydrogenase (3βHSD), which are distributed between the mitochondria and the smooth endoplasmic reticulum. The limitation in steroid hormone biosynthesis is the translocation of substrate cholesterol from the outer mitochondrial membrane to cholesterol side-chain cleavage enzyme (CYP11A), the first enzyme in the steroidogenic pathway, which is located inside the mitochondria as Isola et al. [24] elucidated in their studies. In addition, the nuclei of KTZ group showed pyknosis in the form of patches of heterochromatin with irregular nuclear membranes. These findings were in agreement with previous report of some investigators [25]. The increased lipofuscin pigment noticed in the present work may be the result of increased cellular oxidative stress. It may be explained that damaging mitochondria & SER release oxygen free radicals which lead to lipid oxidation [26].

Another mechanism for KTZ was found to inhibit cholesterol synthesis in a dose-dependent fashion by blocking conversion of lanosterol to cholesterol. Other lipid modifying properties of ketoconazole include decreasing of lipoprotein lipase and 3-hydroxy-3-methylglutaryl coenzyme A reductase
activities, inhibition of intestinal cholesterol absorption and bile acid synthesis [9].

Recently KTZ is used to reverse hypercortisolae- mia and its damaging effects [6]. Currently, surgery offers the only option by removing the source of cortisol excess. Cure is not, however, always possible in some diseases and remission rates following surgery are 40–90% even in specialist centers. Surgery may be unacceptable risk for some patients and it may not be an option for others. This means that significant numbers of patients will need medical therapy to improve clinical and biochemical consequences [6].

In 2013/2014, the use of KTZ as an antifungal agent has been restricted mainly due to concerns of life-threatening hepatotoxicity. However, when KTZ comes to treat patients with hypercortisolae- mia, the risk: benefit ratio differs. Use of KTZ can be justified and has been approved for this purpose by the European Medicines Authority in late 2014 [6]. Ultimately, it seems that the steroidogenesis inhibitor (KTZ) is an important for treatment of resistant diseases but also it has its toxicity in the adrenal cortex.

A histological examination of group III (recovery group) showed that most of the changes of the three zones of the adrenal cortex were improved and similar to those of the control. They showed numerous lipid droplets and many mitochondria. These results were in agreement with Morgan and Laufgraben[17] and John-Price [27] who added that KTZ effects are dose dependent and completely and rapidly reversible upon drug discontinuation.

In conclusion, it is noticed now that the destructive impacts of ketoconazole on the adrenocortical cells reflected on their functions leading to much deficiency in their performance. These effects fortunately are reversible. But, it should be taken in consideration and great concern that this drug must be utilized under restricted precautions in the medical fields.

REFERENCES


