Original Article

The Role of Omega-3 on Diazepam Treated Adrenal Cortex of the Adult Male Albino Rats (Histological and Immunohistochemical Study)

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ABSTRACT

Introduction: Diazepam is a commonly used member of benzodiazepines. Little attention seems to have been paid to the effects of diazepam on endocrine glands. Omega-3 fatty acids exhibit significant antioxidant and anti-inflammatory effects on biological systems.

Objectives: To throw light on the effect of diazepam on the adrenal cortex and the ameliorative effect of Omg-3 on the adrenal cortex.

Materials and Methods: Fifty adult male albino rats were allocated randomly into three classes: Group I (control group) subdivided into three equal subgroups. Group II (diazepam treated group), Group III (diazepam + omg-3 treated group). After 28 days, blood samples were obtained from all groups to measure serum ACTH and cortisone. Tissue samples from adrenal cortex were obtained for evaluating adrenal malondialdehyde, glutathione, and superoxide dismutase. The adrenal cortex were undergone tissue processing for light and electron microscopic analysis. Morphometerical and statistical analysis were done.

Results: Diazepam treated group exhibited significantly elevated levels of serum ACTH and cortisone, elevated levels of MDA, and decreased values of SOD and GSH compared to the control group. Histopathological examination showed thickened capsule, congested blood vessels, many cells with vacuolated cytoplasm, increased lipid droplets, Dilated sinusoids and intrasinusoidal macrophages, many lysosomes, electron-dense mitochondria, dilated SER and dark nuclei. Increased collagen fibers in the capsule and weak PCNA immunostaining. However, concomitant administration of omg-3 had an ameliorating effect on these changes.

Conclusion: Omega 3 exerted ameliorating effects against diazepam-induced damage of the adrenal cortex. Therefore, it will be recommended to add supplement of omega-3 to the patients treated with diazepam.

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Key Words: Adrenal cortex, diazepam, omega-3, ultrastructure.

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INTRODUCTION

A popular benzodiazepine (BDZ), diazepam (Dz) is one of the most regularly prescribed medications globally. It has many clinical and therapeutic applications as a preoperative anesthetic medication to relieve anxiety and provide sedation, as well as its role as an anticonvulsant drug. One of the common uses of diazepam is to relieve anxiety symptoms and tension correlated with the day's stress. Furthermore, diazepam is used to relieve skeletal myospasms caused by pathological conditions including arthritis and myositis with traumatic etiology[1].

The previous epidemiologic data reported that one of four cases with COVID-19 could suffer from depression or anxiety[2] while 15% could experience unstable consciousness which was significantly correlated with higher death risks[3]. Therefore, patients with COVID-19 could sometimes require psychotropic drugs like diazepam but they are facing a high risk in safety because of the potential interaction with medications[4,5].

Benzodiazepines only bind to GABAA receptors in the nervous system, and the potential inhibitory effect in the spinal cord and brain receptors, and amplify their sensitivity to GABA functions including CNS association in sleep elicitation. Benzodiazepines play a potential role in neuronal excitability regulation, anxiety, epilepsy, memory, and hypnosis[6]. Benzodiazepine receptors have been detected in endocrine glands such as the pituitary and adrenal gland. Pituitary hormone influences the density of peripheral binding sites in diverse endocrine organs. Therefore, diazepam could exert its effect directly on the adrenal gland[7].

Diazepam, one of the most used drugs in a wide range for anxiety, is conducted with side effects such as ataxia, cognitive impairments, and sedation, because of its regulation of all synaptic GABAAR subtypes and non-selective binding[8].

Omega-3 (Omg-3) are vital polyunsaturated long-chain fatty acids of marine plants. They incorporate EPA, DHA,

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ALA. Flaxseed is considered a rich source of ALA and omega-3 fatty acid. On the other side, EPA and DHA are the main omg-3 in fish[9].

Omega-3 is a compartment in the cell membrane and possesses a potential role in cellular movement and supporting fluidity and integrity of plasmalemma[10]. In addition, Omg-3 is considered a potential antioxidant, antiarrhythmic, antiatherogenic, anti-hyperlipidemic, anti-inflammatory, antihypertensive, and anticoagulant. According to previous studies, Omg-3 was used for modulating insulin and lipid profile in inflammation and obesity[11,12].

The adrenal gland can adapt to cell injuries by secreting prostaglandins, nitric acid and cytokines[13,14]. In the hypothalamo-pitutary-adrenal (HPA) axis, adrenocorticotropic hormone (ACTH), which is secreted from the anterior lobe of pituitary through corticotropin-releasing hormone (CRH), acts on the zona fasciculata of the adrenal cortex for production of the ''anti-stress hormones'', glucocorticoids, from intracellular cholesterol[15]. Moreover, the free radicals were formed as a result of oxidative stress. The adrenal cortex is specially prepared to handle the expanded hazard of oxidative stress. Many antioxidants, both enzymatic and non-enzymatic, are greatly expressed in the adrenal cortex in comparison to other tissues[16].

Most of the investigators have concentrated their works on effects of diazepam on the central nervous system. But, little attention seems to have been paid to other organs especially the endocrine glands. The purpose of the current investigation is to determine the effect of diazepam on the histological structure of adrenal cortex and to investigate the ameliorating effect of Omega-3 on this organ.

MATERIALS AND METHODS

Chemicals

1. Diazepam (DZ): (Valium®) tablets (5mg) was purcgased from Sigma-Aldrich, Egypt. Each Tablet was powdered and dissolved in 5ml of distilled water, 5% carboxymethylcellulose (CMC) was added as a suspending agent to produce a suspension for different doses. It was administered in a dose of (2mg/kg BW) by once daily by oral gavage[17].
2. Omega-3 fatty acids (Omg-3): Omega-3 Plus (SEDICO, 6 October City, Egypt): used as gelatinous capsules, each contains 1 g fish oil (EPA 70% and DHA 30%). Each capsule was evacuated by syringe carefully and was given orally at a dose of 300 mg/kg once per day by oral gavage[18].

Animals

For this study, we employed fifty adult male albino rats from the animal house at Zagazig University's Faculty of Medicine, weighing between 280 and 320 g. The rats had two weeks of passive prelimination before the start of the experiment to exclude diseased animals to adapt the new environment. Laboratory settings included a temperature range of 22±2ºC, a 12-hour light–cycle, and a relative humidity of 50±5 percent for the rats housed in plastic containers. Drinks and food were given to them ad libitum.

The experiment was approved by the IACUC animal care guidelines of Zagazig University, Egypt, in compliance with the NIH rules for animal research.

Study protocol

The study period was in 8 weeks, the rats were arbitrary aliquoted into three groups:

Group (I); A total of 30 rats were used in the control group, which was divided into three equal subgroups:

- Group (Ia); natural parameters were evaluated in the negative control group, which had no treatment.
- Group (Ib); Rats were fed 1 ml of water and 5 percent carboxymethylcellulose once day via oral gavage in the positive control group.
- Group (1c); Omg-3-treated group): rat received Omega-3 in a dose of 300 mg/kg once per day by oral gavage.

Group (II); DZ-treated group: 10 rats, received (2mg/kg BW) from diazepam suspension once daily by oral gavage.

Group (III); DZ+Omeg-3-treated group: 10 rats; received a combination of diazepam suspension and Omega-3 by oral garage in the same previous doses.

Collecting blood samples

For this study, blood samples were taken in accordance with van Herck *et al*.[19]. For coagulation, the samples were left, then centrifuged for 10 minutes (3000 rpm) to get the serum which were stored (-20º C) for later estimation of serum ACTH and cortisone levels. They were measured using ELISA kits as stated by the manufacturers' method.

Collection of tissue samples

All rats were sacrificed by cutting of the aorta after cervical dislocation[20]. The adrenal glands were carefully dissected and processed for histopathological and ultrastructural examination.

Assessment of Glutathione (GSH), Malondialdehyde (MDA) as well as Superoxide dismutase (SOD):

Samples of adrenal glands were homogenized in phosphate buffer (ice cold) and centrifuged (10,000 X g) for 15 min at 4 °C. Adrenal malondialdehyde and antioxidant enzymes were performed using Biodiagnostic kits (Biodiagnostic, Giza, Egypt). Adrenal malondialdehyde (MDA) level was measured regarding to Ohkawa *et al*.[21]. Superoxide dismutase (SOD) according to the method of Nishikimi *et al*.[22]. GSH was measured using the method of Beutler *et al*.[23]

Light microscopic examination

Samples of adrenal glands were fixed in neutral buffered formalin (10%) for two days, then embedded in paraffin and 4 µm sections were obtained[24]. The following stains were used in this study:

- Haematoxylin and Eosin stain[24]: as a routine method for studying the general architecture of the adrenal gland.
- Masson's trichrome stain[24]: for identification of collagen fibers.

Immunohistochemical examination

Streptavidin biotin-peroxidase technique were used for detection of PCNA as a marker for cell proliferation using a mouse anti PCNA monoclonal primary antibody of IgG type (clone sc-56; product No. AF488) with a minimum working dilution of 1:200.

For IHC examination, the sections were deparaffinized, then rehydration performed in ethanol with descending series and blockage of endogenous peroxidases was done by immersion in 0.3% hydrogen H2O2 for ten minutes. Antigens were extracted in citrate buffer using a microwave oven set to 100°C for 15 minutes (pH 6.0). Blocking negative epitopes using 3% bovine serum albumin for 30 minutes. Two changes of PBS were prepared to rinse the sections in them and then the sections were subjected overnight to primary at 4 °C. The anti-rabbit antibody was administered after washing in PBS for ten minutes followed by a peroxidase-marked streptavidin for another ten minutes. The reactivity was evaluated by DAB. PBS was applied on the negative control sections. Then the counter staine (Mayer's haematoxylin) was added to the Sections which later dehydrated, mounted for examination[25].

Examination and photography

The sections were examined and photographed at the Zagazig University Faculty of Medicine's Department of Medical Histology and Cell Biology.

Transmission Electron Microscopy examination

Samples of adrenal cortex were dissected and immediately immersed in the 2.5% cocodylate buffered glutaraldehyde for 24 h at 4°C. Later on were post-fixed in osmium tetroxide (1.0%) in cacodylate buffer (0.1 mol/l) (pH 7.3) in room temperature for two hours. Then samples were dehydrated in ascending ethanol concentrations. then immersed in propylene oxide, the samples were impregnated in a mixture of propylene oxide and Epon-812 resin overnight, at that point, embedded in Epon-812 resin. Ultramicrotome (Leica Ultracut, Berlin, Germany) was used for get 0.5 µm sections then toluidine blue was applied to select sites to get the 80 nm thickness. Ultrathin sections were counterstained with lead citrate 2% uranyl acetate[26]. In the Electron Microscope Research Laboratory, Faculty of Agriculture, El Mansoura University, the stained sections were examined and micrographs were taken using a JEOL JEM 2100 electron microscope.

Morphometric analysis

Area percentage of both collagen fibres and PCNA immunoreactivity were measured using the Leica Qwin 500 image analyzer in Cairo University's Pathology Department. For each specimen 10 non overlapping histological fields (all fields have the same diameters) were selected, using an objective lens of magnification x 40. For each parameter 10 values were measured in each field. We calculated the average values of each parameter in several groups[27].

Statistical analysis

The obtained data from body weight, biochemical and morphometrical analysis were expressed as mean ± SD and exposed to ANOVA test for differences between the mean of different groups using SPSS (version 20.0) software for analysis. To compare the various groups' parameters with one another, we used the Tukey HSD post-hoc test. For two-tailed tests, a *p value* of 0.05 or less was deemed statistically significant, while a *p value* of 0.001 or less was considered highly significant[28].

RESULTS

Data obtained from all subgroups (Ia, Ib &Ic) of control group regarding biochemical, light and electron microscopic results were nearly similar. So results of subgroup (Ia) were selected for comparison with other groups.

Biochemical results

Table (1) showed that the mean values ACTH and cortisone serum levels in diazepam treated group were significantly higher than control group and diazepam + Omega-3 treated group respectively ($p<0.001$), while Omega-3 co-treatment in group 3 results showed non-significant difference compared to control control ($p>0.05$)

Table (2) showed significant difference regarding the parameters of oxidative stress between MDA and diazepam-treated group compared to the control and Diazepam + Omega-3 -treated group ($p<0.01$) respectively. While Omega-3 co-treatment (in group 3) results showed non-significant difference from the control values ($p>0.05$)

Moreover, mean values of SOD, and GSH serum levels were statistically lower in Diazepam treated group compared to control group and Diazepam + Omega-3 treated group respectively ($p<0.01$), and statistically non-significant difference between control group mean values and omega-3 co-treatment mean values of SOD and GSH ($p>0.05$).

Histological Results

Light microscopic results

Haematoxylin and eosin staining

Light microscopic examination of control adrenal cortex showed connective tissue gland capsule which had many cells with vesicular nuclei. The zona glomerulosa

was formed of arched clusters of cells with a vacuolated cytoplasm and spherical pale nuclei.The cells of zona fasciculate were arranged in long straight parallel cords of polyhedral cells with vesicular rounded nuclei and pale vacuolated cytoplasm separated by longitudinally arranged blood sinusoids. Fat cells adjacent to the capsule were noticed. (Figure 1a). The zona reticularis was formed of anastomosing cords of closely packed polygonal cells which separated by sinusoidal capillaries. Some cells of zona reticularis appeared with pale nuclei and others had dark nuclei. Adrenal medulla formed of anastomosing cords of large chromaffin cells with large vesicular nuclei (Figure 1b).

Light microscopic examination of adrenal cortex of adult male albino rats treated with diazepam showed markedly thickened capsule with congested blood vessels and disorganization of cells of zona glomerulosa. Some cells with flat nuclei were noticed in-between the capsule and zona glomerulosa. Most of zona glomerulosa cells exhibited swollen vacuolated cytoplasm and multiple deeply stained nuclei. Some zona fasciculata cells were obseved with dark pyknotic nuclei and cytoplasmic vacuolation. Others showed deeply eosinophilic cytoplasm and deeply stained nuclei. Intra-sinusoidal macrophage and many cells with karyolitic nuclei were also seen. (Figure 2a). Zona reticularis showed congested blood sinusoids. Some cells had dark stained nuclei (Figure 2b).

Light microscopic examination of adrenal cortex of adult male albino rats treated with diazepam+ omg-3 fatty acids showed apparently normal capsule surrounding the gland. The cells of zona glomerulosa and zona fasciculate had pale rounded nuclei and vacuolated cytoplasm. Some of zona fasciculata cells were still had karyolitic nuclei. (Figure 3a). Zona reticularis cells were separated with blood sinusoids. Ghost cells were noticed with eosinophilic cytoplasm and without nuclei. Medullary chromaffine cells were seen (Figure 3b).

Masson's trichrome stain

Sections which stained with Masson's trichrome in adrenal cortex of adult albino rats showed average amount of collagen fibers within the capsule in control group (Figure 4a). Extensive amount of collagen fibers especially in the capsule were seen in diazepam treated group (Figure 4b). In Omega-3 fatty acids and diazepam treated group mild amount of collagen fibers especially in the capsule were observed (Figure 4c).

Immunohistochemical staining of PCNA

Light microscopic examination of immunohistochemical stained sections for PCNA showed few cells expressed positive nuclear immunoreaction in the control group (Figure 5a), however sections of diazepam treated group showed some cells with positive nuclear immunoreaction (Figure 5b). Numerous cells of diazepam+omg-3 treated group revealed positive immunoreaction in their nuclei mainly at the junctional zone between zona glomerulosa and zona fasciculata (Figure 5c).

Electron microscopic results

Examination of ultrathin sections from a part of the zona glomerulosa of the adrenal cortex of control group showed the cells had large rounded euchromatic nuclei; their cytoplasm contained many spherical mitochondria and multiple lipid droplets (Figure 6a). Diazepam treated group revealed irregular nucleus with abundant heterochromatin formed clumps that frequently adhered to the membrane or appeared dispersed in the karyolymph which was slightly electron dense, electron dense mitochondria and vacuoles. Electron-dense bodies of lysosomes were also seen (Figure 6b). Diazepam+omg-3 fatty acids treated group showed cells had large rounded euchromatic nuclei. However, small nucleus with some heterochromatin was observed. Many spherical mitochondria and lipid droplets were also seen. (Figure 6c).

Examination of ultrathin sections from a part of the zona fasciculata of the adrenal cortex of control group showed many mitochondria with vesicular cristae, smooth endoplasmic reticulum, lipid droplets and a euchromatic nucleus with peripheral clumps of heterochromatin (Figure 7a). Diazepam treated group showed cells with small electron dense nuclei with irregular nuclear membrane and dilated smooth endoplasmic reticulum. Another cells appeared with rounded nucleus with dilated perinuclear space. pleomorphic mitochondria and lipid droplets were observed. Some macrophages with indented nucleus and many lipid granules in its cytoplasm were seen close to dilated blood sinusoids (Figure 7b). Diazepam+omg-3 fatty acids treated group showed many mitochondria, smooth endoplasmic reticulum, lipid droplets and euchromatic rounded nuclei (Figure 7c).

Examination of ultrathin sections from a part of the zona reticularis of the adrenal cortex of control group showed mitochondria with vesicular cristae and rounded euchromatic nuclei. Electron dense lysosomes and smooth endoplasmic reticulum were also seen (Figure 8a). Diazepam treated group (b): showed multiple vacuoles and lipid droplets, rounded euchromatic nuclei, multiple electron dense mitochondria and electron dense lysosomes were also observed (Figure 8b). Diazepam+omg-3 fatty acids treated group revealed rounded euchromatic nuclei, lipid droplets and myelin figures. Many normal mitochondria were seen. Others appeared electron dense (Figure 8c).

Morphometric and statistical results

Area percentage (%) of collagen fibers

The results showed significant increase in the collagen fibers area percentage in diazepam-treated group compared to the control. Diazepam+omg-3 group showed a non-significant increase compared to the control one (Table3).

Area percentage (%) of PCNA immunoreactivity

We detected a significant increased area % of PCNA immunostaining in diazepam treated group regarding the control one and a highly significant increase in diazepam+omg-3 treated group regarding the control one (Table 3).

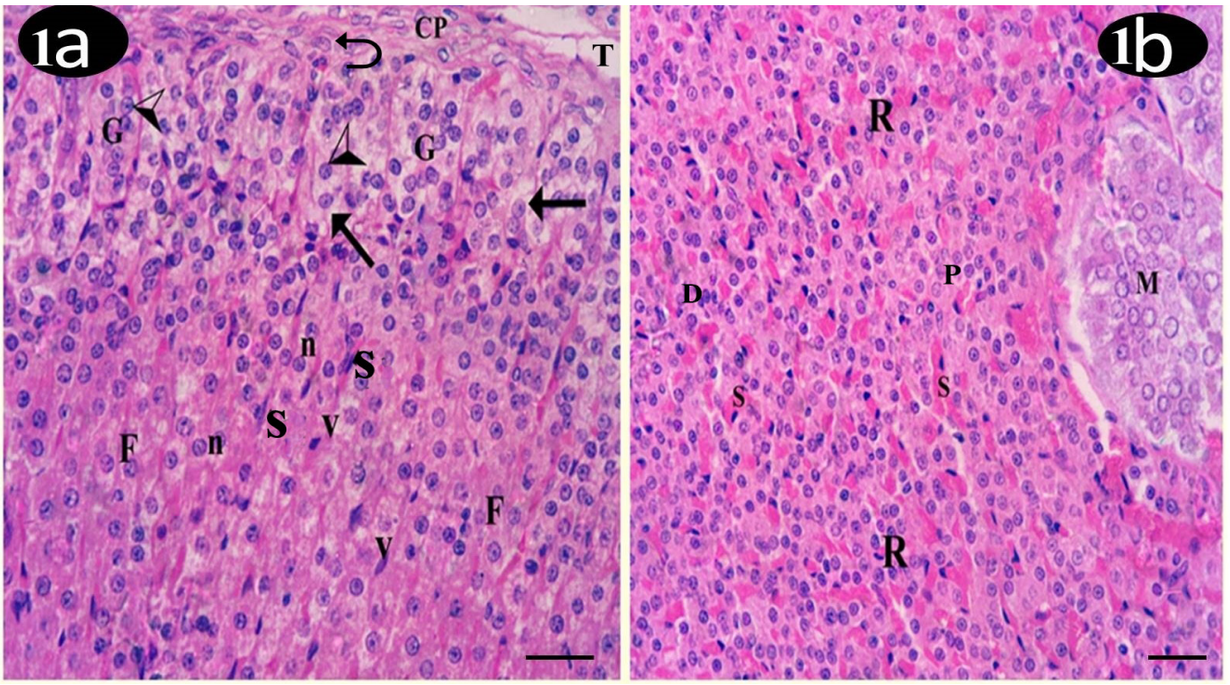

Fig. 1: A photomicrograph of control adrenal cortex showing (a): connective tissue gland capsule (CP) which has many cells with vesicular nuclei (curved arrow). The zona glomerulosa (G) is formed of arched clusters of cells with a vacuolated cytoplasm (arrows) and spherical pale nuclei (arrowheads).The cells of zona fasciculata (F) are arranged in long straight parallel cords of polyhedral cells with vesicular rounded nuclei (n) and pale vacuolated cytoplasm (v) separated by longitudinally arranged blood sinusoids (S). Notice fat cells (T) adjacent to the capsule. (b): zona reticularis (R) is formed of anastomosing cords of closely packed polygonal cells which separated by sinusoidal capillaries (S). Some cells of zona reticularis appear with pale nuclei (P) and others have dark nuclei (D). Adrenal medulla (M) is noticed formed of anastomosing cords of large chromaffin cells with large vesicular nuclei. (H and E, X400, scale bar 20 um).

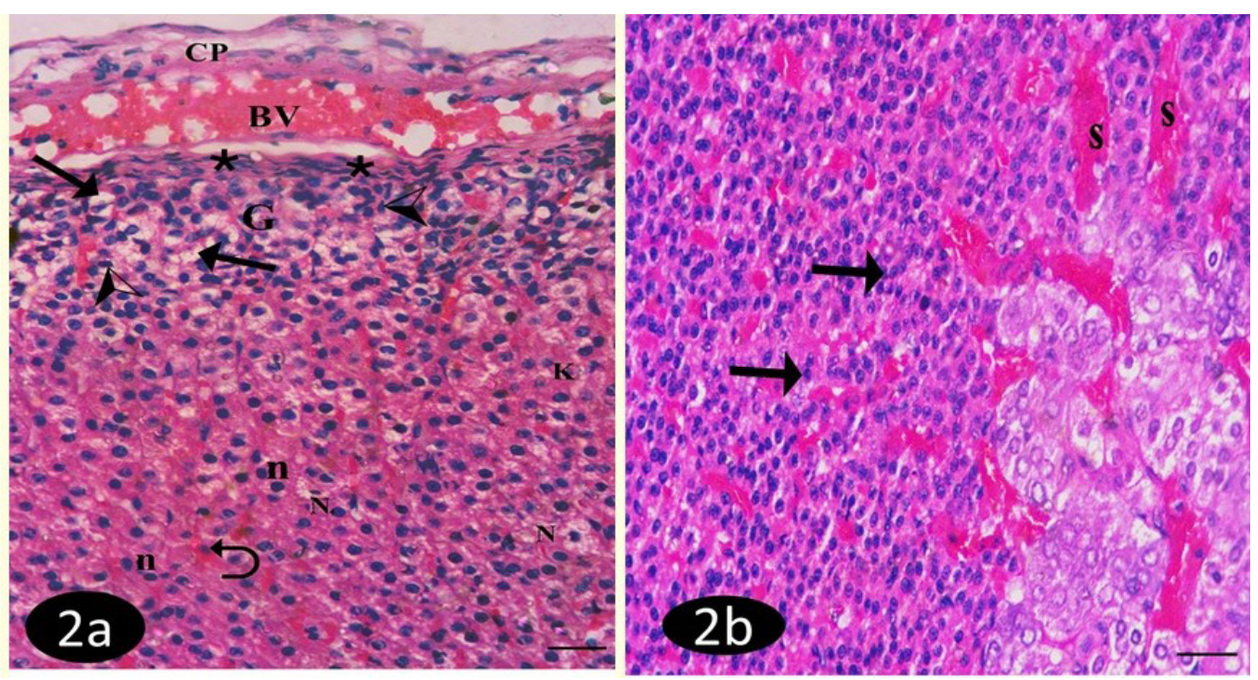

Fig. 2: A photomicrograph of adrenal cortex of adult male albino rat treated with diazepam showing (a): Markedly thickened capsule (CP) with congested blood vessel (BV) and disorganization of cells of zona glomerulosa (G). Some cells with flat nuclei (asterisk) are noticed in-between the capsule and zona glomerulosa. Most of zona glomerulosa cells exhibit swollen vacuolated cytoplasm (arrows) and multiple deeply stained nuclei (arrow heads). Some zona fasciculata cells (N) are obseved with dark pyknotic nuclei and cytoplasmic vacuolation. Others show deeply eosinophilic cytoplasm and deeply stained nuclei (n). Intra-sinusoidal macrophage (curved arrow) and many cells with karyolitic nuclei (K) are also seen. (b): The zona reticularis shows congested blood sinusoids (s). Some cells have dark stained nuclei (arrows). (H and E, X400, scale bar 20 um).

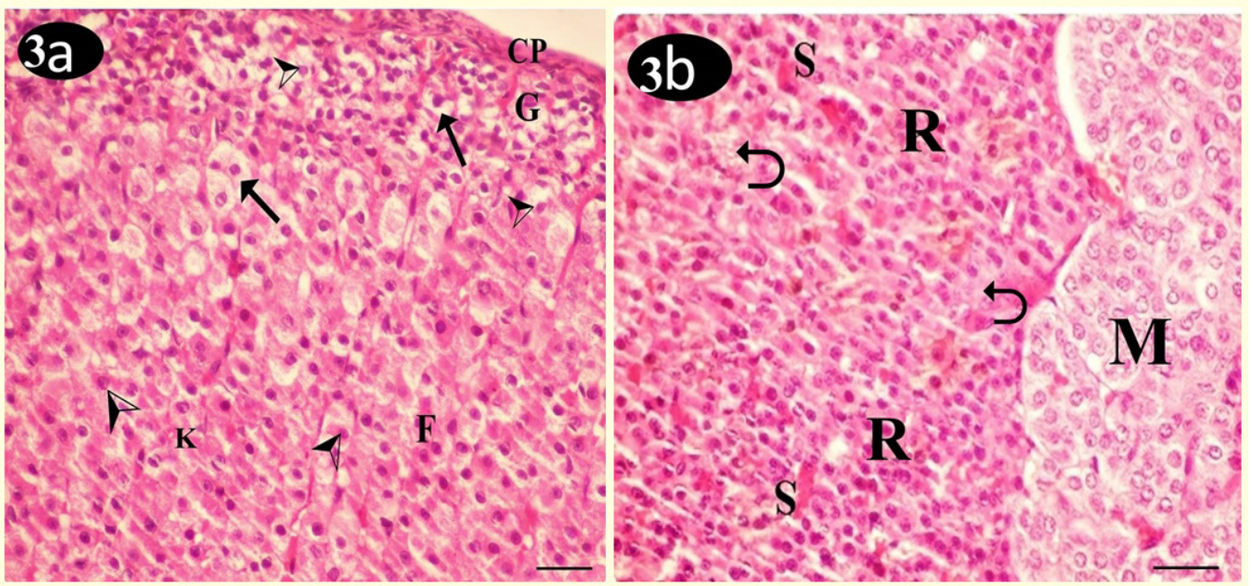

Fig. 3: A photomicrograph of adrenal cortex of adult male albino rats treated with diazepam+omg-3 showing (a): Apparently normal capsule (CP) surrounding the gland. The cells of zona glomerulosa (G) and zona fasciculata (F) have pale rounded nuclei (arrow heads) and vacuolated cytoplasm (arrows). Some of zona fasciculata cells are still have karyolitic nuclei (K). (b): Zona reticularis (R) cells are separated with blood sinusoids (S). Ghost cells (curved arrows) are noticed with eosinophilic cytoplasm and without nuclei. Medullary chromaffine cells are seen (M) (H and E, X400, scale bar 20 um).

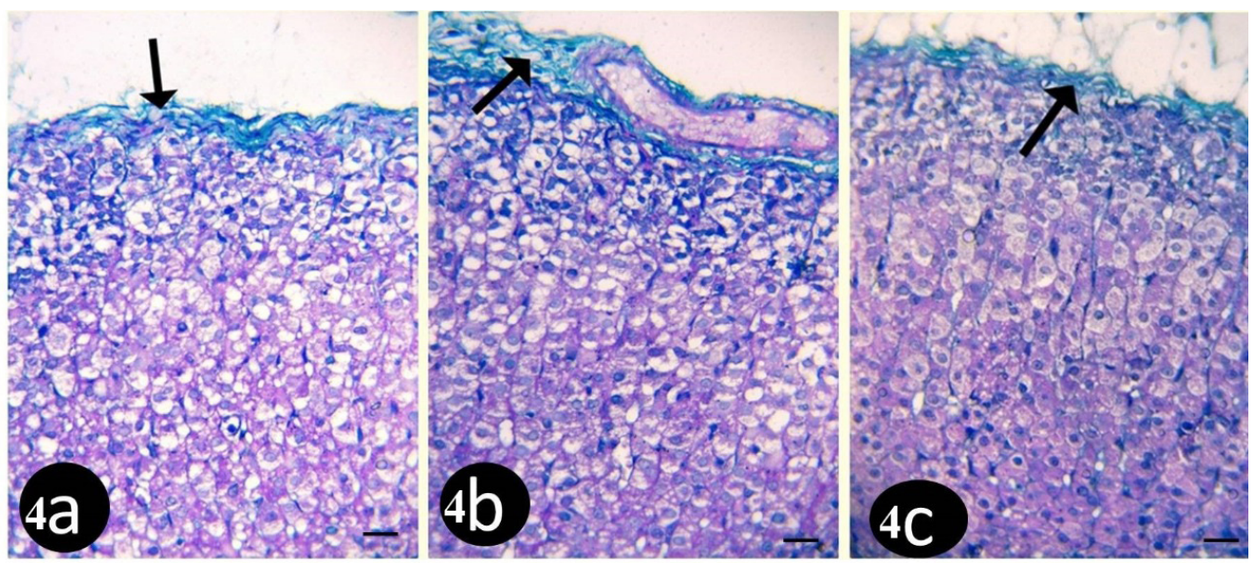

Fig. 4: A photomicrographs of Masson's trichrome stained sections in adrenal cortex of adult albino rats showing (a): average amount of collagen fibers within the capsule (arrow) in control group. (b): extensive amount of collagen fibers especially in the capsule (arrow) are seen in diazepam treated group (c): In diazepam+omg-3 treated group mild amount of collagen fibers especially in the capsule (arrow) are observed (Masson's trichrome, X400, scale bar 20 um).

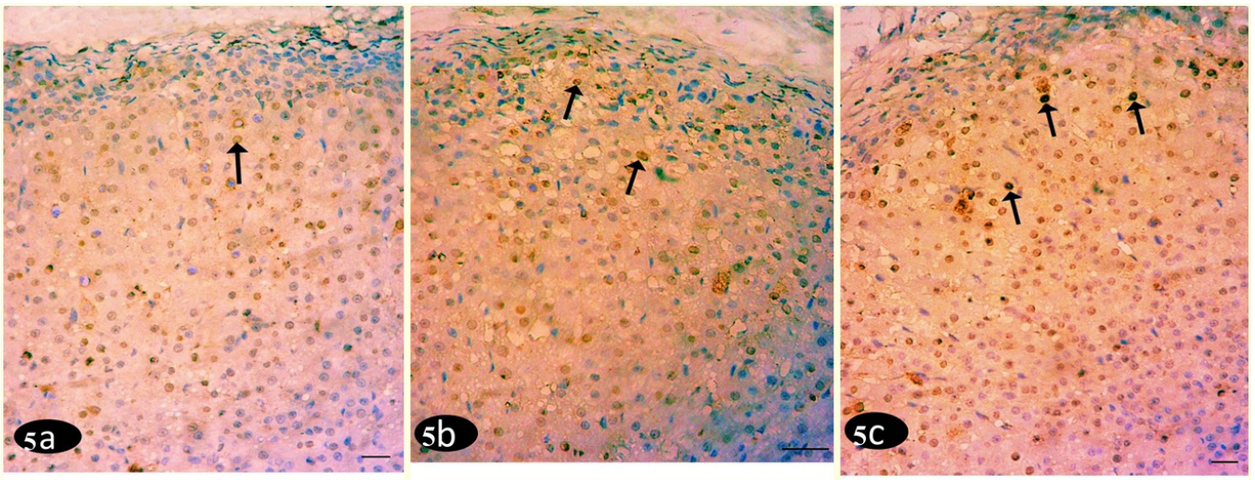

Fig. 5: Photomicrographs of PCNA immunostained sections in adrenal cortex of adult albino rats showing (a): few cells expressed positive nuclear immunoreaction (arrow) in control group. (b): some cells show positive nuclear reaction (arrows) in diazepam treated group (c): In diazepam+omg-3 treated group numerous cells are observed with positive nuclear reaction mainly at the junctional zone between zona glomerulosa and zona fasciculata (arrows) (PCNA immunoreaction, x400 scale bar 20 µm).

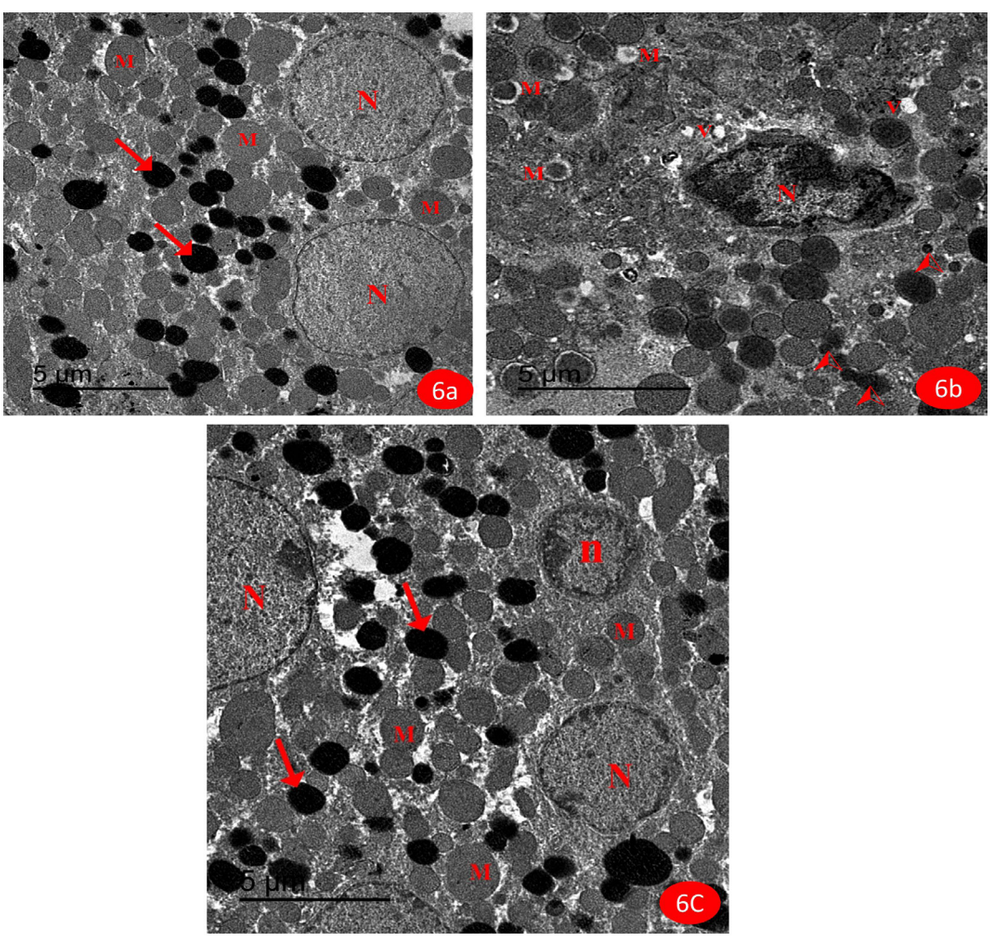

Fig. 6: Electron micrograph of a part of the zona glomerulosa of the adrenal cortex of: control group (a): showing the cells have large rounded euchromatic nuclei (N); their cytoplasm contain many spherical mitochondria (M), and multiple lipid droplets (arrows) (TEM X8400; scale bar 5 µm). Diazepam treated group (b): showing irregular nucleus (N) with abundant heterochromatin formed clumps that frequently adhered to the membrane or appeared dispersed in the karyolymph which is slightly electron dense, electron dense mitochondria (M) and vacuoles (V). Electron-dense bodies (arrow heads) of lysosomes are also seen (TEMX 10000; scale bar 5 µm). Diazepam+omg-3 treated group (c): showing the cells have large rounded euchromatic nuclei (N). However, small nucleus (n) with some heterochromatin is observed. Many spherical mitochondria (M) and lipid droplets (arrows) are also seen. (TEM X 7800; scale bar 5 µm).

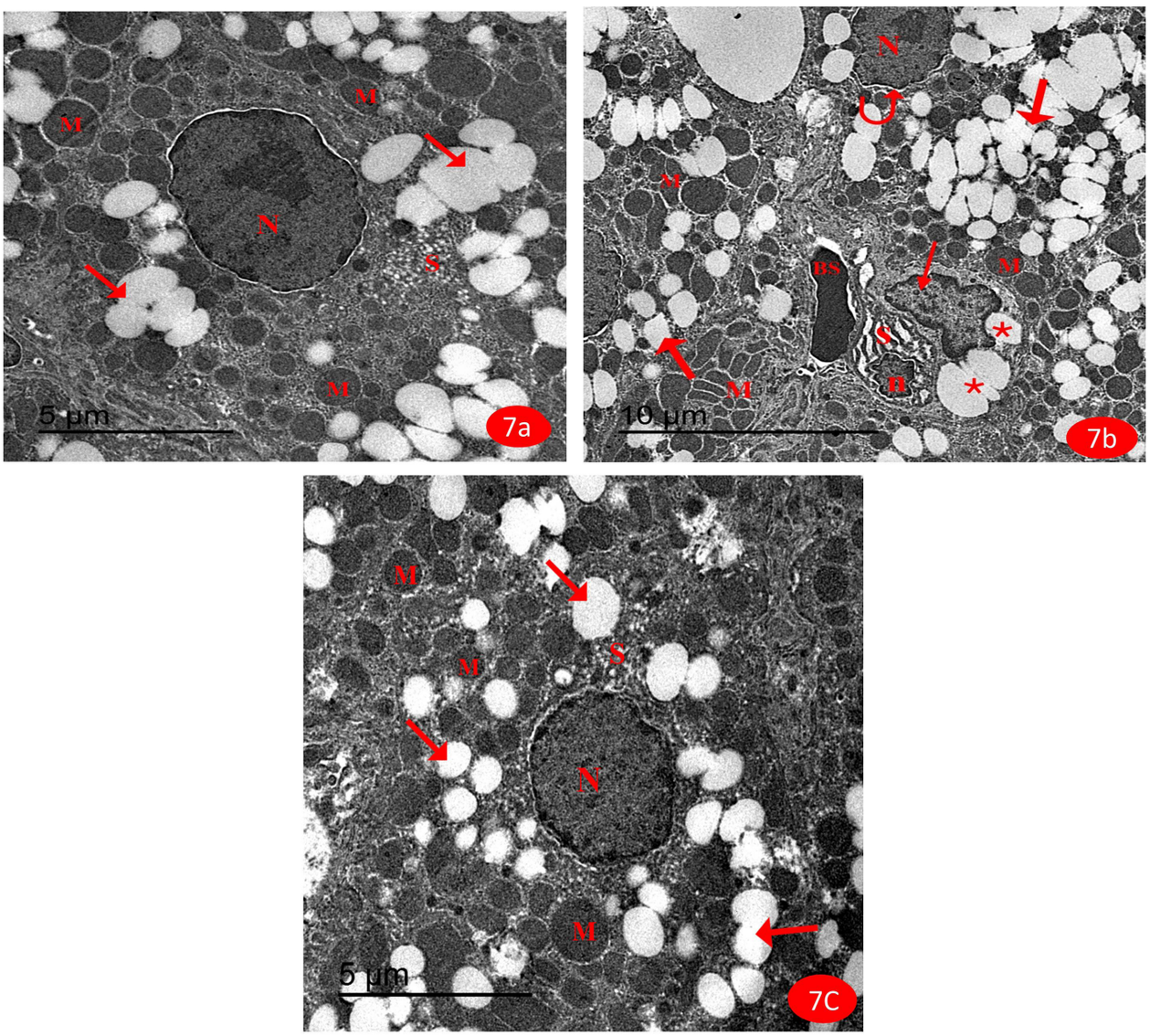

Fig. 7: Electron micrograph of a part of the zona fasciculata of the adrenal cortex of: control group (a): showing many mitochondria with vesicular cristae (M), smooth endoplasmic reticulum (S), lipid droplets (arrows), and a euchromatic nucleus with peripheral clumps of heterochromatin (N) (TEM X 10000; scale bar 5 µm). Diazepam treated group (b): showing one cell have small electron dense nucleus (n) with irregular nuclear membrane and dilated smooth endoplasmic reticulum (S). Another cell appears with rounded nucleus (N) with dilated perinuclear space (curved arrow). Macrophage (thin arrow) with indented nucleus and many lipid granules (asterisk) in its cytoplasm is seen. Dilated blood sinusoid (BS), pleomorphic mitochondria (M) and lipid droplets (thick arrows) are observed. (TEMX 6300; scale bar 10 µm). Diazepam+omg-3 treated group (c): showing many mitochondria (M), smooth endoplasmic reticulum (S), lipid droplets (arrows), and a euchromatic rounded nucleus (N) (TEM X10000; scale bar 5 µm).

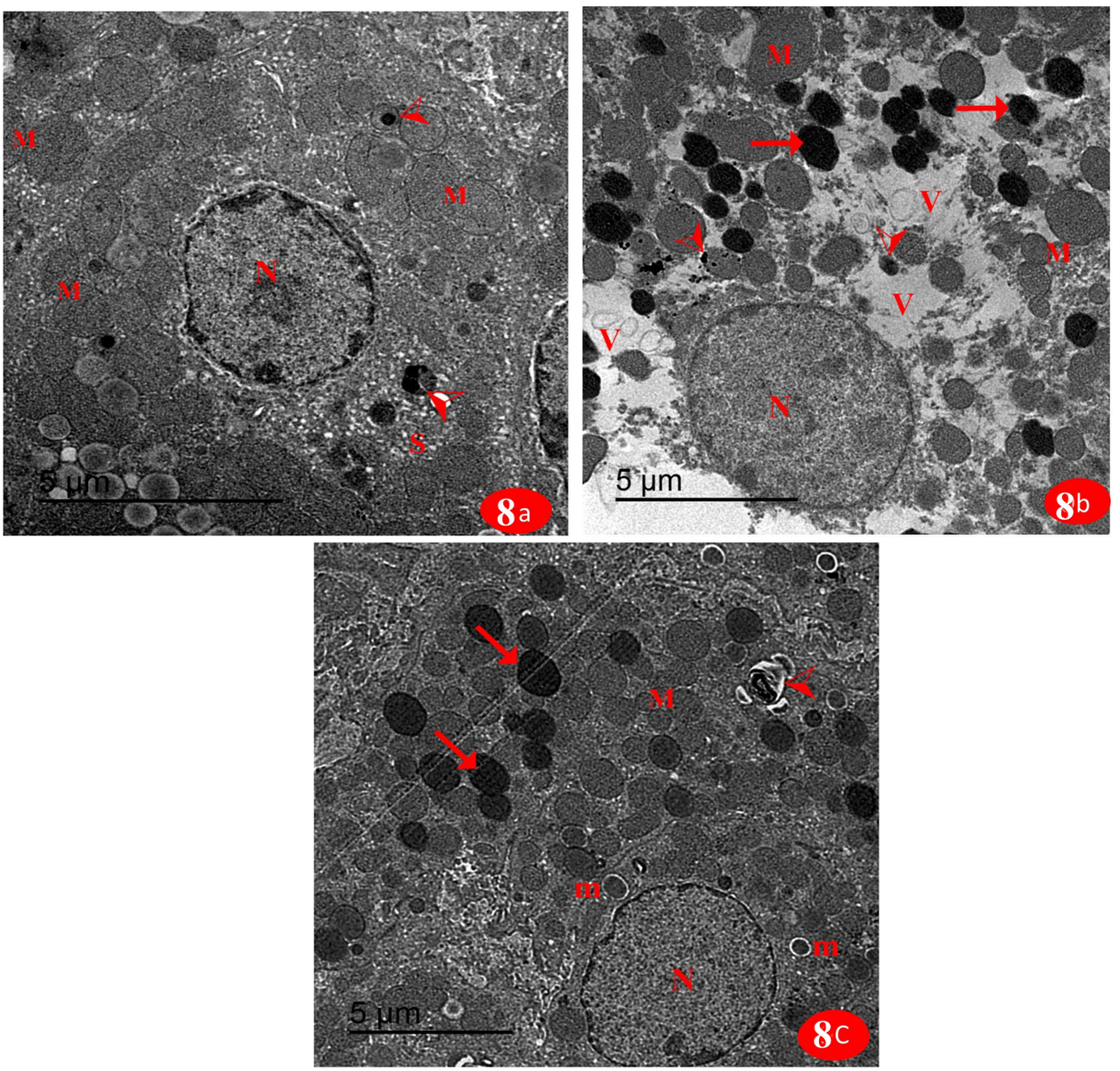

Fig. 8: Electron micrograph of a part of the zona reticularis of the adrenal cortex of: control group (a): showing mitochondria with vesicular cristae (M) and a rounded euchromatic nucleus (N). Electron dense lysosomes (arrow heads) and smooth endoplasmic reticulum (S) are also seen (TEM X12200; scale bar 5 µm). Diazepam treated group (b): showing multiple vacuoles (V) and lipid droplets (arrows). A rounded euchromatic nucleus, multiple electron dense mitochondria (M) and electron dense lysosomes (arrow heads) are also observed (TEMX 10000; scale bar 5 µm). Diazepam+omg-3 treated group (c): showing a euchromatic rounded nucleus (N), lipid droplets (arrows) and myelin figures (arrow heads). Many normal mitochondria (M) are seen. Others appear electron dense (m) (TEM X 8200; scale bar 5 µm).

Table 1: Statistical comparison among different studied groups of plasma levels of hormones (ACTH and Cortisone) by one way ANOVA with Tukey HSD post-hoc test

Group / Plasma levels of Hormones	Negative control group	Diazepam-treated group	Diazepam+ Omega-3 treated group	*p-value*
	Mea n± SD			
ACTH (pg/ml)	350±3.2	700.0±5.5[a]	350.0±6.3[b]	<0.0001***
Cortisone (ng/ml)	300.0±3.4	800.0±8.4[a]	460.0±8.1[b]	<0.0001***

SD: standard deviation ***: very highly significant
a: versus control b: versus Diazepam -treated group

Table 2: Statistical comparison among different studied groups of serum oxidative stress parameters (MDA,SOD and GSH) by one way ANOVA with Tukey HSD post-hoc test

Group / Oxidative parameters stress	Negative control group	Diazepam-treated group	Diazepam+ Omega-3 treated group	*p-value*
	Mea n± SD			
MDA (nmol/g tissue)	4.4±1.2	12.3±0.20[a]	5.4±0.32[b]	<0.0001***
SOD (Units/mg protein)	1.3±0.056	0.42±0.23[a]	0.73±0.05[b]	<0.0001***
GSH (µg/mg protein)	0.009±0.003	0.005±0.001[a]	0.03±0.006[b]	<0.0001***

SD: standard deviation ***: very highly significant
a: versus control b: versus Diazepam -treated group

DISCUSSION

Benzodiazepines (BZDs) are used to decrease anxiety, enhance effects of opioid and decrease symptoms of cravings and withdrawal. Diazepam is one of BZDs, which is very popular, approved, as well as consequently molecules that can be mismanaged[29]. Therefore, the current search has been done to to throw light on the effect of diazepam on the adrenal cortex and the ameliorative effect of Omg-3.

In the present study, histological examination of adrenal cortex in the control group revealed Presence of many cells with rounded vesicular nuclei in the capsule. This could be explained by Pignatelli *et al*.[30] who reported that undifferentiated cells capable of adrenocortical regeneration are found in the connective tissue capsule of adrenal gland. These cell clusters are likely to represent homologous groups of adrenocortical stem and progenitor cells.

Histological examination of adrenal cortex treated with diazepam showed many degenerative changes in the form of markedly thickened capsule with congested blood vessels and disorganization of cells of zona glomerulosa. Many cells with flat nuclei, mostly fibroblasts, were observed between the capsule and zona glomerulosa. Most of zona glomerulosa cells exhibited vacuolated swollen cytoplasm and multiple deeply stained nuclei. Some zona fasciculata cells were obseved with dark pyknotic nuclei and cytoplasmic vacuolation. Others showed deeply eosinophilic cytoplasm and deeply stained nuclei. Intra-sinusoidal macrophages and many cells with karyolitic nuclei were also seen. Zona reticularis showed dilated congested blood sinusoids. Some cells had dark stained. These findings were in agreement with Anber *et al*.[17]. They noticed degeneration and necrosis of hepatocytes and congestion of central veins in the group treated with diazepam.

Vascular changes, including congestion and dilatation, that have been noticed in diazepam treated group were also in line with the findings of Labib *et al*.[31] who reported that therapeutic dose of BDZ derivatives has shown congested and dilated vessels in the cerebral and lung tissue.

Masson's trichrome stained sections of diazepam treated group revealed a significant increased collagen fibers deposition especially in the capsule. Researchers reported that diazepam administration stimulates the production of profibrogenic mediators from inflammatory cells that lead to fibrosis[32]. In addition, the elevated level of ACTH in diazepam treated group could stimulate proliferation of capsular fibroblasts leading to induction of fibrosis[30]. This agreed with the proliferation of fibroblasts observed in H&E examination.

We used PCNA as an indicator of cell proliferation and as a component of the DNA replication and repair mechanism. Diazepam treated group showed a significant decrease of PCNA immunostaining compared to diazepam+omg-3 group. This was in agreement with Rinaldi *et al*.[33] who found that chronic BZD administration induced pro-apoptotic and anti-proliferative effects on acinar cells of the salivary glands. Consistent with our results, some researchers have shown that BDZs inhibit the growth and function of cancer cells in the prostate due to their anti-growth and pro-apoptotic effects[34]. We have confirmed

this finding by the electron microscope examination of the DZ-treated group as we detected many cells with irregular nuclei containing abundant heterochromatin.

Numerous cells of diazepam+omg-3 treated group revealed positive immunoreaction for PCNA in their nuclei mainly at the junctional zone between zona glomerulosa and zona fasciculata. This could be explained by Elisabeth[35] who mentioned that in rats, an undifferentiated zone containing clusters of undifferentiated stem cells exists between the zona glomerulosa and the zona fasciculata, and is referred to as the zona intermedia. We hypothesized that administration of Omega-3 fatty acids could activate stem cell proliferation with subsequent regeneration of cells of adrenal cortex. Our hypothesis was supported by Hilgendorf and his colleagues who found that omega-3 fatty acids could prompt the fat stem cells to divide, leading to the creation of more fat cells[36]

Electron microscopic examination of cells of adrenal cortex of diazepam treated group confirmed the light microscopic results. we detected cytoplasmic vacuolation, increased lipid droplets, many lysosomes, dilated SER and electron dense mitochondia. Some sinusoids associated macrophages were seen. These results were in accordance with Yousif[37] who examined the ultrastructure of the liver of BDZs- derivatives treated mice and found many phenotypical alterations including, degenerated nuclei and fragmentation of nucleoli, damaged and decreased mitochondria, dilated rER, decreased glycogen content, hypertrophied Golgi apparatus, dilatation of sER and increase of lipid droplets.

Cytoplasmic vacuolations and increased lipid droplets detected in diazepam treated group may be due to increased demand for lipids which form the corner stone for steroid hormones synthesis. This result also found by Vargas *et al*[38]. They described stimulatory effect of diazepam on the HPA axis. This agreed with our biochemical results which revealed a significant increasead serum ACTH as well as cortisone levels in Diazepam treated group regarding control groups also when compared with Diazepam + Omega-3 treated group.

Although many previous studies have demonstrated that classical benzodiazepines such as diazepam decrease the HPA axis activity in stressful conditions, under basal conditions they have been shown diverse results on the HPA axis activity. Such diverse results might be related to several factors such as the dose and sex. BDZs tend to exert inhibitory effects on female adrenal cortex and stimulatory effect on male[39].

On top of that, Papadopoulo observed that the peripheral type of benzodiazepine receptors is present in hypothalamus, pituitary and adrenal gland. These receptors have vital role in transport of cholesterol to the inner membrane of mitochondria in steroid biosynthesis[40]. They proved that diazepam exerts a stimulatory effect on steroidogenesis through an action on mitochondrial benzodiazepine receptors. Lazzarini and his colleagues confirmed that diazepam causes stimulation of the peripheral benzodiazepine receptors in the suprarenal gland resulting in increasing the serum corticosterone level[41].

Moreover, our work revealed that many mitochondria appeared more electron dense after diazepam administration. This was in line with Abdel-Maksoud who reported that mitochondria present in large number and more electron dense after diazepam treatment for 15 days indicating the activity of the cells in steroidogenesis. They mentioned that mitochondria vary in their internal conformation in different respiratory state. They assume the condensed form with increased oxidative phosphorylation[42].

Although oxidative phosphorylation is a vital part of metabolism, it produces reactive oxygen species such as superoxide and hydrogen peroxide, which lead to propagation of free radicals, damaging cells ElSokkary[43]. This was in line with our results which showed significantly increased levels of MDA (lipid peroxidation product) and significantly decreased values of both SOD (antioxidant enzyme) and GSH (non-enzymatic antioxidant) in diazepam treated group. Also, this was in agreement with Musavi & Kakkar[44] who reported that diazepam causes free radical-mediated changes.

These pathological lesions may be due to the direct toxicant and adverse effects of diazepam and its active metabolites and its accumulative ability during repeated administration. Moreover, oxidative stress induced by diazepam administration could disrupt proteins, lipids and DNA, induce apoptosis and damage of cells. Also, oxidative stress induces damage of cellular calcium channels and increases inflammation[44,45].

Usage of omega-3 in the current study ameliorated both the biochemical and the morphological changes induced by diazepam administration. These results were in agreement with Moghadamnia and his colleagues who proved that omega-3 supplementation had protective effects against lipid and renal dysfunction in male rats[46].

Omega-3 has by immunomodulation activity such as genetic mutations, protein synthesis, and cellular signaling pathways. In addition, Omg-3 has anti-inflammatory properties, enforces the effect of antioxidant enzymes by regulating expression of genes and has a great role in stabilizing oxidative stress in the cell[47].

CONCLUSION

In agreement with the biochemical findings, which were supported by the histopathological changes in the cortex of adrenal gland of the diazepam treated group, current results have highlighted the risk of adrenal cortex injury due to diazepam administration. In addition, combination therapy with Omega-3 had ameliorating effects against diazepam. Therefore, it would be appropriate to include omega-3 supplements in patients treated with diazepam.

ETHICAL APPROVAL

All rats received care in accordance with the standards of the National Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The Institutional Animal Care and Use Committee (IACUC), Zagazig University, Egypt approved the design of

ABBREVIATIONS

DZ: diazepam, **BDZs:** Benzodiazepines, **COVID-19:** coronavirus disease, **GABA:** gamma-aminobutyric acid, **NO:** nitric oxide, **PGs:** prostaglandins, **HPA:** hypothalamo-pituitary-adrenal, **CMC:** carboxymethylcellulose, **GSH:** Glutathione, **H2O2:** hydrogen peroxide, **DAB:** diaminobenzidine, **ALA:** α-linolenic acid, **NO:** nitric oxide, **PGs:** prostaglandins, **HPA:** hypothalamo-pitutary-adrenal, **ACTH:** adrenocorticotropic hormone, **CRH:** Corticotropin-releasing hormone, **CMC:** carboxymethylcellulose, **NIH:** National Institutes of Health, **IACUC:** Institutional Animal Care and Use Committee, **ELISA:** enzyme-linked immunosorbent assay, **MDA:** Malondialdehyde, **SOD:** Superoxide dismutase, **GSH:** Glutathione, **PCNA:** Proliferating Cell Nuclear Antigen, **H2O2:** hydrogen peroxide, **DAB:** diaminobenzidine, **ANOVA:** one-way analysis of variance, **SPSS:** Satistical Package for the Social Sciences.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nat Rev Drug Discov. 2011; 10:685–97.
2. Kong X, Kong F, Zheng K, Tang M, Chen Y, Zhou J, Li Y, Diao L, Wu S, Jiao P, Su T and Dong Y. Effect of Psychological–Behavioral Intervention on the Depression and Anxiety of COVID-19 Patients. Front. Psychiatry. 2020; 11:586355. doi: 10.3389/fpsyt.2020.586355
3. Mao L, Jin H, Wang M, *et al*. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. JAMA Neurology. 2020; 77(6):1–9. https://doi.orgl/10.1001/jamaneurol.2020.1127.
4. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, Ding C, Zhang X, Huang J, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 26: 368:m1091. doi: 10.1136/bmj.m1091.
5. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. JAMA. 2020. https://doi.org/10.1001/jama.2020.2648. Online ahead of print.
6. Calogero AE, Kamilaris TC, Bernardini R, Johnson EO, Chrousos G P, Gold PW. Effect of peripheral benzodiazepine receptor ligands on hypothalamic-pituitary-adrenal axis function in the rats. J. Pharmacol. Exp. Ther. May.1990; 253 (2): 729-737.
7. Mohler H. The legacy of the benzodiazepine receptor:from flumazenil to enhancing cognition in Down syndrome and social interaction in autism. Adv Pharmacol. 2015; 72: 1–36.
8. Fox C, Liu H, Kaye AD. Manchikanti L, Trescot AM, Christo PJ, *et al*, eds. Clinical Aspects of Pain Medicine and Interventional Pain Management: A Comprehensive Review. Antianxiety agents; 2011; 543–552.
9. Abdel A, Yacoub S. Omega-3 polyunsaturated fatty acids attenuate radiation-induced oxidative stress and organ dysfunctions in rats. Egyptian Journal of Radiation Sciences and Applications. 2013; 26(1-2), 41– 54.
10. Valentine RC, Valentine DL. Omega-3 fatty acids in cellular membranes: a unified concept. Prog Lipid Res. 2004; 43: 383–402.
11. Kasim-Karakas S. Omega-3 fish oils and insulin resistance. In: Wildman R, editor. Handbook of nutraceuticals and functional foods. 2nd ed. Boca Raton, FL: CRC Press. 2006; 155–164.
12. Komal F, Nisa MU, Khan MK, Ashfaq UA, Manzoor F, Masroor A, Nadeem M, Amir RM, Kausar R, Huda NU. Evaluation of the efficacy of different sources of omega-3 fatty acids in polycystic ovarian syndrome (PCOS) induced rats. Pak J Pharm Sci. 2019; 32(4):1781–8.
13. John CD, Buckingham JC. Cytokines: Regulation of the hypothalamopituitary–adrenocortical axis. Current Opinion in Pharmacology. 2003; 3:78–84.
14. Ehrhart-Bornstein M, Bornstein SR. Cross-talk between adrenal medulla and adrenal cortex in stress. Annals of the New York Academy of Sciences . 2008; 1148:112–117.
15. Hayashia T, Ikematsub K, Abec Y. Temporal changes of the adrenal endocrine system in a restraint stressed mouse and possibility of postmortem indicators of prolonged psychological stress .Legal Medicine .2014; 16: 193-196.
16. Prasad R, Kowalczyk JC, Meimaridou E, Storr H L, Metherell LA. Oxidative stress and adrenocortical insufficiency. J Endocrinol. 2014; 221(3): R63–R73. PMID: 24623797.
17. Anber ZNH, Fadhil AA, Anber SA. (). THE BIOCHEMICAL AND HISTOLOGICAL EFFECT OF DIAZEPAM ON THE LIVER OF ALBINO MALE RATS. International Journal of Academic Research and Reflection. 2018; (6) 3.

18. Meganathan M, Gopal KM, Sasikala P, Mohan J, Gowdhaman N, Balamurugan K, Nirmala P, Santhakumari S, Samuel V. Evaluation of hepatoprotective effect of omega 3-fatty acid against paracetamol induced liver injury in albino rats. Global J of Pharmacol. 2011; 5 (1): 50-53.
19. van Herck H, Baumans V, Brandt CJWM, Hesp APM, Sturkenboom JH, van Lith HA, van Tintelen G, Beynen AC. Orbital sinus blood sampling in rats as performed by different animal technicians: the influence of technique and expertise. Lab Animals. 1998; (32):377-386.
20. Prakoso Y, Kurniasih K. Effects of aloe vera cream on skin wound healing in sprague dawley rats: the role of CD4+ and CD8+ lymphocytes. Advances in Health Sciences Research (AHSR).2017; 5:58-63.
21. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem.1979; 952: 351358. https://doi.org/10.1016/0003-2697 (79)90738-3.
22. Nishikimi M, Roa NA, Yogi K. Colorimetric determination of superoxide dismutase in tissues. BiochemBioph Res Common.1972; 46:849854. https://doi.org/10.1016/S0006-291X (72)80218-3.
23. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 1963; 61:882–888.
24. Suvarna KS, Layton C, Bancroft JD.Bancroft's Theory and Practice of Histological Techniques E-Book. 8th ed. 2018; 126-139 and 434-475. Elsevier Health Sciences, China.
25. Dabbs DJ. Diagnostic immunohistochemistry E-Book: Theranostic and Genomic Applications. 5th ed. 2019; 542-580. Elsevier, China.
26. Hayat M. Principles and Techniques of Electron Microscopy Biological Applications. 4th ed., 2000; 70-92. Maac Millan Press, London.
27. Jensen EC. Quantitative Analysis of Histological Staining and Fluorescence Using Image J. Anat Rec.2013; 296: 378381. https://doi.org/10.1002/ar.22641.
28. Field A. Discovering statistics using IBM SPSS statistics. Sage, New Delhi. 2013; 395-420.
29. Chevillard L, Declèves X, Baud F, Risède P, Mégarbane B. Respiratory effects of diazepam/methadone combination in rats: a study based on concentration/effect relationships. Drug Alcohol Depend. 2013; 131:298–307.
30. Pignatelli D, Ferreira J, Vendeira P, Magalhães MC, Vinson GP. Proliferation of capsular stem cells induced by ACTH in the rat adrenal cortex. Endocr Res. 2002 Nov;28(4):683-91. doi: 10.1081/erc-120016987.
31. Labib MM,Kandil AM, Saleh HD. Histopathological effects of flunitrazepam on some organs of pregnant rat and their fetuses II. Lung and Brain. Egypt. J. Zool.2000; 35: 413-430.
32. Sanchez-Valle V, C. Chavez-Tapia N, Uribe M, Mendez-Sanchez N. Role of Oxidative Stress and Molecular Changes in Liver Fibrosis: A Review. Current Medicinal Chemistry. 2012; 19(28):4850-4860.
33. Rinaldi M, Johann ACBR, Rocha F, Ignácio SA, Rosa EAR, de Azevedo- Alanis LR, Sari Y, da Silva S, de Lima AA, do Prado AM, Bettega PV, Gregio AM. Histomorphometric analysis of salivary gland in wistar rats treated chronically with two benzodiazepines. Curr. Pharm. Biotechnol. 2015; 16(6):1–6. doi: 10.2174/1389201016061504071152 58.
34. Fafalios A, Akhavan A, Parwani AV, Bies RR, McHugh KJ, Pflug BR. Translocator receptor blockade reduces prostate tumor growth. Clin. Cancer Res. 2009; 15(19):6177–6184. doi: 10.1158/1078-0432.CCR-09-0844.
35. Elisabeth M. Walczak and Gary D. Regulation of the adrenocortical stem cell niche: implications for disease. Nat Rev Endocrinol. 2015 Jan; 11(1): 14–28. doi: 10.1038/nrendo.2014.166.
36. Hilgendorf KI, Johnson CT, Mezger A, Rice SL, Norris AM, Demeter J, Greenleaf WJ, Reiter JF, Kopinke D, Jackson PK. Omega-3 Fatty Acids Activate Ciliary FFAR4 to Control Adipogenesis. Cell. 2019 Nov 27;179(6):1289-1305.e21. doi: 10.1016/j.
37. Yousif B. Microscopic Studies on the Effect of Alprazolam (Xanax) on the Liver of Mice. Pakistan Journal of Biological Sciences.2002; 5: 1220-1225.
38. Vargas ML, Abella C, Hernamdez J. Diazepam increases the hypothalamic - pituitary - adrenocortical (HPA) axis activity by a cyclic AMP - dependent mechanism. British J. Pharmacol. 2001; 133(8): 135561.
39. Švob Štrac D, Muck-Šeler D, Pivac N. The involvement of noradrenergic mechanisms in the suppressive effects of diazepam on the hypothalamic-pituitary-adrenal axis activity in female rats. Croat Med J. 2012 Jun;53(3):214-23. doi: 10.3325/cmj.2012.53.214.
40. Papadopoulo V.Peripheral benzodiazeprine receptor: structure and function in health and disease. Ann. Pharm. Fr. 2003; 61 (1): 30-50.
41. Lazzarini R, Malucelli BE, Muscara MN, de Nucci G, Palmera-Neto J.Reduction of inflammation in rats by diazepam: tolerance development. Life. Sci. 2003; 72 (21): 2361-68.
42. Abdel-Maksoud SA. Effected of diazepam administration and withdrawal on the structure of suprarenal gland zona fasciculata cells of adult male albino rats. AAMJ, 2003; Vol.1, N. 3.

43. El-Sokkary GH. Melatonin and vitamin C administration ameliorate diazepam-induced oxidative stress and cell proliferation in the liver of rats. Cell Prolif. 2008; 41(1): 168–176.

44. Musavi S, Kakkar P. Effect of diazepam treatment and its withdrawal on pro/antioxidative processes in rat brain. Mol Cell Biochem. 2003; 245:51–56.

45. Girgis N, Kamel S, Labib B *et al*. Cellular and DNA changes due to clonazepam abuse in brains of albino rats and role of clonidine during withdrawal period. Mansoura J. Forensic Med. Clin. Toxicol. 2010; 18: (1).

46. Moghadamnia D, Mokhtari M, Khatamsaz S. The Protective Effect of Omega-3 Against Thioacetamide Induced Lipid and Renal Dysfunction in Male Rats, Zahedan J Res Med Sci. 2016; 18(11):e4781. doi: 10.17795/zjrms-4781

47. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002; 21:495–505.

الملخص العربى

دور أوميجا ٣ على قشرة الغدة الكظريةالمعالجة بالديازيام لدى ذكور الجرذان البيضاء البالغة(دراسة هستولوجيه وهستوكيميائية مناعيه)

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المقدمة: الديازيبام هو دواء شائع الاستخدام من البنزوديازيبينات التي هي من بين الفئة الأكثر انتشارا من الأدوية في جميع أنحاء العالم. يبدو أن القليل من الاهتمام قد تم إيلاءه لآثار الديازيبام على الغدد الصماء. أظهرت الأبحاث السريرية والتجريبية أن الاحماض الدهنية أوميجا ٣ تظهر تأثيرات كبيرة مضادة للأكسدة ومضادة للالتهابات على النظم البيولوجية .

الأهداف: لإلقاء الضوء على تأثير الديازيبام على تركيب الغدة الكظرية وكذلك التأثير الوقائي لأحماض أوميجا ٣ الدهنية علي الغدة الكظرية

مواد وطرق البحث : تم تصنيف خمسين من الجرذان البيضاء الذكور البالغة إلى ثلاث مجموعات هم: المجموعة الاولي) ةمجموعة المراقبة، والتي تم تقسيمها إلى ثلاث مجموعات فرعية متساوية، المجموعة الثانيه وهي المجموعة المعالجة بالديازيبام، والمجموعة الثالثة المجموعة المعالجة بالديازيبام مع أوميجا . بعد ٢٢ يوما، تم جمع عينات الدم من جميع المجموعات لقياس هرمونات الأدرينوكورتيكوتروفيك والكورتيزون. واستخدمت عينات الأنسجة من الغدد الكظرية لقياس مالوندیلدهايد ، سوبرأكسيد الديسميوتيس والجلوتاثيون. وتمت معالجة الغدد الكظرية للفحص المجهري للضوئي والإلكتروني.

النتائج: أظهرت المجموعة المعالجة بالديازيبام زيادة كبيرة في مستويات هرمونات الأدرينوكورتيكوتروفيك والكورتيزون في الدم. وزيادة مستويات المالوندیلدهايد ونقص قيم سوبرأكسيد الديسميوتيس والجلوتاثيون مقارنة بالمجموعة المراقبة. كما أظهر الفحص الهستوباثولوجي العديد من الخلايا ذات سيتوبلازم مفرغ , زيادة قطرات الدهون، والعديد من الليسوسومات, وميتوكوندريا داكنة وأنوية داكنة. زيادة ألياف الكولاجين في الغلاف، وضعف الصبغة المناعية PCNA ومع ذلك أدي تناول أحماض أوميجا ٣ مع الديازيبام إلي تراجع هذه التغيرات.

الاستنتاج: أحماض أوميجا ٣ لها آثار تحسينيه ضد الضرر الناجم عن الديازيبام في قشرة الغدة الكظرية. لذلك، سيكون من المستحسن إضافة أوميجا ٣ للمرضى الذين يعالجوا بالديازيبام.