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Original Research Article

Midazolam impact on the histological and ultrastructural characteristics of mice adrenal cortex

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Abstract

Midazolam is a member of the benzodiazepine group of tranquilizers. The present study aimed to investigate the histological and ultrastructural alterations induced by administration of midazolam on the adrenal cortex of male albino mice. Twenty adult male CD-1 mice were used in the present study. They were divided into two even groups. The first group served as control. The second group received daily double the therapeutic dose (0.26 mg/kg b.wt.) of midazolam for 28 days. The animals were sacrificed and adrenal samples were obtained and processed for histological and ultrastructural examination. Histologically, adrenal cortex sections of midazolam-treated mice revealed cytoplasmic vacuolation of the cells of three cortical zones; zona glomerulosa, fasiculata and reticularis, beside the nuclei of some of these cells showing signs of pyknosis, karyorrhexis and karyolysis. Ultrastructurally, cortical cells of midazolam-treated mice revealed conspicuous alterations, represented by deformed mitochondria with abnormal type of cristae (i.e. lamelliform). Besides, the rough endoplasmic reticulum was fragmented into small stacks and the smooth endoplasmic reticulum was dilated. The vacuolated cytoplasm contained numerous lysosomes, in addition to numerous lipid droplets. The nuclei showed clear signs of pyknosis and possessed irregular nuclear envelopes. In conclusion, it seems that the destructive impacts of midazolam on the adrenocortical cells reflected on their functions leading to much deficiency in their performance. So, it should be taken in the consideration and great concern that much drug must be utilized under restricted precaution in the medical fields to protect the human health from its hazardous impact.

Key words: Adrenal cortex, histology, mice, midazolam, ultrastructure

INTRODUCTION

Benzodiazepines are among the most widely prescribed tranquilizing drugs which are widely used for the treatment of many neurological and psychiatric disorders such as acute depression, muscular convulsions, gastric stress ulcers, sleeping disorders and skeletal muscles relaxation (Harro *et al.,* 1993; Shader and Greenblatt, 1993; Iqbal *et al*., 2002; Martire *et al.,* 2002). Despite their therapeutic benefits, benzodiazepines may be a cause of intoxication cases due to abuse by addicts (Wang, 2002). In this respect, Hertiz and Knight (2006) reported that the use of these agents is limited by the development of tolerance to their effects and the risk of developing dependence.

Dependence to benzodiazepines can also be manifested by a withdrawal syndrome which may include symptoms such as tremors, sweating, sleep disturbance, increased tension, anxiety and difficulty of concentration (Jonson *et al.,* 2007). Several investigators reported the health hazards of the illegal use of these groups of drugs in many countries (Roger *et al.,* 1997; Kapczinski *et al.,* 2001; Hertz and Knight, 2006).

Midazolam is a benzodiazepine with sedative, amne-

sic, anxiolytic, musle relaxant, and anticonvulsant properties in humans (North and Clark, 1997; Bulmer, 1998). At higher doses, midazolam induces sleep (Hogskilde *et al.,* 1987). Midazolam is mainly metabolized by cytochrom P_{450} 3A (CYP3A) isoenzymes (Wandel *et al.,* 1994). Hoen *et al.* (2001) analyzed the mRNA levels and enzyme activities of the major CYP isoforms in the rat liver after intraperitoneal injection of midazolam for 3 consecutive days. They found that CYP3A mRNA level were increased 4-fold in midazolam-treated animals compared with controls. The findings of Tan *et al.* (2009) indicated that midazolam and ketamine could induce widespread neural apoptosis in immature rat brain if they were administered during synaptogenesis. The authors added that repeated exposure of neonatal mice to ketamine or midazolam impaired dendritic maturation of the pyramidal neurons immediately, but this influence disappeared during further postnatal development.

El Rawi and Yousif (2007) found that high doses of the benzodiazepine, diazepam caused alterations in sartorius muscle fibers of mice, including distortion of their normal architecture and degenerated myofibrils. So *et al.* (2010) reported, in their *in vitro* study, that midazolam could induce Leydig tumor cells steroidogenesis in mice. More recently, Sedkey *et al*. (2012) showed that liver injury induced by benzodiazepine is rare and is classified as an unpredictable or idiosyncratic reaction.

Therefore, the present study has been designed to investigate the potential histopathological and ultrastructural alterations in the adrenal cortex cells induced by the administration of double the therapeutic dose of benzodiazepine midazolam in mice.

MATERIALS AND METHODS

The experimental animals

Twenty adult CD-1 male albino mice (Mus musculus) with body weights ranging from 27-30 g and aging about 3 months, obtained from Egyptian Organization for Biological Products and Vaccines, were used in the present study. The animals were acclimated to laboratory conditions and were allowed feed and water *ad libitum.*

The applied drug

Midazolam (marked under brand name Midathetic) is available in the medical market as ampoules; each ampoule containing 5 mg/ml of the active ingredient. It is produced by Amoun Pharmaceutical company, S.A.E., El- Obour City, Cairo, Egypt. The therapeutic dose of this drug for mice was calculated according to Paget and Barnes (1964). Animals were injected intraperitonealy (i.p.) with double the therapeutic dose (the therapeutic dose was 0.13 mg/kg b.wt. and double the therapeutic dose was 0.26 mg/kg b.wt.).

The experimental design

The mice were divided into two groups, each of 10 animals. The animals of the first group saved as control group and were injected intraperitonealy with 0.5ml 0.9% of saline solution for 28 days. The animals of the second group were injected (i.p.) daily with midazolam (0.26 mg/kg b.wt.) for 28 days. Twenty four hours after the last dose, the mice were killed by decapitation.

Histological preparations

The excited adrenal glands were fixed in Bouin's fixative for 24 hours. The specimens were then dehydrated, cleared in terpineol and embedded in paraffin wax. Serial transverse sections of 5 µm thickness were stained with haematoxylin and eosin (Bancroft and Gamble, 2002), microscopically examined and photographs were made as required.

Ultrastructural preparations:

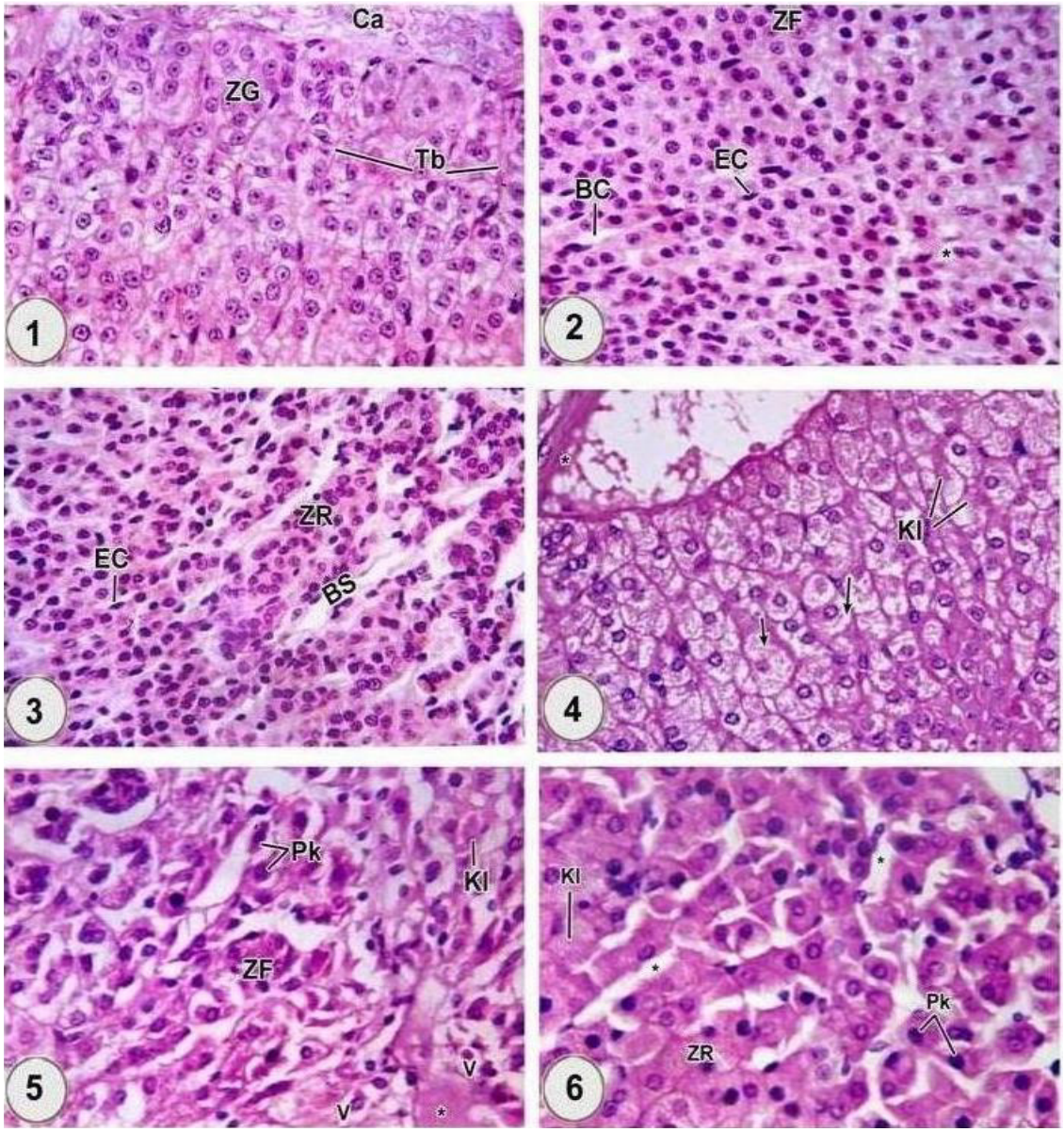
For ultrastructural evaluation by transmission electron microscopy as described by Dykstra *et al.*(2002), freshly excised adrenal glands were cut into small blocks fixed directly in cold 4FIG(i.e. 4% formalin +1% glutaraldehyde adjusted at pH 2.2) for 24 hours then were post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3), dehydrated in an ethanolic series culminating in 100% acetone, and infiltrated with epoxide resin. After polymerization overnight at 60 C. Semi-thin sections (0.5µm) were stained with 1%toluidine blue in sodium borate and examined with light microscope. Areas of cortical cells were selected and blocks trimmed accordingly. Ultrathin sections were cut, mounted on 200 mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). The stained grids were examined and photographed by a JEOL –JEM-1400 EX-electron microscope, at the Regional Center for Mycology and Biotechnology (RCMB) Al-Azhar University.

RESULTS

A-Histological observations

Group I-The control group

The adrenal cortex of control mice showed the common characteristic organization of the mammalian adrenal cortex. It is differentiated into three zones; zona



Photomicrographs (1-3) of adrenal cortex of the control mice group.

Figure 1. Showing the glomerular organization of zona glomerulosa cells (ZG), being separated by trabeculae (Tb) extend from the capsule (Ca), which is formed of fibrous elements. X 200

Figure 2. Showing zona fasciculata cells (ZF), which is long radial cords, separated by narrow blood capillaries (BC) lined with endothelial cells (EC). Binucleated cells (*) are also seen. X 400

Figure 3. Showing zona reticularis cells (ZR) arranged in irregular network of intermingled cords, separated by numerous wide blood sinusoids (BS) lined with endothelial cells (EC). X 400

Photomicrographs (4-6) of adrenal cortex of midazolam treated mice.

Figure 4. Showing zona glomerulosa cells which exhibit cytoplasmic vacuolation (arrows) and their nuclei showing karyolysis (Kl). Congested blood vessel with stagnant blood (*) is also detected in these figure. X 400

Figure 5. Showing hypertrophied fasciculata cells (ZF) possessing cytoplasmic vacuolation (V) with variable size and these vacuolated cells possessing necrotic nuclei revealing signs of pyknosis (Pk) and karyolysis (Kl). Notice, dilated and congested blood sinusoids with stagnant blood (*). X 400

Figure 6. Showing enlarged reticularis cells (ZR), and possessing necrotic nuclei revealing pyknosis (Pk) and karyolysis (Kl). Notice, dilatation of blood sinusoids in between these cells (*). X 400

Electron micrographs (7-10) of adrenal cortex of the control mice group.

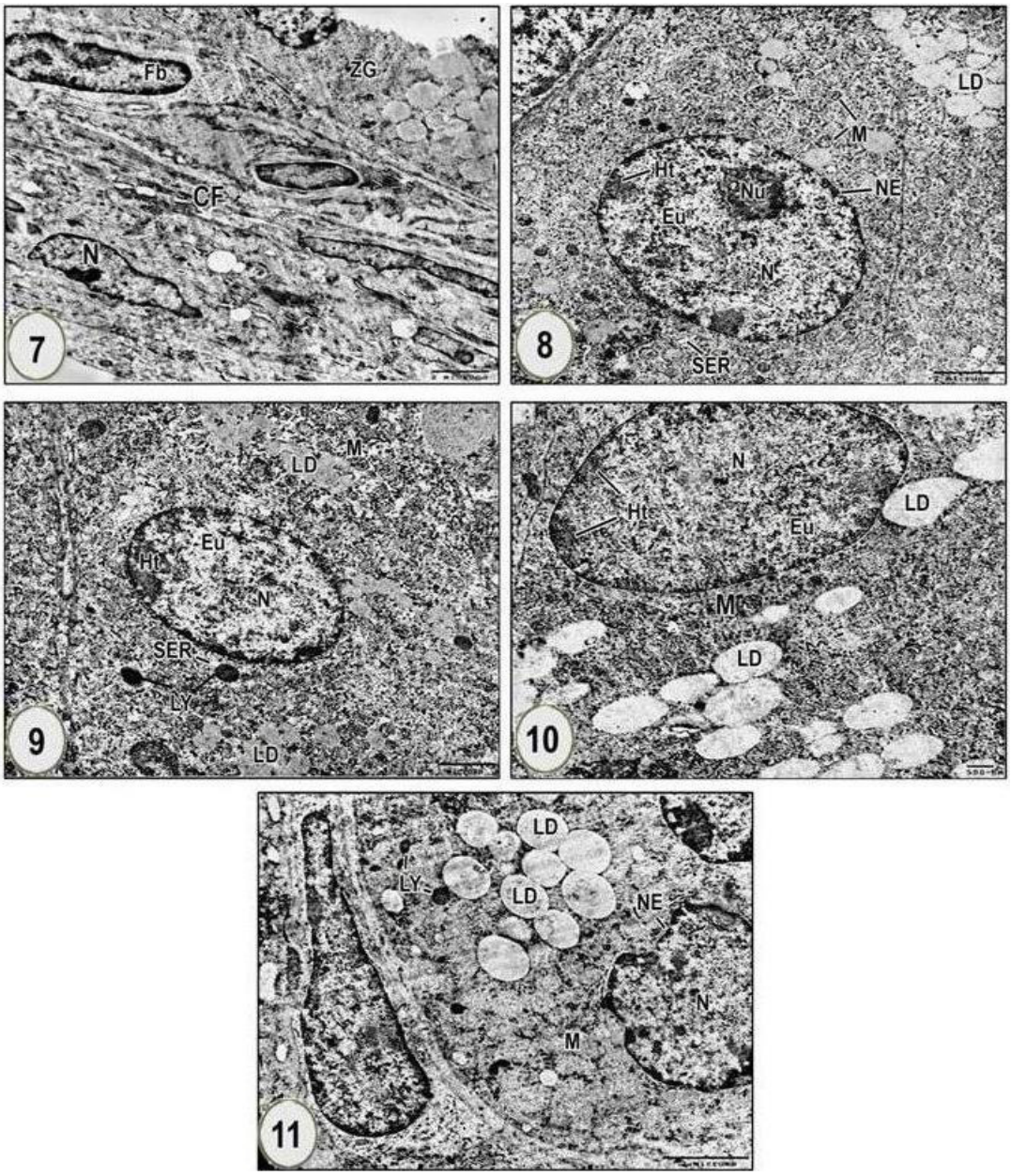


Figure 7. Illustrating part of adrenal cortex capsule consists of mainly of fibroblasts (Fb) with their distinct elongated nuclei (N) and collagen fibers (CF). Part of zona granulose (ZG) is also observed in this figure. X 8000

Figure 8. Showing fine structure of zona glomerulosa has abundant lipid droplets (LD), mitochondria (M) with tubule-saccular cristae, smooth endoplasmic reticulum (SER), spherical nucleus (N) surrounded by nuclear envelope (NE), having nucleolus (Nu), marginated heterochromatin (Ht) and euchromatin (Eu). X 10000

Figure 9. Fasciculate cell loaded with numerous mitochondria (M) that characterized by tubular cristae, smooth endoplasmic reticulum (SER), lysosomes (Ly) and lipid droplets (LD). Distinct spherical nucleus (N) containing peripheral heterochromatin (Ht) and flocculent euchromatin (Eu) is clearly observed. X 10000

Figure 10. Reticularis cell illustrating prominent rounded mitochondria (M) with tubular cristae, lipid droplets (LD) and oval nucleus (N) possessing peripheral heterochromatin (Ht) and euchromatin (Eu). X 15000

Electron micrographs (11-16) of adrenal cortex of midazolam treated mice.

Figure 11. Glomerulosa cell illustrating hypertrophied mitochondria (M), lysosomes (Ly), lipid droplets (LD) and pyknotic nucleus (N), being shrunken ensheathed by irregular nuclear envelope (NE). X 12000

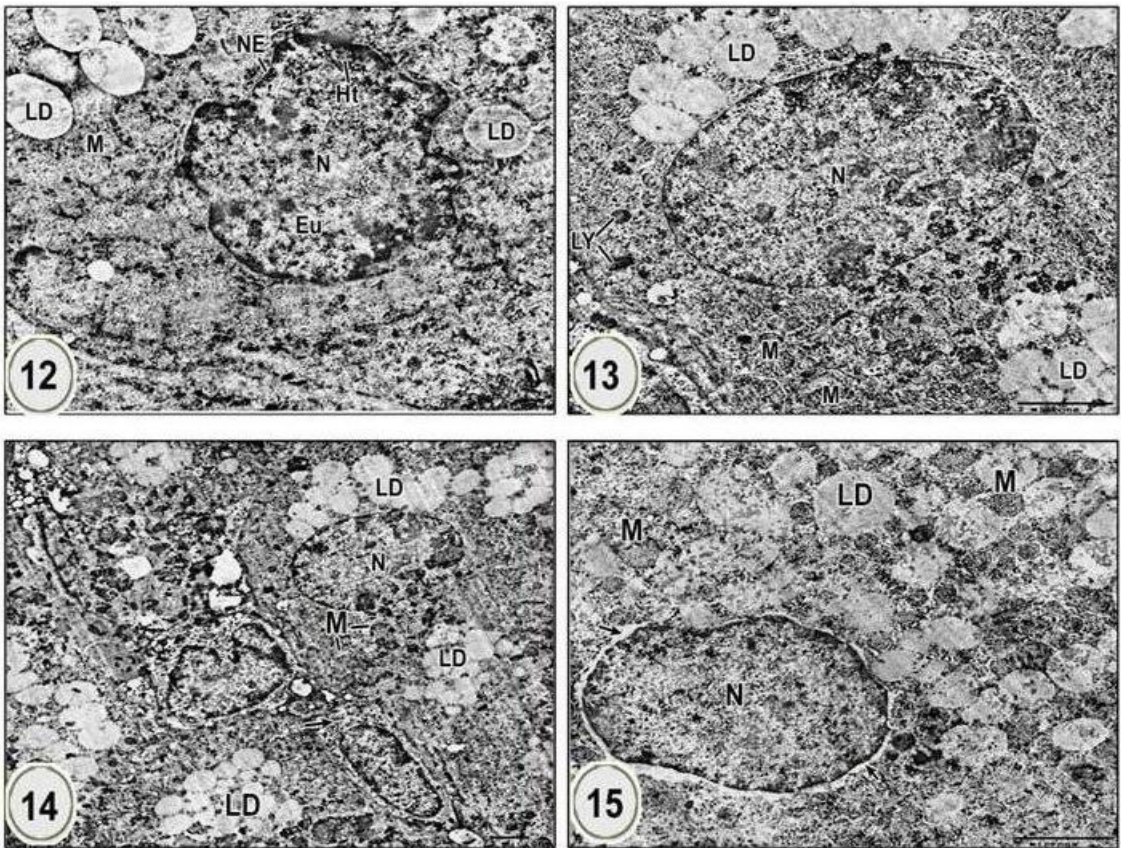


Figure 12. A highly magnification part of glomerulosa cell showing hypertrophied mitochondria (M), lipid droplets (LD) and pyknotic nucleus (N), being shrunken ensheathed by irregular nuclear envelope (NE) and having electron dense heterochromatin (Ht) and euchromatin (Eu). X 15000

Figure 13. Illustrating fasciculata cell with lipid droplets (LD) of variable sizes, hypertrophied mitochondria (M), some of them possessing cavitation with loss their cristae (*) and numerous lysosomes (Ly). X 12000

Figure 14. Zona reticularis cells over loaded with lipid droplets (LD), having deformed mitochondria (M) and deformed some nuclei (N), in addition to blood sinusoid (arrow). X 5000

Figure 15. Showing reticularis cell overloaded with lipid droplets (LD) of variable sizes, hypertrophied mitochondria (M) and deformed nucleus (N) detached itself from the outer nuclear envelope (arrow). X 12000

glomerulosa, fasiculata and reticularis respectively. The adrenal gland is surrounded by a fibrous connective tissue capsule as shown in figure (1). Zona glomerulosa is formed of columnar or rather pyramidal cells arranged in glomeruli- like structure, which are separated by delicate trabeculae extending from the capsule. Its cells contain acidophilic cytoplasm with fairly rounded to oval basophilic nuclei having distinct nucleoli (Figure 1). Zona fasiculata is composed of polyhedral or columnar cells arranged in one or two cells thick in long radial cords or fasiculae and they are separated by narrow blood capillaries lined with endothelial cells. The cells have granulated eosinophilic cytoplasm embodying spherical basophilic nuclei showing distinct nucleoli (Figure 2). Zona reticularis characterized by an irregular anastomoising network of intermingled cords separated by numerous wide blood sinusoids lined with endothelial cells. The cells of these cords are columnar cells possessing moderately eosinophilic cytoplasm, containing granules and have spherical nuclei possessing centrally located nucleoli (Figure 3).

Group II- Midazolam-treated group

The examination of adrenal cortex sections of mice treated daily with double dose (0.26 mg/kg b.wt.) of midazolam for 28 days revealed the outer cortex hypertrophy in its three zones. Glomerulosa, fasiculata and reticularis cells exhibited hypertrophy with large vacuoles in their cytoplasm. The nuclei showed clear signs of pyknosis, karyorrhexis and karyolysis as observed in figures (4-6). Blood sinusoids of zona fasiculata became dilated and loaded with stagnant blood in their lumina which lined with pyknotic endothelial cells (Figure 5).

B- Ultrastructural observations

Group I-The control group

Electron microscopic examination of the adrenal cortex of the control mice revealed the fine structure of zona glomerulosa cells with different mitochondrial configuration vary from oval or spherical shapes with specific tubule-saccular cristae. In addition small Golgi vesicles and abundant number of lipid droplets in the form of rounded bodies are evident. The nuclei of these cells are spherical or oval in shape; sometimes wavy in appearance ensheathed by nuclear envelope and possessing nucleoli, peripheral dense heterochromatin and homogenous euchromatin material (Figure 8).

Figure (9) exhibits the fine characteristic feature of fasiculata cells including; abundance rounded mitochondria with obvious tubular cristae, smooth endoplasmic reticulum, fair amount of lysosomes and richness of lipid droplets. The nuclei are large, spherical possessing prominent nucleoli, dense peripheral heterochromatin. Blood capillaries lined with endothelial cells are located between these fasiculata cells.

Zona reticularis cells are distinguished by their riches of rounded mitochondria with electron dense tubular cristae, smooth endoplasmic reticulum, lysosomes and lipid droplets with varying sizes. Their nuclei are spherical or ovoid in shape containing condensed heterochromatin, euchromatin and prominent nucleoli (Figure 11). Widened and clear blood sinusoids lined with endothelial cells are illustrated in figure (10).

Group II- Midazolam-treated group

Marked ultrastructural changes of zona glomerulosa cells were illustrated in figures (12 and 13), their cytoplasm contained hypertrophied mitochondria and some of them possessing small vacuolar degeneration, in addition to lysosomes and lipid droplets of variable sizes. The nuclei being electron dense, showing shrinkage, and sings of pyknosis. They were surrounded by irregular nuclear envelopes and containing electron dense heterochromatin and euchromatin.

Zona fasiculata cells showing hypertrophied mitochondria filled with tightly packed tubular cristae, some of them having cavitations.

Extensive accumulation of various sized lipid droplets. Fair numbers of lysosomes are seen (Figure 14).

Similarly, zona reticularis cells having hypertrophied mitochondria with some of them with degenerated cristae and cavitations, extensive accumulation of various sized lipid droplets, some of them become so large thus occupying almost the entire cytoplasm (Figure 16), few number of lysosomes were present and deformed nuclei surrounded by irregular nuclear envelops (Figures 15 and 16).

It is worthy to mention that smoth endoplasmic reticulum, was scanty, sometimes almost absent in all examined cells of the adrenocortical zones, suggesting that it may be disintegrated under the influence of midazolam.

DISCUSSION

Benzodiazepines have been prescribed for more than three decades to patients of all ages for a wide array of physical and psychological disorders (Reed *et al.,* 2001). Depending on the administrated doses, benzodiazepines produced, as previously mentioned in the introduction, their clinical effects (North and Clark, 1997). Nearly all these effects results from their actions on the gamma-aminobutric acid (GABA) receptors within the central nervous system (Hobbs *et al.,* 1996). According to Rey *et al.* (1999) benzodiazepines exerted their effects by binding to the benzodiazepine binding site, which leads to an allosteric modification of GABA receptors and enhanced activity of the inhibitory neurotransmitter GABA.

The adrenal glands reported to be the most common endocrine organ associated with chemically induced lesions (Ribelin, 1984). The adrenal cortex plays a tremendous number of vital activities in the human body. This importance of being out from the fact that the adrinocortical zones synthesize and secret steroid hormones, which fall into three major categories; mineral corticoid, exemplified by aldosterone which is secreted by zona glomerulosa. Aldosterone is an important regulator of salt homeostasis which is responsible for increasing sodium resorption and stimulating potassium excretion by the kidneys and thereby indirectly regulating extracellular fluid volume. Loss of this zona or the inability to secrete aldosterone may result in death due to retention of high levels of potassium with excess loss of sodium, chloride, and water, it can also potently influence the blood pressure, and it is a major control unit of acid/ base balance (Bielohuby *et al*., 2007). While, zona fasiculata secrets glucocorticoid, exemplified by cortisol, which is essential for life since it has a major role in responding to environmental stimuli; it decreases protein synthesis. Therapy increasing he circulating level of amino acids; it elevates blood glucose by stimulating the enzymes involved in gluconeogenesis in the liver; increasing the activity of urea cycle, and it mobilizes fatty acids and glycerol from adipose cells. It has also anti-inflammatory effects; it stabilizes lysosomal membrane, reducing release of damaging proleolytic enzymes at sits of inflammation; and it decreases capillary permeability, minimizing local swelling. These attributes make cortisol a valuable medication (Fawcett and Jensh, 2002; Compbell, 2005). By zona reticularis, small amounts of androgens are secreted. The two principal adrenal androgens are; and rostenedione (Andro) hormone and

dehydroepiandrosterone (DHEA), which is farless potent than testosterone and has little physiological significance. Both hormones can serve as substances for the conversion into testosterone and estradiol (Fawcett and Jensh, 2002; Keegan and Hammer, 2002).

The results of the present investigation clearly demonstrated that the application of midazolam in double the therapeutic dose to adult male mice induced conspicuous alterations in the histological structure of adrenal cortex tissue. Such lesions were more prominent in outer cortex hypertrophy in its three zones; glomerulosa, fasiculata, and reticularis cells exhibit hypertrophy with large vacuoles in their cytoplasm. The nuclei showing clear signs of pyknosis, karyorrhexis and karyolysis. These findings are in accordance with earlier studies that investigated the side effects of benzodiazepines including midazolam (Andrade *et al.,* 2000; Calarasu *et al.,* 2004; Yilmaz *et al.,* 2014). The effects of different treatments on the adrenal gland has been investigated by different researchers, as Lorente *et al*. (2002) who elucidated the change in the rat adrenal zone glomerulosa under the effect of chronic hypoxia, Pereira *et al.* (2007) studied the toxicity of Clophen A60 and diethyl phthalate on the adrenal cortex, Hermenean *et al*. (2008) demonstrated that adrenal glands morphofunctional damages in rats post chemotherapy administration, Dogaru *et al.* (2009) examined the impacts of pulsed short wave on the rat adrenal glands, Guerrero *et al*. (2010) detected the changes caused by the brown widospider venom in mice adrenal glands, as well as Florea and Cracium (2011) who evaluated the abnormal mitochondrial cristae generated by high doses of Apis mellifera (hony bee) venom (AmV) in rat adrenal cortex.

The adrenal cortex, and in particular zona fasiculata has been reported by Rosol *et al.* (2001) to be among the most common site lesions in the endocrine system. The factors which predispose this organ to such lesions include: its disproportionately large blood supply per unit mas; its high content of lipids and the susceptibility of its unsaturated fatty acids to peroxidation damage; and its high levels of cytochrome P450 which metabolize xenobiotics to reactive intermediates. In addition, the adrenal expresses several of the pathways for steroid production present in the testes and ovaries. Therefore, toxic chemicals can affect the adrenal or its axis directly or indirectly in a manner similar to the testes and ovaries.

The current results showed that administration of midazolam induced vasculature changes in the adrenal cortex of male mice. These alterations included dilation and congestion of blood vessels and sinusoids. Comparable results were obtained in a study carried out by Labib *et al*. (2000) who reported that the therapeutic and double the therapeutic doses of benzodiazepines derivative flunitrazepam initiated several vascular alterations in the lung and cerebral tissues of pregnant rats. Similar changes have been reported, in human subjects, following intrarterial (Knill and Evans, 1975) and intravenous (Graham *et al.,* 1977) injection of diazepam. More recently, Rabei (2011) investigated the effect of long-term treatment of ketamine on the liver tissue of albino rats. The author reported that ketamine induced some histological changes including dilated and congested portal areas and inflammation reaction in the peripheral area; the hepatocytes displayed also severe vacuolation. In the present investigation, vascular congestion was the first inflammatory histological changes observed in the adrenal tissue of the treated mice. This change may be resulting from the disturbance in the permeability of the blood vessels due to direct toxic action of midazolam. This explanation agrees with Sandritter *et al.* (1977) who reported that fibroid degeneration or necrosis, develops after severe acute disturbance of vascular permeability with subsequent sudden leakage of blood plasma into the vessel wall and the surrounding connective tissue. Because of these changes, the vascular tissues are either congested or destroyed.

Ultrastrucurally, conspicuous deleterious alterations in the cytoplasmic organelles of the three zones were seen, especially, the occurrence of an abnormal type of mitochondrial cristae (i.e. lamelliform cristae) and increased density of its matrix. Such abnormalities were also demonstrated in the rat adrenal cortex after the acute treatment with Apis mellifera (hony bee) venom (AmV) as reported by Florea and Cracium (2011). These authors mentioned that, this abnormal lamella mitochondrial cristae resulted by a complex fusion of the tubular mitochondrial cristae, and it may represent a first level of alteration of the cristae, which can be next, either destroyed, or closed into circular, concentric mitochondrial dysfunction. The profound lesions observed in mitochondria and endoplasmic reticulum by Guerrero *et al.* (2010) might be sufficient to cause impairment of steroid synthesis, in accordance of the fact that these organelles play an important role in steroidogenesis within the cortex, through, they involve in the coordinated actions of cytochrome P450 and enzyme 3ß-hydroxysteroid dehydrogenase (3ß HSD), which are distributed between the mitochondria and the smooth endoplasmic reticulum. The rate-limiting step in steroid hormone biosynthesis is the translocation of substrate cholesterol from the outer mitochondrial membrane to cholesterol side- chain cleavage enzyme (CYPIIA), the first enzyme in the steroidogenic pathway, which is located inside the mitochondria as Rainey *et al.* (2004) and Isola *et al*. (2010) elucidated in their studies.

The accumulation of lipid droplets observed in this study has been reported in dexamethasone- treated rats particularly in cells of zona fasiculata and zona reticularis (Almeida *et al.,* 2001). Much of the cholesterol used in steroid synthesis is stored in lipid droplets of the steroid-forming cells. The cholesterol ester in these droplets is transported to the inner mitochondrial membrane where it

enters the pathway to steroid hormones as free cholesterol (Hall. 1995). lipid accumulation inside adrenocortical cells may be considered as a secondary phenomenon due to the inhibition of the sequence of reactions leading from cholesterol to progesterone (Hemmaid, 2009). This explanation is in harmony with that of Tarantio *et al.* (2009) who reported that the pathogenesis of drug-induced liver injury takes part through the hepatotoxic compounds which are formed by metabolism (formation of neo-substances that react abnormally), mainly by cytochrom P450 (CYP), with further pathways, such as mitochondrial dysfunction and apoptosis, also playing a role. In this context, it was reported by Colleoni *et al.* (1996) that midazolam primarily acts as mitochondrial electron transfer inhibitor.

The present electron microscopic observations of adrenal cortex cells of midazolam-treated mice showed marked nuclear pyknosis in some necrotic cells, these results are also in agreement with those obtained by Almeida *et al.* (2006) who found nearly the same results of destructive alterations in the ultrastructural features of the nuclei in their study of dexamethasone influence.

CONCLUSION

The present study showed that administration of benzodiazepine, midazolam, has produced conspicuous histopathological and ultrastructural alterations in the adrenocortical cells of male albino mice. Accordingly, these results seem to prove adrenotoxic effects of high doses of the benzodiazepine midazolam in mice.

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