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Vitamin D3 May Ameliorate the Ketoconazole Induced Adrenal Injury: Histological and Immunohistochemical Studies on Albino Rats

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Ketoconazole (KZ) is used widely for treating the superficial, systemic fungal activities and hyperandrogenemic states. Its uses are limited by its deleterious effect on histological structure and function of the adrenal cortex. This study investigates whether vitamin D3 supplement can ameliorate the morphological changes induced by KZ.

Thirty four adult male albino rats were randomized into control group (Group I) which was subdivided into: control 1 (n=7) and control 2 (n=7): In control 1, rats were intraperitoneal (I.P) injected once with 1 ml of polyethylene glycol-400 for 15 consecutive days and control 2 rats were injected I.P with (1 µg/kg) of vitamin D3 for the same period. Group II (n=10): rats were I.P injected with KZ (10 mg/100 g of body weight) once daily for 15 days; Group III (n=10): rats were I.P concomitantly injected with KZ and vitamin D3 similar doses to animals in groups II and control 2 respectively. Blood samples were collected to determine plasma ACTH, corticosterone and aldosterone levels. The right adrenal specimens sections were stained with Haematoxylin & Eosin and Masson Trichrome for histological studies and treated with Bax, Ubiquitin and vitamin D receptors for immunohistochemical studies.

KZ induced adrenal cortical morphological changes in forms of disturbed adrenocorticocyte cytological architecture, nuclear changes, and intracellular lipid accumulation. KZ also increased adrenal Bax and Ub but decreased the vitamin D receptors immunopositive staining expression, in addition to increased plasma ACTH as well as decreased corticosterone and aldosterone levels. These changes were ameliorated by supplementing with vitamin D3.

Key words: ketoconazole, vitamin D3, adrenal cortex, rats

I.   Introduction

Ketoconazole (KZ), an imidazole derivative that has a wide range of superficial and systemic antifungal activities [21, 54]. The mechanism of action of KZ is mediated by inhibition of 14-demethylation of lanosterol and subsequently reducing the production of ergosterol; a characteristic constituent of yeast cell membranes [9]. KZ has also been used also for the treatment of hyperandrogenemic states which are found in advanced prostatic cancer [7],

postmenopausal breast cancer [27], androgen-producing tumors [63], and hirsutism [30]. KZ has been also utilized for the treatment of Cushing's syndrome [60]. KZ diminishes cortisol production through inhibition of deoxycortisol hydroxylation. In some patients, KZ induces symptomatic adrenal insufficiency, which presents with hyponatremia, hyperkalemia, hypotension, lethargy, depression, and malaise [19]. KZ-induced adrenal insufficiency leads to increased plasma ACTH levels to compensate for the adrenal blocking [19].

KZ, a lipophilic drug; is distributed in the fatty tissues and organs including the adrenal gland [13]. The steroid biosynthesis begins in cells of the adrenal gland where the cholesterol, the initial product in sterol, is converted into

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adrenal steroid hormones such as aldosterone, hydrocortisone, and corticosterone by a series of P450-mediated hydroxylation steps [4]. KZ interferes with the production of adrenal and gonadal steroids [48] through inhibition of the cytochrome P450 isoenzyme CYP-3A4 necessary for metabolizing steroids [22, 32]. In the meantime, KZ inhibits adrenal and gonadal steroidogenesis through the inhibition of CYP17 activity. At higher doses, it also inhibits CYP11A1 and consequently blocks steroidogenesis in all primary steroidogenic tissues. The inhibition of CYP17 and CYP11A1 activities produce depletion of Cytochrome P450 [14, 36, 49, 55].

KZ has been shown, in an *in-vitro* study, to inhibit the 11-hydroxylase step. This step is carried out by a P450-dependent mitochondrial enzyme, a metabolic step between deoxycorticosterone (DOC) and corticosterone in rats [37]. KZ induces adrenal injury by apoptosis which occurs through increased the expression Bax as a results of increased P450 in rats. Bax is a protein of the Bcl-2 gene family, which promotes apoptosis by competing with Bcl-2 proper [35].

Vitamin D3 modulates mitochondrial energy transduction, which may represent the bio-energetic basis for the fatigue experienced by vitamin D3 deficient adolescents and adults. Biologically the vitamin D_3 action is mediated by the vitamin D receptor (VDR) which facilitates mitochondrial oxidative phosphorylation, which is considered as an alternative pathway for formation of P450 [33]. The supplementation of vitamin D3 also increases the availability of extracellular calcium. This calcium is used by the mitochondria of the adrenals to stimulate adrenal cortical hormone formation through using endogenous substrates and potentiating of P450 [40, 57]. In addition to cholesterol, 7-dehydrocholesterol, vitamin D3 and ergosterol are used as substrates for P450scc [25, 58, 59] to form adrenal hormones. Vitamin D3 is also considered as an antioxidant which inhibits iron-dependent liposomal lipid peroxidation [31, 64].

In the current study, KZ induced adrenal functional, using plasma ACTH, corticosterone and aldosterone levels, histological and immunohistochemical changes were evaluated with and without the administration of vitamin D3.

II.   Materials and Methods

A total of 34 adult male wistar albinos rats (200–250 g) were obtained from the animal house of King Saud University and housed in polypropylene cages. They were maintained with water and food ad libitum under well ventilated animal house conditions (temperature: 28–31°C). The animals were randomized into 3 groups.

Group I (Control groups): control 1 (n=7): rats were injected once daily with 1 ml of polyethylene glycol-400 (Sigma-Aldrich, CA) for 15 days intraperitoneal (I.P); control 2 (n=7): rats were injected I.P with 1,25-dihydroxy-vitamin D_3 (1 μg/kg) for 15 days [26]. The 1,25-dihydroxy-

vitamin D_3 (Sigma-Aldrich, CA) was dissolved in ethanol and diluted in saline (20:80 v/v) before being injected into rats.

Group II (KZ treated group) (n=10): rats were injected I.P with 1 ml of 10 mg/100 g of the body weight with KZ (Santa Cruz Biotechnology, USA) dissolved in polyethylene glycol-400 once daily for 15 days [31, 52].

Group III (KZ and vitamin D3 treated group): (n=10) rats were concomitantly treated with similar dose of vitamin D3 and KZ as for groups I and II for 15 consecutive days.

By the end of the treatment period, the animals were anaesthetized by ether inhalation before exsanguinations. Blood samples were collected from heart puncture to determine plasma ACTH, corticosterone and aldosterone levels. The right adrenal glands were removed and prepared for histological and immunohistochemical studies.

The animal manipulations were performed in the Laboratory Animal Center of College of Medicine, King Saud University (Riyadh, KSA) in accordance with the institutional and national guide for the care and use of laboratory animals. The experiment was approved by the Ethical Committee of College of Medicine, King Saud University.

The plasma ACTH activity levels were determined by chemiluminescence method (IMMULITE automatic analyzer, DPC, Los Angeles, USA) by using diagnostic kit (Abnova, USA). The ACTH values were expressed as pg/ml. Moreover, plasma concentrations of corticosterone and aldosterone were measured by using a Corticosterone Enzyme Immunoassay Kit (Assay Designs/Stressgen, Ann Arbor, Michigan, USA) and an Aldosterone enzyme immunoassay (BioVendor, Asheville, NC, USA) respectively. The corticosterone and aldosterone values were expressed as ng/ml and pg/ml respectively.

The right adrenal specimens were fixed in 10% neutral buffered formalin solution and processed to prepare 4 μm-thick paraffin sections. The sections were stained for histological and immunohistochemical studies.

Haematoxylin & Eosin (H&E) [17] and Masson trichrome [2] were used to verify histological details and the presence of collagen fibers respectively in the adrenal's sections.

For the immunohistochemistry studies paraffin sections were deparaffinized in xylene and processed for Bax (B-9, Santa Cruz), ubiquitin (Ub) (A-5, Santa Cruz) and vitamin D receptor (VDR) antibody (Novus Biologicals, UK) immunohistotochemistry. Immunohistochemistry was performed using a 3-step indirect process based on the labelled avidin biotin peroxidase complex (ABC) method. Sections were rehydrated in descending grades of alcohol. Following blocking of endogenous peroxidase activity with 3% H_2O_2 in methanol and non-specific binding sites with a protein blocker, the sections were incubated for 32 min with a 1:100 dilution of Bax, ubiquitin (Ub) (Rabbit anti-rats Bax and Ub polyclonal antibody, Santa Cruz, USA) and VDR (Rabbit monoclonal, Novus Biologicals, UK)

Table 1. *Plasma concentrations of ACTH, corticosterone and aldosterone*

Animal groups	ACTH (Mean in pg/ml±SEM)	Corticosterone (Mean in ng/ml±SEM)	Aldosterone (Mean in pg/ml±SEM)
Group I	38±3	151±15	240±24
Group II	355±23*	61±7**	122±12***
Group III	50±6	102±10	160±13

ANOVA test values comparing the different groups: *=(ANOVA=0.001); **=(ANOVA=0.01); ***=(ANOVA=0.04).

primary antibodies. Then, biotinylated secondary antibody was added at a concentration of 2% for 30 min (37°C) followed by addition of the avidin-biotin complex (ABC). Visualization of the reaction was performed using 3,3-diaminobenzidine (DAB) as the chromogen, which produces a dark brown precipitates that is readily detected by light microscopy. The sections were then counterstained with Mayer's hematoxylin, dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX (the Bax cytoplasmic and membranes sites of reaction were stained brown and nuclei were stained blue/brown; Ub cytoplasmic and nuclear sites of reaction; VDR nuclear, membranous and cytoplasmic sites were stained brown). The negative control included sections which were incubated in the absence of Bax, Ub and VDR primary antibodies.

The adrenal cortices were examined for changes in the adrenal cellular organization, cellular size, cytoplasmic vacuolation and presence of intracellular lipid vacuoles; nuclear changes (polymorphic, pyknosis, karyolysis and karyorrhexis) as well as the presence of engorged blood vessels.

Semi-qualitative analysis of the cytoplasmic density of Bax, Ub and VDR immunopositive staining were determined as 0, negative; 1, weak; 2, moderate; and 3, marked [56].

Quantitative measurements were also carried out using image analyzer (Super eye-Heidi soft), Histology Department, Faculty of Medicine, King Saud University, Saudi Arabia. Ten random high-power fields (Objective, ×400) in each slide were selected and captured for each group. The mean optical density of cytoplasmic Bax, Ub and nuclear VDR brown color reactions of the adrenal cortex were measured. The mean color area percentage of the blue color (collagen) in Masson's trichrome stained sections was also measured. The image analyzer was calibrated for color measurement before using.

The data were analyzed using SPSS-11 (Chicago, USA) statistical software. Comparison of the means of the above-mentioned, histological and immunohistochemical parameters among the studied groups were done using ANOVA-test. The significance level was considered at P≤0.05.

III. Results

The means plasma levels of ACTH were significantly higher in group II compared to other groups. The means plasma levels of corticosterone and aldosterone were significantly lower in group II compared to other groups (Table 1).

The H&E histological staining of group I (controls groups 1&2) showed normal adrenal gland (Fig. 1A, B and C).

In KZ-treated group (II), the zona glomerulosa showed a disturbed cortical cytological architecture. The zona glomerulosa noted atrophic and replaced by fasciculata cells. The zona fasciculata showed a disturbed architecture due to loss of the radial placed cell columns. Hypertrophied cells were also overloaded with well demarcated cytoplasm vacuoles. Some fasciculata cells cytoplasms were almost occupied by the vacuoles, thus displacing the nuclei eccentrically. The nuclear changes in the forms of variation in size and stain, pyknosis and karyolysis were also seen (Fig. 2A).

Zona reticularis showed unclear network distribution of its cells. Reticularis cells were enlarged and contained large well demarcated vacuoles. Reticularis cells also showed diversity of the nuclear size and stain in addition to pyknosis and karyolysis. Vascular network histological structure showed remarkable engorged blood vessels especially in the zona reticularis (Fig. 2B).

In group III, the adrenal cortex showed an almost preserved adrenocorticocyte of glomerulosa and fasciculata zones (Fig. 3A). The majority of cellular cytoplasm and nuclei were almost normal.

The network of the reticularis cells of group III was almost preserved. Vascular network was almost normal in the adrenal cortex (Fig. 3B).

The Masson Trichrome histological staining of group II showed a markedly stained collagen of the trabeculae in between fasciculata and reticularis cells (Fig. 2C) compared to groups I (Fig. 1D) and III (Fig. 3C) which were mild stained.

The quantitative analysis of Masson Trichrome staining showed that the mean color area percent of the zona glomerulosa, fasciculata and reticularis was significantly higher in group II compared to groups I and III (Table 2).

The Bax immunopositive staining of the controls was mild to moderate detected (brown color) in the capsule, subcapsular cells, some of the glomerulosa, fasciculata cytoplasm (Fig. 1E) which were markedly detected in the majority of glomerulosa, fasciculata (Fig. 2D) and reticularis cellular cytoplasm and nuclei of group II animals. The immunopositive staining of the sections of rats of group III was approximately similar to controls (Fig. 3D).
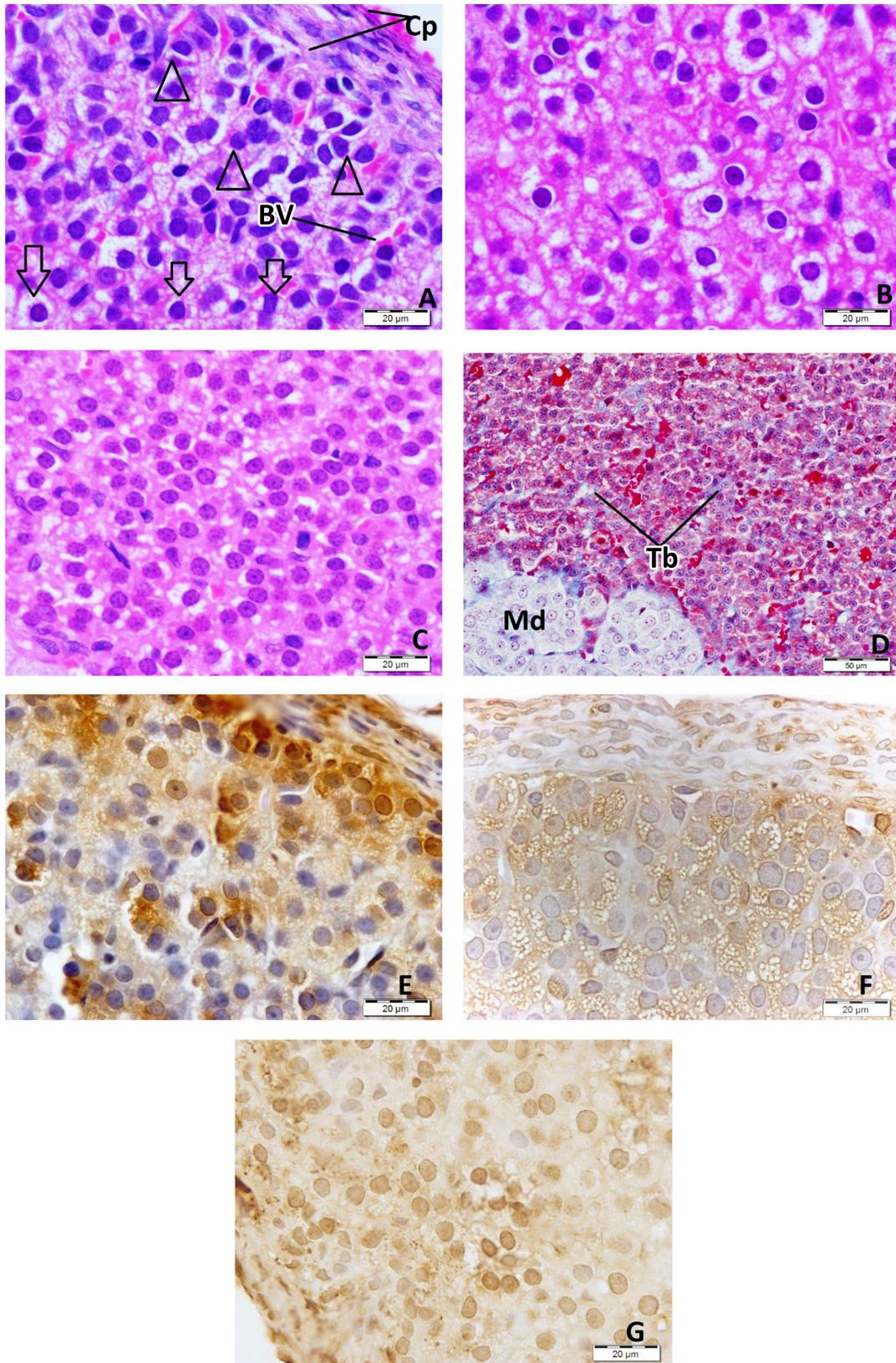
Fig. 1. Control (**A–G**) adrenal cortex. **A)** Blood sinusoids (BV); Capsule (Cp); glomerulosa cells (arrow heads); fasciculata cells (arrows); (H&E). **B)** The zona fasciculata cells (H&E). **C)** Zona reticularis cells (H&E). **D)** Trabeculae (Tb); adrenal medulla (Md); (Masson Trichrome). **E)** Immunopositive Bax staining (DAB&H). **F)** Immunopositive Ub staining (DAB&H). **G)** Immunopositive vitamin D receptors staining (DAB&H).
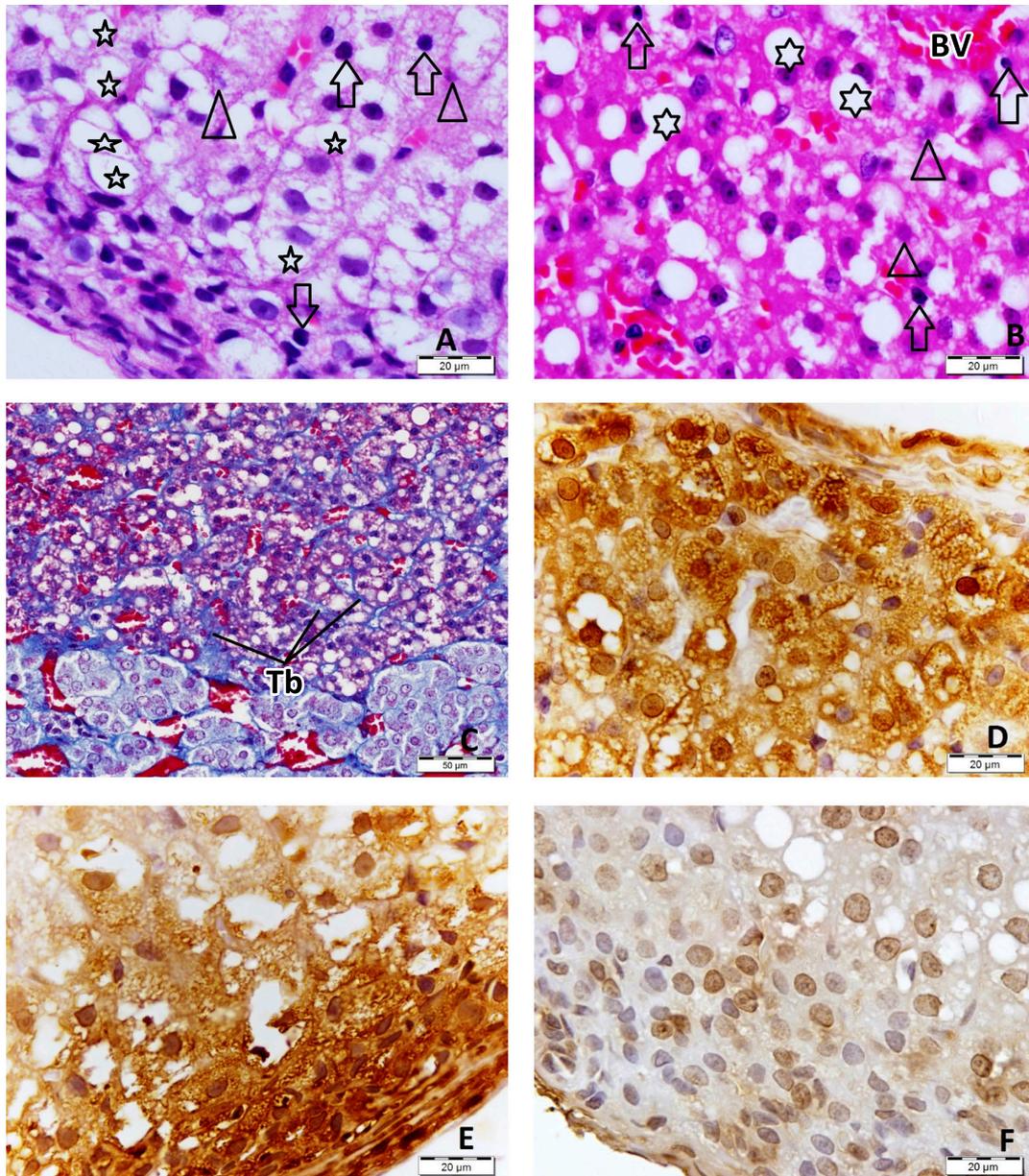
Fig. 2. KZ (**A–F**) treated group. **A)** Zona glomerulosa and fasciculate cells contains nuclear pyknosis (arrows) and karyolysis (arrow heads). Zona fasciculate contains well demarcated cytoplasm vacuoles (stars). (H&E) **B)** Zona reticularis cells contains nuclear pyknosis (arrows), and karyolysis (arrow heads), well demarcated cytoplasm vacuoles (stars) and dilated blood sinousoids (BV); (H&E). **C)** Zona reticularis cells contains trabeculae (Tb) (Masson Trichrome). **D)** Bax immunostaining of glomerulosa and fasciculate cells (DAB&H). **E)** Ub immunostaining of glomerulosa and fasciculate cells (DAB&H). **F)** VDR immunostaining of glomerulosa and fasciculate cells (DAB&H).

In Bax immunostaining the semi-quantitative analysis showed that the intensity of the cytoplasmic immunopositive reaction of the Bax immunostaining was markedly higher in the zona glomerulosa of group II (2.7) compared to groups I and III (1.7 and 2.1) respectively. The intensity of the immunopositive reaction was also markedly higher in the zona fasciculata of group II (2.2) compared to groups I and III (1.3 and 1.2) respectively. The intensity of the immunopositive reaction was also significantly (ANOVA <0.005) markedly higher in the zona reticularis of groups II (2.8) compared to groups I (1.1) and III (1.3) (Fig. 4).

The quantitative analysis showed that the mean optical density of Bax immunoreactions of the zona glomerulosa, fasciculata and reticularis was significantly higher in group II compared to groups I and III (Table 2).

The Ub immunopositive staining (brown color) of the controls was weakly to moderately detected in capsule, subcapsular cells, some of glomerulosa, fasciculata cellular cytoplasm and nuclei (Fig. 1F). Weak immunopositive staining was also detected in a few of reticularis cellular cytoplasm. The immunopositive staining of group II was markedly detected in almost all glomerulosa, fasciculata
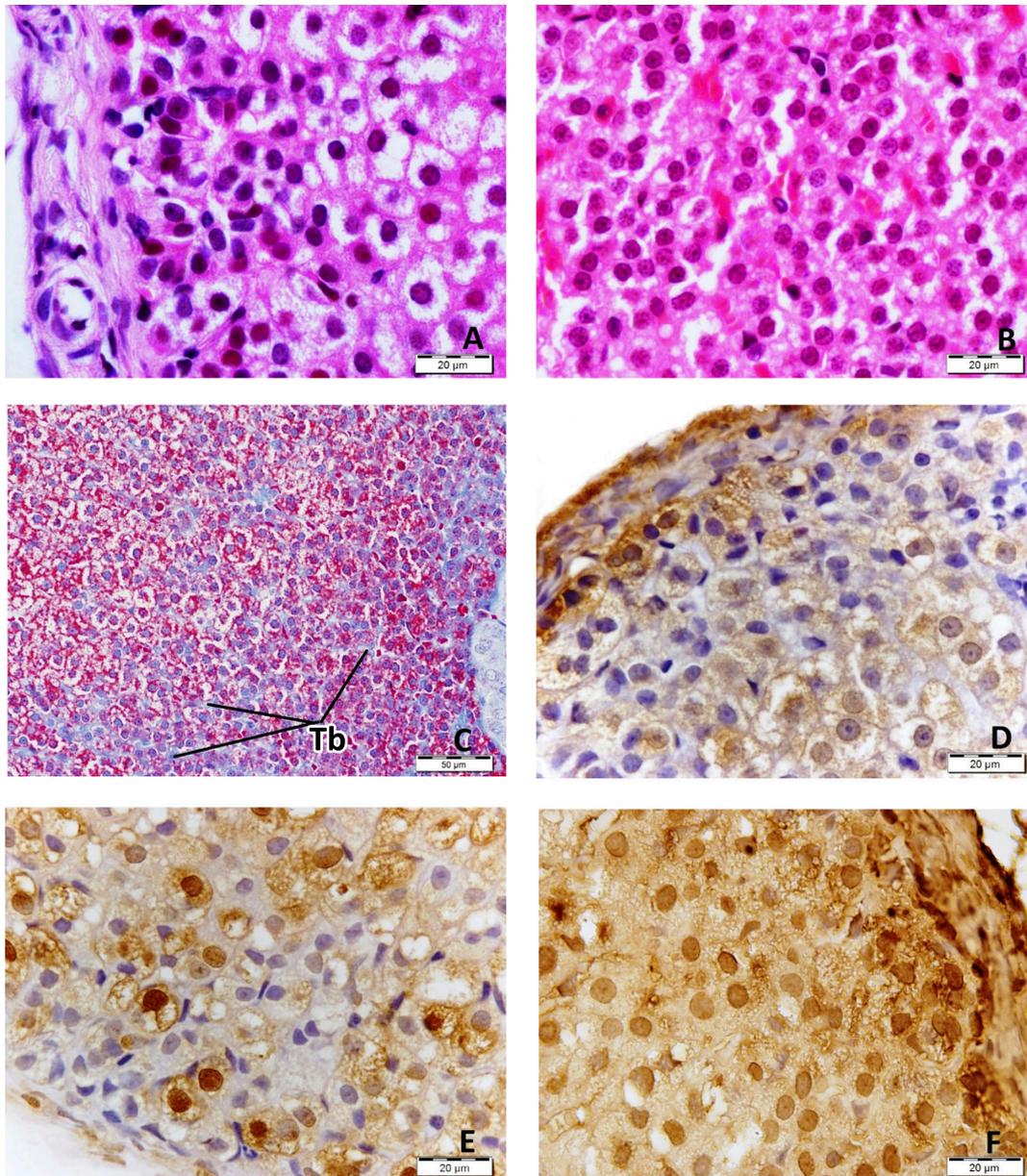
Fig. 3. KZ+vitamin D (**A–F**) treated group. **A)** Zona glomerularis cells and zona fasciculate cells (H&E). **B)** Zona reticularis cells (H&E). **C)** Trabeculae (Tb) (Masson Trichrome). **D)** Moderate glomerulosa and fasciculate immunopositive Bax staining (DAB&H). **E)** Imunopositive Ub staining of glomerulosa and fasciculate cells (DAB&H). **F)** Imunopositive VDR staining of glomerulosa and fasciculate cells (DAB&H).

(Fig. 2E) and reticularis cellular cytoplasm and nuclei.

However, the immunopositive staining of group III was moderately detected in some of glomerulosa fasciculata (Fig. 3E) but weakly detected in reticularis cellular cytoplasm and nuclei.

The semi-quantitative analysis showed that the intensity of the cytoplasmic immunopositive reaction of the Ub immune-staining was markedly higher in the zona glomerulosa of group II (2.8) compared to in groups I and III (1.8 and 1.1) respectively. The intensity of the immunopositive reaction was also markedly higher (ANOVA <0.005) in the zona fasciculata of group II (2.2) compared to groups I and III (1.3 and 1.2) respectively. The intensity of the

immunopositive reaction was significantly markedly higher (ANOVA <0.005) in the zona reticularis of group II (1.8) compared to groups 1 (0.9) and III (1.1) (Fig. 5).

The quantitative analysis showed that the mean optical density of Ub immunoreactions of the zona glomerulosa, was significantly higher in group II compared to groups I and III (Table 2). The mean optical density was also significantly higher in zona fasciculata and reticularis of groups II compared to groups I and III (Table 2).

The VDR immunopositive staining (brown color) of the controls was markedly detected in nucleus, membranes and cytoplasm of the adrenal capsule, subcapsular cells, glomerulosa, fasciculata (Fig. 1G) and reticularis cells. The

Table 2. *Quantitative measurement of the immunostaining optical densities (AU) and the color area percent (means±SD) of the Masson Trichrome*

|		Group I (Mean±SD)	Group II (Mean±SD)	Group III (Mean±SD)	P value for ANOVA
Zona glomerulosa	Bax	0.117±0.003	0.298±0.009	0.12±0.002	0.0003
	Ub	0.124±0.01	0.385±0.01	0.09±0.001	0.0001
	VDR	0.324±0.009	0.109±0.003	0.355±0.01	0.0001
	Masson Trichrome	9.33±2.11	23.35 ± 4.12	10.11±3.99	0.01
Zona fasciculata	Bax	0.046±0.002	0.247±0.005	0.108±0.001	0.001
	Ub	0.04±0.002	0.15±0.009	0.067±0.004	0.005
	VDR	0.36±0.003	0.12±0.004	0.38±0.003	0.0001
	Masson Trichrome	10.98±3.44	22.21±3.12	12.37±5.78	0.02
Zona reticularis	Bax	0.009±0.001	0.217±0.003	0.05±0.004	0.0001
	Ub	0.01±0.001	0.155±004	0.054±0.006	0.0004
	VDR	0.33±0.005	0.09±0.001	0.29±0.009	0.0005
	Masson Trichrome	11.43±4.56	24.52±4.51	15.13±4.22	0.04
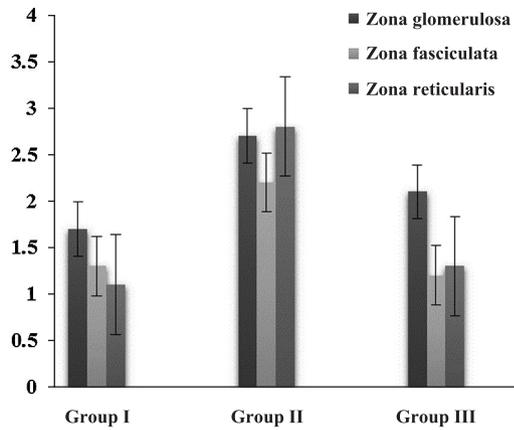
Fig. 4. The semi-quantitative data (means±SEM) of the color intensity of Bax immunostaining.
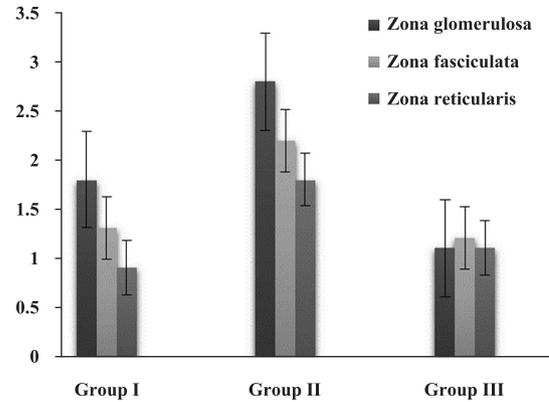

Fig. 5. The semi-quantitative data (means±SEM) of the color intensity of Ub immunostaining.
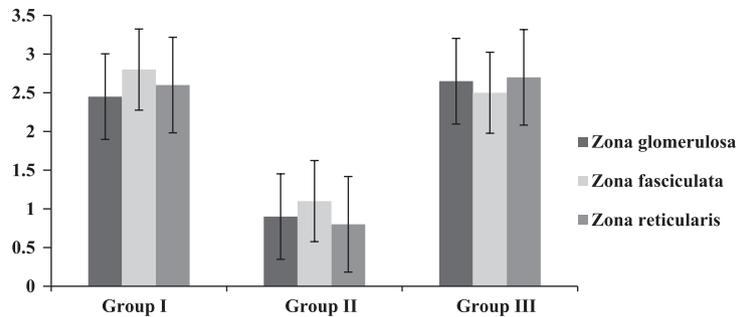

Fig. 6. The semi quatitative values (means±SEM) of the color intensity of VDR immunopositive staining.

immunopositive staining of group II was weakly detected in some of glomerulosa, fasciculata (Fig. 2F) and reticularis cellular cytoplasm and nuclei. Moreover, the immunopositive staining of group III was markedly detected in glomerulosa, fasciculata (Fig. 3F) and reticularis cellular cytoplasm and nuclei.

The semi-quantitative analysis showed that the intensity of the cytoplasmic immunopositive reaction of the VDR immune-staining was markedly higher in groups I (2.45, 2.8, 2.6) and III, (2.65, 2.5, 2.7) compared to group II (0.9, 1.1, 0.8) in zona glomerulosa, fasciculate and reticularis of respectively (ANOVA <0.005) (Fig. 6).

The quantitative analysis showed that the mean optical density of VDR immunoreactions of zona glomerulosa, fasciculate and reticularis were significantly higher in groups I and III compared to group II (Table 2).

IV. Discussion

The effect of systemic administration of KZ on adrenal cortex morphology was investigated in this study. The results showed significant increased plasma levels of ACTH and significant decreased corticosterone and aldosterone. The increased ACTH level was associated with a fall in plasma corticosterone and aldosterone when KZ was given to normal rats [16]. This study demonstrated marked morphological changes in the adrenal cortex of the KZ treated rats in forms of enlarged outer cortex, disturbed adrenocorticocyte cytological architecture, with shrinkage glomerulosa and enlarged fasciculate regions. Nuclear changes, large well demarcated cytoplasmic vacuoles and engorged blood vessels were also noted.

Mitochondria and smooth endoplasmic reticulum, which play the main role in steroidogenesis within the adrenal cortex, are the main cellular affected organelles [24, 68]. KZ induces low P450 secretion which leads to increased collagen deposition and hepatic fibrosis in mice [46].

KZ induced dilatation and engorged blood sinusoids mainly in zona fasciculata and reticularis [28]. This finding is attributed to the fact that KZ decreases the stroidogenesis and consequently increases the production of ACTH. The ACTH increases adrenal blood flow that is mediated through the release of neurotransmitters such as histamine and 5-HT [28]. These neurotransmitters are released by the mast cells which are present in the adrenal capsule particularly in the region where adrenal arteries enter the capsule [28]. The acute stimulation of the ACTH has only major effects on the zona fasciculata and zona reticularis [23]. Giving ACTH alone for 10 days leads to disrupt the cord like arrangement of fasciculta region, transformation of glomerulosa cells into fasciculata-type and red blood cells infiltration [51]. Moreover, KZ induces hypertrophy and vacuolation of adrenal mitochondria due to inhibition of the conversion of cholesterol to pregnenolone and consequently cholesterol may accumulate within the mitochondria [24].

Although ACTH does play a role in the regulation of zona glomerulosa function, angiotensin II is the most important regulator of aldosterone secretion [61]. The zona glomerulosa however, is under the control of the peptide angiotensin II that binds to a specific receptor on the surface of the zona glomerulosa cells [41].

Down-regulation of cytochrome P450 increases the nitric oxide (NO) production, a short-lived free-radical gas, which also increases blood flow and has a deleterious effect on the tissues [10]. KZ also releases pro-inflammatory cytokines such as interleukin l alpha (IL-1α) and tumor necrosis factor (TNF) either from the adrenal cortical cells or infiltrating neutrophils which destruct the adrenal cortical cells [13]. Moreover KZ induced microvascular injury through induction and potentiating the effect of interferon which also released by adrenal cortex and infiltrated leuko-cytes [18, 26]. This blood vessel destruction could explain the presence of the extravasted blood which has been found in this study. The KZ induced proinflammatory cytokines inhibit the expression and/or the activity of the cytochrome P450 [26].

The well demarcated large vacuoles which were found in the different cells of the adrenal cortex may be due to the accumulation of intracellular lipids which could be attribute to the breakdown of the steroidogenic acute regulatory protein (StAR) by KZ. Cholesterol transports to the outer mitochondrial membrane via the steroidogenic StAR [38]. Cytochrome P450scc (CYP11A1) then initiates steroidogenesis by converting cholesterol to pregnenolone on the inner mitochondrial membrane [39]. Thus KZ leads to accumulation of intracellular lipids by disruping the StAR and Cytochrome P450scc [62].

The immunohistochemical results of the current study showed that Bax and Ub were expressed in subcapsular cells and in also the medulla (data not shown). The subcapsular cells are chromaffin cells which spread in the subcapsular space of the zona glomerulosa and have a paracrine role in neuroregulation of the adrenal cortex [8].

Bax and Ub were maximally however, VDR is minimally expressed in KZ treated rats group (group II).

Bax is mostly found in the cytosol, but when apoptotic is initiating signaling, Bax undergoes a conformation shift. Upon induction of apoptosis, Bax becomes organelle membrane-associated, particularly mitochondrial [43, 47]. Bax interacts with, and induces opening of the mitochondrial voltage-dependent anion channel [11, 66]. This result in the release of cytochrome C and other pro-apoptotic factors from the mitochondria often referred to as mitochondrial outer membrane permeabilization, leading to activation of caspases which is the key factor in apoptoic process [12].

Ubiquitin, a small regulatory protein, is found in almost all tissues. It attaches to proteins and labels them for destruction via directed proteins to the proteasome. The increased Ub immunostaining can be attributed to KZ induced P450 damage. It is processed by several proteolytic pathways including endoplasmic reticulum, lysosomal, and 20S and the 26S-ubiquitin system [3, 15, 42]. The damaged P450 is initially cross-linked to ubiquitinated proteins and forms a large protein complex [67]. This complex may be degraded by either an auto-phagic mechanism or by the proteasome.

KZ inhibits the vitamin D3 by damaging P450 27B1 (CYP27B1) which is known as 1-hydroxylase enzyme [45]. The decreased vitamin D3 leads to decreased VDR expression which has been noted in group II of the current study.

Vitamin D3 supplementation of the current study resulted in increased VDR expression in the adrenal glands of group III. Increased VDR expression Vitamin D3 supplementation resulted in increased occupied and total VDR. This up-regulation was associated with reduced vitamin D3 catabolism [53].

Vitamin D3 and its precursors have immune and neuroendocrine activities as well as tumorostatic and anticarcinogenic properties. These properties affect proliferation, differentiation, and apoptosis in cells of different lineages as well as protect DNA against oxidative damage [6, 29]. The immunostaining findings were matched with other studies that found that proteasomal enzyme activities, expression of the E_2 ubiquitin conjugating enzyme and ubiquitin conjugates were increased in the vitamin D3 and calcium deficient rats compared to controls. Supplementation with vitamin D3 and/or calcium was found to decrease the proteasomal enzyme activities, expression of the E_2 ubiquitin conjugating enzyme and ubiquitin conjugates [5]. The apoptotic Bcl-2 member Bax is regulated by ubiquitination [69] this fact may explain the decreased expression of Bax in group III of the current study.

The beneficial effect of vitamin D3 injection could be attributed to the fact that KZ might decrease adrenal calcium levels and consequently decrease the production of P450 by blocking the formation of vitamin D3. This process is reversed by giving active vitamin D3 which increases the availability of calcium for achieving the mitochondrial function [20] and consequently increases the production of P450. Moreover, the 1α, $25(OH)_2$ D_3 upregulated *P450c24* gene expression [44] assumes to safe the mitochondria and consequently the adrenal function. Furthermore, vitamin D3 inhibits apoptosis via inhibition of ubiquination of vitamin D3 receptors [34].

Supplementation of the rats with vitamin D3 may also increase the vitamin D3 nuclear receptors, a process by which the production of P450 increases [65]. Moreover, the decreased accumulated lipid in the adrenal cortex of this group might be attributed to the elimination of cholesterol from cells by vitamin D3 [1]. Vitamin D3 serves as a substrate for P450scc and consequently the production of adrenal hormones [50] and utilization of intracellular lipids and consequently decreases the intracellular lipids. It was revealed that vitamin D3 incorporates into the bilayer of phospholipid membranes and can exchange between membranes at a faster basal rate than cholesterol. As for cholesterol, the transfer between membranes can be stimulated by the StAR protein. This is the mechanism by which vitamin D3 may enter the inner mitochondrial membrane of steroidogenic tissues such as the adrenal cortex, to be metabolized by P450scc. An *in vitro* study showed the production of hydroxyvitamin D3 metabolites when vitamin D3 is added to isolated rat adrenal mitochondria containing cholesterol supports the view that vitamin D3 can be metabolized by P450 enzyme in competition with cholesterol [59] and consequently produced the adrenal cortical hormones without entrance and accumulation of intracellular lipids.

Vitamin D3 also increases the production of cytochrome b5 [25] which have an important role in stimulation and augmentation of P450 reactions [50]. Taken together, the supplementation of vitamin D3 decreased accumulation of intracellular lipids could attribute to decreases the

entrance of the cholesterol in the adrenal cortical cells and/or increasing the production of P450 enzymes which increases the utilization of intracellular lipids during the production of adrenal cortical hormones.

In addition to its antioxidant effect, vitamin D3 also inhibits the pro-cytokines such as IL-6 and TNF-α which might be induced by KZ [70].

As far as we know, no extensive histological study has been done on the adrenal gland and investigated if the deleterious effects of KZ can be ameliorated. Moreover this is the first study which investigates the beneficial effect of vitamin D3 on the KZ induced adrenal changes.

More histological studies on the effects vitamin D3 on KZ induced adrenal injury by using special stains to verify the intracellular lipids and electron microscopic are recommended.

In conclusion KZ is used for the treatment of a variety of diseases but its use is limited by its side effects, which includes functional and morphological adrenal cortical changes. Vitamin D3 ameliorates the deleterious functional, microstructure and apoptotic changes induced by KZ. The results of this study may encourage studying the effects of vitamin D3 on the KZ induced adrenal injury on more functional basis. If proven, this may open the door for the safe and wide usage of KZ in treating the hyper-androgenemic states, prostatic cancer, and postmenopausal breast cancer, androgen secreting tumors, hirsutism and Cushing's syndrome in addition to its antifungal activities.

V.   Author Contribution

The author brought the idea, designed the work, did the practical part, took the slight pictures, read the findings in the histological and immunohistochemical sections, did the statistics and wrote the paper.

VI.   Conflict of Interests

The author has no financial conflicts of interest.

VII.   Acknowledgments

VIII.   References

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