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Role of Omega-3 Fatty Acids and Vitamin C on the Suprarenal Gland of Adult Male Albino Rats Subjected to Cadmium

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Abstract:

Background: There is solid evidence linking cadmium to several types of cancer. Ascorbic acid and omega-3 polyunsaturated fatty acids have been studied to modify oxidative stress processes. The aim of this study is to declare histopathological & ultrastructural effects of cadmium on the suprarenal gland of male albino rats and to declare the possible modulation by omega-3 polyunsaturated fatty acids and ascorbic acid. **Materials and methods:** 60 Male Albino rats were equally assigned into: control group, cadmium group (cadmium chloride 1 mg/kg/day SC), omega-3 polyunsaturated fatty acids exposed group, ascorbic acid exposed group, omega-3 polyunsaturated fatty acids and ascorbic acid exposed group, withdrawal group. Four weeks after the experiment started, euthanasia was carried out, and the adrenal glands were evaluated biochemically, histologically, ultra-structurally, and immunohistochemically. **Results:** The outcomes of the control, omega-3 polyunsaturated fatty acid, and ascorbic acid exposure groups were essentially the same. Adrenal cortical and medullary apoptosis, as well as ultrastructural disruption of cell organelles, were caused by cadmium. Swollen, degenerated mitochondria with broken cristae, a few lipid droplets, an expanded lysosome, and a considerable modulation of the adrenal gland hormones glutathione, superoxide dismutase, and malondialdehyde were all indicators of cadmium-induced damage. Cadmium plus omega-3 polyunsaturated fatty acids and ascorbic acid significantly reduced caspase-3 immunoexpression in comparison to the cadmium group. **Conclusions:** Cadmium is highly toxic to adrenal tissue, while Omega-3 and ascorbic acid, especially in combination, can significantly ameliorate its damaging effects.

Key words: Cadmium, Adrenal, Omega-3, Ascorbic acid, Rats

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Introduction

The outer cortex and the inner medulla are the two separate tissues that make up the adrenal gland. Because it tends to be fattier, the adrenal cortex is more yellow in color. More reddish-brown is the color of the adrenal medulla. The adrenal glands are enclosed in a dense capsule of connective tissue [1,2].

One naturally occurring heavy metal that possess serious risks to human health is cadmium (Cd). Human exposure to cadmium typically results from smoking cigarettes, inhaling cigarette smoke, and consuming tainted food or water [3,4].

Because of its incredibly low rate of elimination in the body, it builds up in soft tissues and can cause endocrine, reproductive, hepatotoxic, neurotoxic, and nephrotoxic effects. Acute or chronic intoxications brought on by exposure can interfere with DNA repair, produce reactive oxygen species, and trigger apoptosis [5,6].

Lipid peroxidation and oxidative strain in neurons are reduced when omega-3 unsaturated fatty acids, which have neurobehavioral components, are incorporated into cell membranes [7].

With special focus on the omega-3 polyunsaturated fatty acids PUFAs, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). ALA is found in nuts and seeds whereas EPA and DHA are the main components of fish oil [8].

In its capacity as an antioxidant, ascorbic acid protects the body from the detrimental effects of free radicals and pollutants. In addition, it has been demonstrated to mitigate the development of protein aggregation and oxidative stress in neurodegenerative diseases. According to our understanding, these two antioxidants' roles in protecting against Cd-induced behavioral changes in the suprarenal gland have not yet been evaluated, despite numerous studies documenting their protective effects against Cd-induced nephrotoxicity, hepatotoxicity, and reproductive toxicity in animals [9]

This study assesses how well ascorbic acid and omega-3 unsaturated fatty acids protect the adult male albino rat's suprarenal gland against cadmium-induced damage.

Materials and methods:

Type of study: Experimental study

Duration of study: from April 2024 to October 2024.

1) Chemicals:

- **Cadmium**:

Sigma Chemical Co. (St. Luis, Missouri, USA) was the supplier of the cadmium chloride salt. It was administered daily by SC injection (1 mg/kg/day) after dissolving in saline [4].

100 mg of CdCl2 will be added to 100 ml of saline to create a solution of Cadmium chloride salt with a concentration of 1 mg of CdCl2/ml. One milliliter of cadmium chloride was included in each milliliter of the resultant solution.

- **Omega-3 fatty acids:**

For fatty acids with omega-3; we purchased 500 mg pills from Abbott (GmbH, Germany), dissolved in ten milliliters of olive oil to get 50 mg of omega-fatty acids in each milliliter of the resulting solution, which was administered orally by gastric tube at a dose of 100 mg/kg b.w [7]

- **Ascorbic acid:**

Ascorbic acid tablets 500 mg were purchased from Sigma Pharma, Egypt, were dissolved in 100 ml saline. Each one ml of the formed solution contained 5mg of ascorbic acid and was given at dose (10 mg/kg b.w orally by gastric tube) [9].

2) Animals:

A total of sixty male albino rats housed at the anatomy department's experimental animal unit at the Benha Faculty of Medicine were used in this study. These rats had access to food and water and were kept in a regulated setting. Their average weight was between 200 and 250 grams, and their median age at the start of the trial was 8 weeks. The treatment of

these animals was authorized by Benha Medical University's experimental animal committee and adhered to national research council regulations (MD 8-7-2023).

1) Experimental design:

60 male Albino rats were divided into 6 equal groups (10 rats each) as follows:

- **Group I** (control group) was subdivided into:

Negative control (subgroup Ia)

Five male albino rats were selected at random to serve as the negative control group (normal, healthy rats) and were not given any medication for four weeks.

Positive control (subgroup lb)

It was formed of 5 male albino rats that were chosen randomly as the positive control group (normal healthy rats), which were given olive oil, that was offered every day orally by gastric tube for 4 weeks.

- **Group II (**cadmium group): Consisted of 10 male albino rats which were given cadmium, after being dissolved in saline, it was offered every day by SC injection (1 mg/kg/day), for a period of 4 weeks **[(4)].**
- **Group III** (cadmium + Omega-3 fatty acids): Consisted of 10 male albino rats which were treated with cadmium as in group II accompanied by oral supplementation of omega-3 fatty acids with a daily dose of 100 mg/kg weight for a period of 4 weeks through gastric tube **[(7)].**
- **Group IV** (cadmium + Ascorbic acid**):** Consisted of 10 male albino rats which were treated with cadmium as in group II accompanied by oral supplementation of ascorbic acid with a daily dose of 10 mg/kg weight for a period of 4 weeks by gastric tube **[(9)].**
- **Group V** (cadmium + Omega-3 fatty acids+ Ascorbic acid):
  This group consisted of ten male albino rats which were administered oral supplements of omega-3 fatty acids at a dosage of 100 mg/kg weight per day and oral supplements of ascorbic acid at a dosage of 10 mg/kg weight per day for four weeks. The rats received the same treatment as group II in terms of cadmium.
- **Group VI (**withdrawal group**):**
  This sample consisted of 10 male albino rats that were administered cadmium. After being disintegrated in saline, it was administered daily via SC injection at a rate of 1 mg/kg day, for a period of 4 weeks. Then this group was leaved for another 4 weeks without any drug intake.

2) Preparation of suprarenal glands specimens [(10)].:-

All rat groups were weighed just before scarification. They were anesthetized after 4 weeks (except withdrawal group scarified after 8 weeks) from the beginning of the experiment by Pentobarbital (Nembutal, 30 mg/kg of body weight). The viscera were exposed by generating a long midline abdominal incision and inserting two retractors after the animals were anesthetized. Retraction of the colon was implemented. The inferior vena cava, abdominal aorta, suprarenal ducts, and kidneys are available for observation. The suprarenal glands were dissected free. The left suprarenal glands were fixed in 10 % buffered formalin saline, underwent paraffin blocking for histological evaluation and the right ones were fixed in 2.5% glutaraldehyde solution for electron microscopy processing. Ethical protocols for animal treatment were followed according to Benha Faculty of Medicine Ethical Committee (MD 8-7-2023).

3) Histopathological study:

3.1) Hematoxylin and Eosin stain.

Preparation of paraffin sections

The specimens were preserved in a 10% formalin solution for 48 hours. Xylol was utilized as a cleaning agent, and dehydration was done in an increasing alcohol grades. Following paraffin embedding, the fixed samples were

sectioned at (5 μm thick) on slides coated with gelatin. After additional deparaffinization, they were stained with hematoxylin and eosin stain, which gives the cytoplasm a pink hue and the nuclei a blue hue. Slides examined by using a light microscope fitted with an automated photomicrographic camera system at Benha University's Faculty of Medicine's Anatomy and Embryology Department [11].

3.2) Immunohistochemistry

The identification of antigens in tissue sections using particular antibodies is the basic idea of immunohistochemistry. Both antigens and other antibodies can bind to the immunoglobulin molecule. When antigen-antibody binding occurs, a colorful histochemical reaction that may be seen with light or fluorescence microscopy is produced [12].

Protocol of Caspase 3 Antibody Staining as a marker of apoptosis [13].

Tissue sections were first heated to 65 °C to deparaffinize them before being submerged in xylene, ethanol, and buffer washes for immunohistochemistry. Sections were cleaned in phosphate buffered saline(PBS) after the antigen was extracted using a microwave in retrieval solution. Slides were treated with rabbit polyclonal anti-active caspase-3 antibody (1:200) either at room temperature or overnight at 4 °C after endogenous peroxidase activity was inhibited with 3% hydrogen peroxide. Following PBS washing, slices were subjected to horseradish peroxidase (HRP)-conjugated secondary antibody treatment, diaminobenzidine tetrahydrochloride (DAB) substrate development, hematoxylin counterstaining, alcohol dehydration, xylene clearing, and mounting. Cytoplasmic brown staining was an indication of positive immunoreactivity..

3.3) Electron microscopic study [14].

Specimens were double-fixed in 4% glutaraldehyde and then 1% osmium tetroxide for transmission electron microscopy. Propylene oxide was used to clarify them after they had been dehydrated in increasing ethanol and cleaned in sodium cacodylate buffer. Epon resin made from dodecenyl succinic anhydrite (DDSA), methylnadic anhydrite (MNA), and accelerator mixes was used to implant the samples. Ultrathin sections were placed on copper grids, while semithin sections were oriented using toluidine blue stain. The sections were desiccated and inspected using a Joel electron microscope calibrated to 80 kV. Representative fields were obtained on camera after uranyl acetate and lead citrate staining.

The specimens were subsequently photographed at the Faculty of Medicine, Tanta University, Tanta, Egypt, using transmission electron microscope JOEL JSM-52500 LV from Japan.

4) Morphometric study: [15].

The area percent of caspas-3 immunoreactivity was evaluated at the Pathology Department, Faculty of Medicine, Benha University, using the Leica Qwin-500 LTD software image analysis computer system Ltd. (Cambridge, UK).

Assessment of the area percentage of caspase-3 immunostaining: The interactive measurements menu was used to measure them in ten non-overlapping high-power fields. A blue mask (binary image) immediately masked the immunoreaction's brown hue. Next, the area of this binary image which showed the cells that were positively labeled for caspase 3 was computed.

5) Biochemical analysis:

Samples of blood were obtained from the retroorbital plexus of veins at the conclusion of the experiment using a capillary pipette. Subsequently, the samples were collected in heparinized receptacles that contained 5000 I.U./ml of heparin sodium and centrifuged at 3000 revolutions per minute (r.p.m.) for 15 minutes. After being separated, plasma was kept at -20°C until it was needed. Every sample was utilized to assess the adrenal gland' hormone levels, as detailed in [16].

Centrifuged at 4000 r.p.m. for 20 minutes, adrenal gland homogenates were stored at 4°C. Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) quantitative activities were estimated using the supernatant, as detailed in [17]

Approval code: MD 8-7-2023

Statistical analysis:

The data, which were later computed as the mean ± SD, were analyzed using SPSS version 19 (SPSS Inc., Chicago, Illinois, USA). We utilized analysis of variance and Tukey's multiple comparison tests to find the statistical significance. "Significant" was determined as $p < 0.05$.

Results:

Light Microscopic Results

Hematoxylin and Eosin-Stained Sections

Group I (Control group):

The control group's adrenal gland histological analysis showed comparable histological findings to those of the positive and negative control groups.

The adrenal cortex and adrenal medulla are the two components visible in the sections of the suprarenal gland tissues of normal control rats. Three zones were visible in the cortex: zona reticularis (ZR), zona fasciculata (ZF), and zona glomerulosa (ZG). ZG layer cells with highly pigmented nuclei form rounded or oval clusters. Blood capillaries divided the straight cords of zona fasciculata (ZF) cells. There were several big, polyhedral cells with vesicular, rounded nuclei and acidophilic cytoplasm. Small cells with highly acidophilic cytoplasm were seen in Zona Reticularis (ZR). These cells were grouped in a distinct network of branching cords and lipofuscin pigments. The center was the adrenal medulla. Regularly spaced out in distinct clusters, the medullary cells were divided by broad blood sinusoids. The medullary cells featured spherical vesicular nuclei and basophilic cytoplasm **(Figs.1 A, B & C)**.

Group II (cadmium treated group):

Sections of the group II adrenal gland tissues showed that the normal architecture of the ZG, ZF, ZR, and adrenal medulla were lost. Some cells had pyknotic nuclei and vacuolated cytoplasm, while others had shrunk and had cytoplasm that was deeply acidophilic. Other cells had frequent karyolysis. The adrenal medulla and ZR's blood capillaries were clogged. **(Figs.1 D, E & F)**.

Group III (Omega-3 fatty acids + cadmium treated group):

Sections of the group III adrenal gland tissues indicated a moderate improvement over group II, ZG, ZF, ZR, and the adrenal medulla. The cells looked almost identical to the control group, and a moderate number of them had pyknotic nuclei and vacuolated cytoplasm. Remarkably, a large number of mitotic figures were seen **(Figs.1 G, H & I)**.

Group IV (Ascorbic acid + cadmium treated group):

Sections of group IV's adrenal gland tissues, ZG, ZF, ZR, and the adrenal medulla, showed a moderate improvement over group II, with cells looking almost identical to those in the control group. Lipofuscin pigments were seen in Zona Reticularis, and a small number of cells with pyknotic nuclei and noticeable vacuolated cytoplasm were seen **(Figs.1 J, K & L)**.

Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group):

Examining sections of group V's adrenal gland tissues revealed significant improvement when compared to group II, ZG, ZF, ZR, and the adrenal medulla, with cells that looked almost identical to those in the control group. Large polyhedral cells with vesicular nuclei and acidophilic cytoplasm were seen in ZR cells, which were grouped in a well-defined network of branching cords divided by wide blood sinusoids. ZG cells were found in oval or rounded clusters, whereas ZF cells were found in columns. The medullary cells were grouped in distinct clusters and were

oriented regularly. The rounded vesicular nuclei and basophilic cytoplasm were the characteristics of medullary cells. A small number of cells had pyknotic nuclei and vacuolated cytoplasm, while Zona Reticularis showed lipofuscin pigments. It's interesting that a lot of mitotic figures were seen **(Figs.1 M, N & O)**.

Group VI (withdrawal group):

Sections of group VI's adrenal gland tissues showed a little improvement when compared to group II. The normal architecture of the ZG, ZF, ZR, and adrenal medulla was lost, and some cells had pyknotic nuclei and moderately vacuolated cytoplasm; others had shrunk cells with pyknotic nuclei and deeply acidophilic cytoplasm; and some cells had frequent karyolysis. **(Figs.1 P, Q & R)**.

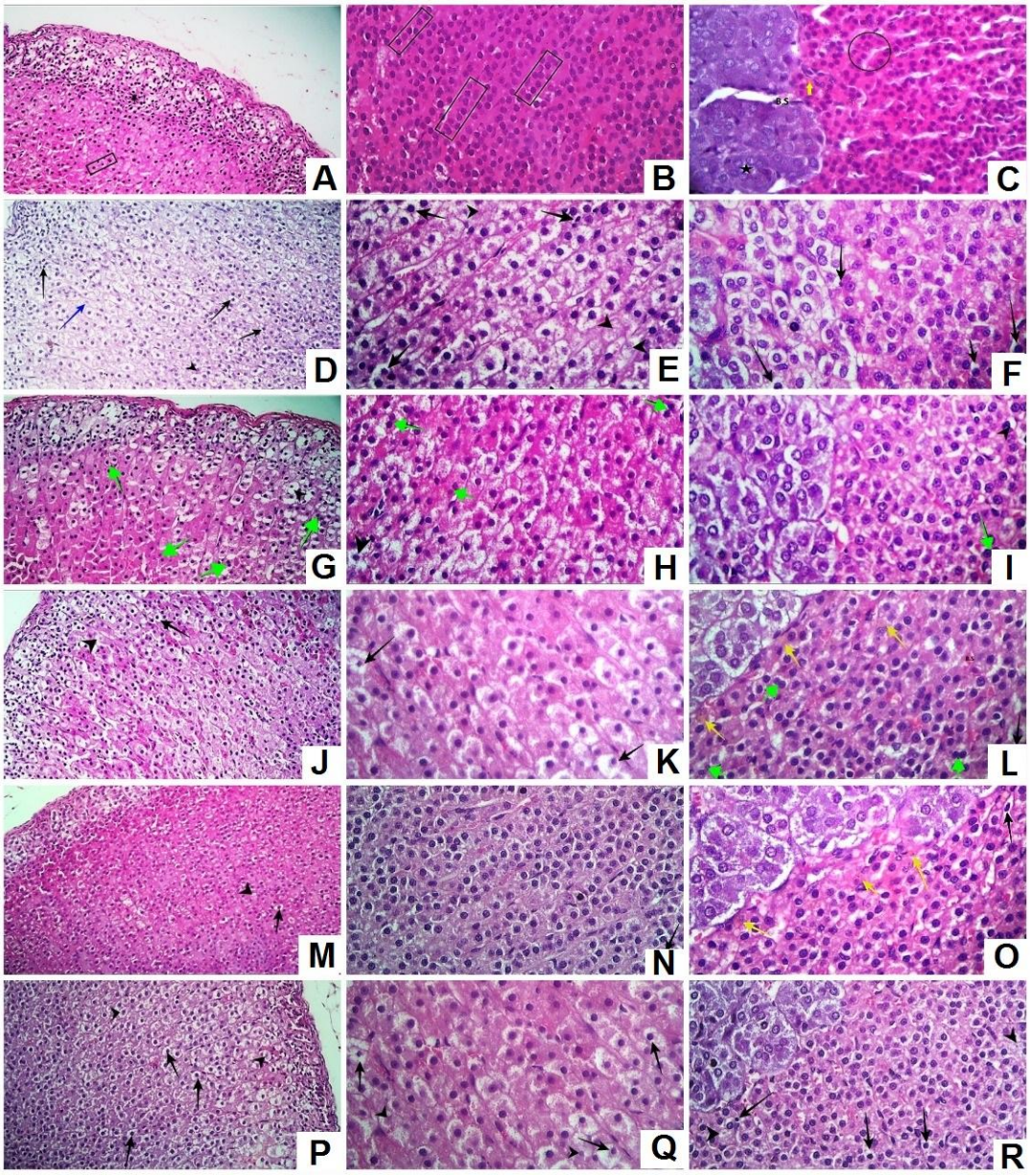


Figure 1. A photomicrograph of the adrenal gland showing the control group (Group I) (figs: A (zona glomerulosa and zona fasciculata), B (zona fasciculata) and C (zona reticularis and medulla): The sections of the adrenal gland tissues of the control group showing normal architecture with well-organized cortical zones (ZG, ZF, ZR) and medulla. In contrast, the cadmium-treated group **(Group II) (figs: D (zona glomerulosa and zona fasciculata), E (zona fasciculata) and F (zona**

reticularis and medulla): demonstrating severe structural changes; such as vacuolated cytoplasm, pyknotic nuclei (black arrows), karyolysis (blue arrows), and others with pyknotic nuclei, profoundly acidophilic cytoplasm (arrow head) and congested blood capillaries. **Group III (Omega-3 fatty acids + cadmium treated group) (figs: G (zona glomerulosa and zona fasciculata), H (zona fasciculata) and I (zona reticularis and medulla):** showing some retained vacuolated cytoplasm, pyknotic nuclei (arrow head) and mitotic figures (green arrows). **Group IV (Ascorbic acid + cadmium treated group) (figs: J (zona glomerulosa and zona fasciculata), K (zona fasciculata) and L (zona reticularis and medulla):** showing some retained vacuolated cytoplasm, pyknotic nuclei (black arrows), karyolysis (arrow head), lipofuscin pigment (yellow arrows) in the ZR of this group and mitotic figures (green arrows). **Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) (figs: M (zona glomerulosa and zona fasciculata), N (zona fasciculata) and O (zona reticularis and medulla):** showing adrenal histology nearly restored to the control architecture, cells with vacuolated cytoplasm, pyknotic nuclei (black arrows), karyolysis (arrow head) and lipofuscin pigment (yellow arrows). **Group VI (withdrawal group (figs: P (zona glomerulosa and zona fasciculata), Q (zona fasciculata) and R (zona reticularis and medulla):** showing minimal improvement, with persistent structural damage and frequent nuclear changes; vacuolated cytoplasm, pyknotic nuclei; (black arrows) and karyolysis (arrow head). Figs (A,D,G,J,M,P) [H&E X200], Figs (B,C,E,F,H,I,K,L,N,O,Q,R) [H&E X400]

Immune histochemical results

Caspase 3 Immune histochemical results (were used to evaluate the extent of apoptosis of adrenal gland cells)

Group I (Control group):

The sections of the adrenal gland tissues of the control group showed few scattered brownish cytoplasmic immune reactive colorations **(Figs. 2 A, B & C)**.

Group II (cadmium treated group):

The sections of the adrenal gland tissues showed a **strong positive** brownish cytoplasmic immune reaction **(Figs.2 D, E & F)**.

Group III (Omega-3 fatty acids + cadmium treated group):

The sections of the adrenal gland tissues show **mild positive** cytoplasmic immunoreactivity of caspase 3 **(Figs.2 G, H & I)**.

Group IV (Ascorbic acid + cadmium treated group):

The sections of the adrenal gland tissues showed **mild positive** cytoplasmic immunoreactivity of caspase 3 **(Figs.2 J, K & L)**.

Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group):

The sections of the adrenal gland tissues showed **minimal positive** cytoplasmic immunoreactivity of caspase 3 **(Figs .2M, N & O)**.

Group VI (withdrawal group):

The sections of the adrenal gland tissues showed **moderate positive** cytoplasmic immunoreactivity of caspase 3 **(Figs.2 P, Q & R)**.

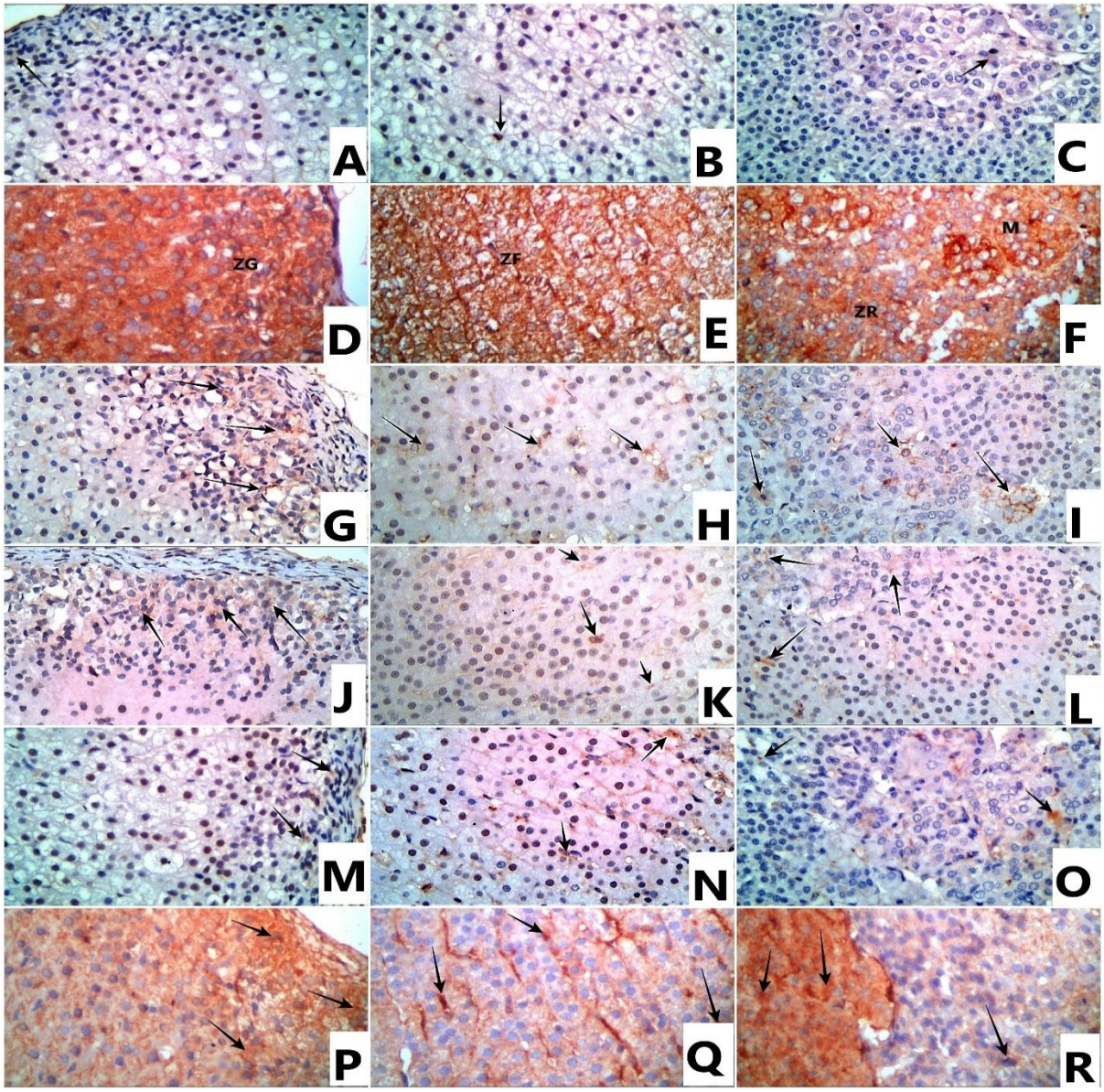


Figure 2: A Photomicrographs of the adrenal gland showing the control group **(Group I) (figs: A (zona glomerulosa), B (zona fasciculata) and C (zona reticularis and medulla):** The sections of the adrenal gland tissues of the control group showing few scattered brownish cytoplasmic immune reactive colorations (arrows). **(Group II) (figs: D (zona glomerulosa), E (zona fasciculata) and F (zona reticularis and medulla):** The sections of the adrenal gland tissues showing a **strong positive** brownish cytoplasmic immune reaction. **Group III (Omega-3 fatty acids + cadmium treated group) (figs: G (zona glomerulosa), H (zona fasciculata) and I (zona reticularis and medulla):** The sections of the adrenal gland tissues showing **mild positive** cytoplasmic immunoreactivity of caspase 3 (arrows). **Group IV (Ascorbic acid + cadmium treated group) (figs: J (zona glomerulosa), K (zona fasciculata) and L (zona reticularis and medulla):** The sections of the adrenal gland tissues showing **mild positive** cytoplasmic immunoreactivity of caspase 3 (arrows). **Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) (figs: M (zona glomerulosa), N (zona fasciculata) and O (zona reticularis and medulla):** The sections of the adrenal gland tissues showing **minimal positive** cytoplasmic immunoreactivity of caspase 3 (arrows). **Group VI (withdrawal group (figs: P (zona glomerulosa), Q (zona fasciculata) and R (zona reticularis and medulla):** The sections of the adrenal gland tissues showing **moderate positive** cytoplasmic immunoreactivity of caspase 3 (arrows). [caspase-3 X400]

Transmission electron microscopic (TEM) results:

Group I (Control group):

The ultrastructural analysis of the adrenal gland tissues of the control group demonstrated a high concentration of mitochondria with cristae and lipid droplets in the zona glomerulosa, zona fasciculata, and zona reticularis cells. Heterochromatin is present on the periphery of cells in the zona glomerulosa, zona fasciculata, and zona reticularis; the nucleus appeared spherical, irregular, or euchromatic. The medulla showed the chromaffin cells with rounded euchromatic nuclei, granules of variable electron density and blood sinusoid **(Figs.3 A, B, C & D)**.

Group II (cadmium treated group):

Group II cells in the zona glomerulosa, zona fasciculata, and zona reticularis exhibited expanded lysosomes, vast intercellular gaps, few lipid droplets, and abundant enlarged, degraded mitochondria with damaged cristae. Pyknotic irregularly shaped nuclei that contracted into a larger perinuclear region. The medulla showed the chromaffin cells with apparent decrease in the secretory granules. The nuclei of these cells were irregular and the cytoplasm exhibited distorted mitochondria with ruptured cristae and multiple vacuoles. **(Figs.3 E, F, G & H)**.

Group III (Omega-3 fatty acids + cadmium treated group):

Group II cells in the zona glomerulosa, zona fasciculata, and zona reticularis exhibit expanded lysosomes, vast intercellular gaps, few lipid droplets, and abundant enlarged, degraded mitochondria with damaged cristae. Pyknotic, has irregularly shaped nuclei that contract into a larger perinuclear region, a small number of swollen mitochondria with ruptured cristae, and roughly the same number of lipid droplets and mitochondria. The medulla shows the chromaffin cells with apparent increase in the secretory granules. The nuclei of these cells are irregular and the cytoplasm exhibits some vacuoles. **(Figs3. I, J, K & L)**.

Group IV (Ascorbic acid + cadmium treated group):

The euchromatic nuclei of the cells from the zona glomerulosa, zona fasciculata, and zona reticularis exhibited regular outlines, an apparent increase in the lipid droplets, and mitochondria with intact cristae. A small number of enlarged mitochondria with ruptured cristae, and an abundance of lipid droplets and mitochondria that was similar to or even greater than that of the control were observed. The medulla showed the chromaffin cells with apparent increase in the secretory granules. The nuclei of these cells were irregular and the cytoplasm exhibited some vacuoles similar to group III **(Figs.3 M, N, O, & P)**.

Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group):

The zona glomerulosa, zona fasciculata, and zona reticularis cells contained lipid droplets, mitochondria with intact cristae, and rounded euchromatic nuclei. A small number of swollen mitochondria with ruptured cristae, as well as a large number of lipid droplets and mitochondria, that look almost identical to those in the control group were noted. The medulla showed the chromaffin cells with apparent increase in the secretory granules. The nuclei of these cells were regular and the cytoplasm exhibited few vacuoles**. (Figs 3 Q, R, S, & T)**.

Group VI (withdrawal group):

Cells of the zona glomerulosa, zona fasciculata and zona reticularis showed irregular pyknotic nuclei and perinuclear spaces. The cytoplasm contained many swollen mitochondria with ruptured cristae and few lipid droplets. The medulla showed the chromaffin cells with apparent decrease in the secretory granules and swollen mitochondria with ruptured cristae. **(Figs 3 U, V, W & X)**.

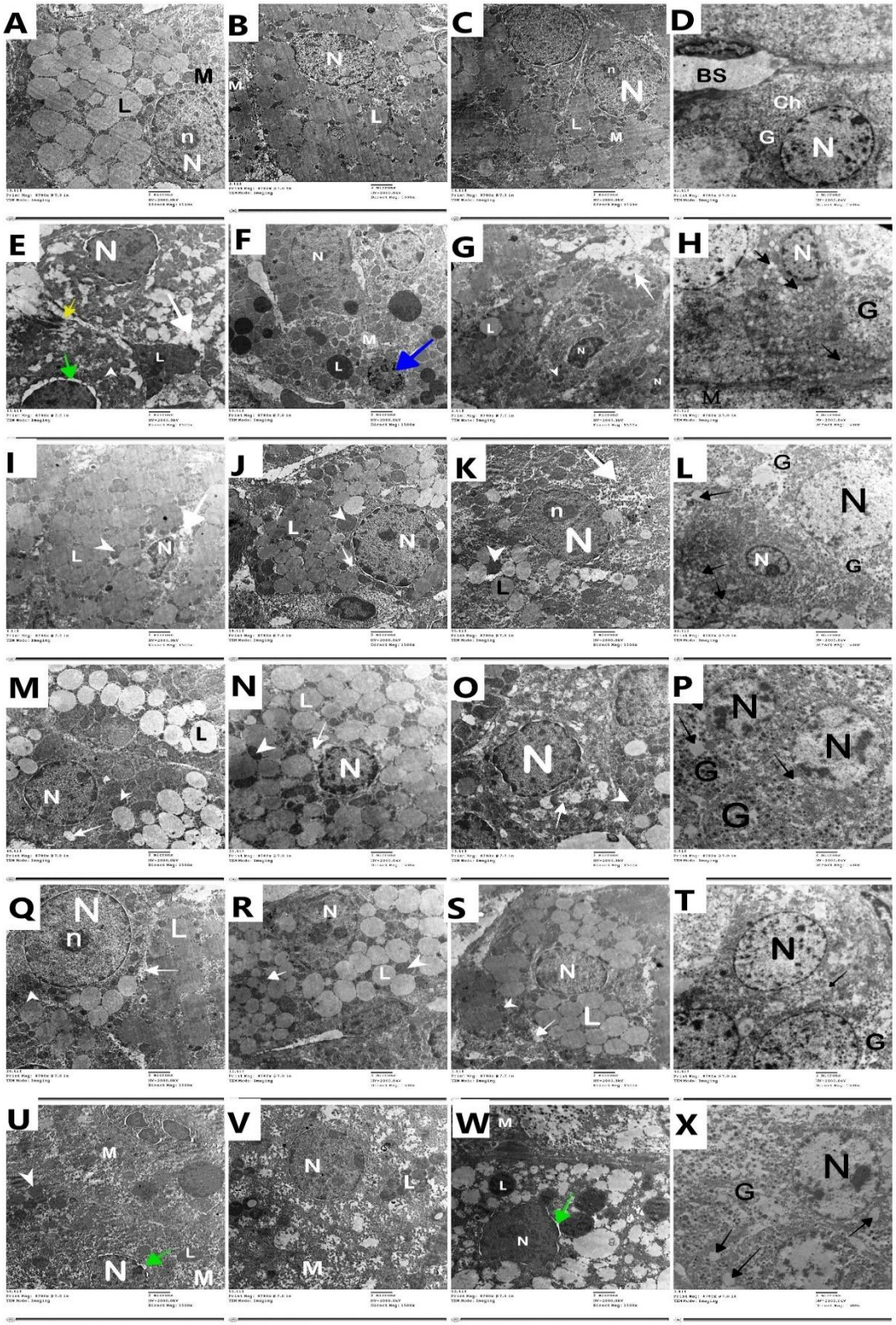


Figure 3: An Electron micrographs of the adrenal gland showing the control group **(Group I**) **(figs : A (zona glomerulosa), B (zona fasciculata) , C (zona reticularis) and D (medulla):** showing normal cellular features in cortical zones with numerous mitochondria (M) possessing intact cristae, abundant lipid droplets (L) with distinct boundaries, and euchromatic nuclei (N), while chromaffin cells in the medulla contains rounded nuclei (N) and secretory granules (G) of variable electron density. **(Group II) (figs: E (zona glomerulosa), F (zona fasciculata), G (zona reticularis) and H (medulla):** showing severe degenerative changes, including swollen mitochondria (white arrows) with disrupted cristae, enlarged lysosomes (blue arrow), wide intercellular spaces, irregular pyknotic

nuclei with perinuclear dilation (green arrows), and reduced secretory granules (G) in the medulla accompanied by vacuolated cytoplasm (black arrows). **Group III (Omega-3 fatty acids + cadmium treated group) (figs: I (zona glomerulosa), J (zona fasciculata) , K (zona reticularis) and L (medulla):** showing restoration of mitochondria (arrow heads), increased lipid droplets (L), and partial recovery of medullary secretory granules (G); although some irregular nuclei (N) and cytoplasmic vacuoles persisted. **Group IV (Ascorbic acid + cadmium treated group) (figs :M (zona glomerulosa), N (zona fasciculata) , O (zona reticularis) and P (medulla):** showing restoration of mitochondria (arrow heads), increased lipid droplets (L), and partial recovery of medullary secretory granules (G); although some irregular nuclei (N) and cytoplasmic vacuoles persisted. **Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) (figs: Q (zona glomerulosa), R (zona fasciculata), S (zona reticularis) and T (medulla):** showing data resembling the control architecture, with intact mitochondria (arrow heads), abundant lipid droplets (L), regular euchromatic nuclei (N), and preserved medullary secretory granules (G), with minimal vacuolar changes. **Group VI (withdrawal group (figs: U, V, W, X): (figs: U (zona glomerulosa), V (zona fasciculata), W (zona reticularis) and X(medulla):** showing persistent ultrastructural damage, including swollen mitochondria (white arrows) with ruptured cristae, irregular nuclei (N), reduced lipid droplets (L), and decreased medullary secretory granules (G).
[TEM X1500].

Morphometric and statistical results:

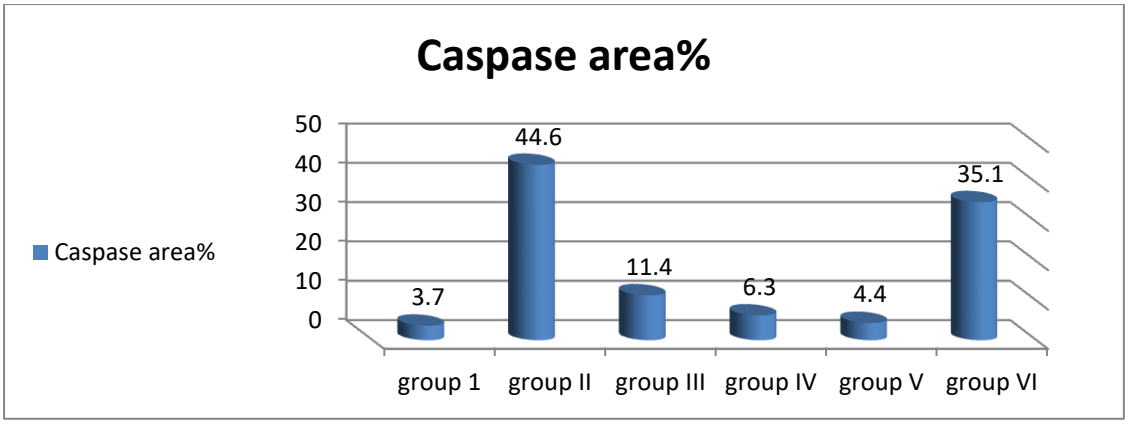
- **The mean area % of caspase-3 immune reactivity for apoptosis (Table 1 and Histogram 1):**

Compared to the Control and Group III (Omega-3 fatty acids + cadmium treated group), Group II (cadmium treated group) and Group VI (withdrawal group) exhibit a substantial increase in caspase expression., Group IV (Ascorbic acid + cadmium treated group) and Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) **(Table. 1 and Histogram. 1)**

Table (1) Show the mean area % of caspase-3 immune reactivity for experimental animals in different groups and comparison between all groups (Post Hoc test).

	Control Group	**Group II (cadmium treated group)**	**Group III (Omega-3 fatty acids + cadmium treated group)**	**Group IV (Ascorbic acid + cadmium treated group)**	**Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group)**	**Group VI (withdrawal group)**
Caspase area%	3.7± 1.5	44.6±7.6 [a, c, d & e]	11.4±1 [b&f]	6.3±0.9 [b & f]	4.4±1.1 [b & f]	35.1±6.5 [a, c, d & e]

Data expressed as mean±SD, *: significance ≤ 0.05
a: Significance vs Control, b: Significance vs group II, c: Significance vs group III, d: Significance vs group IV, e: Significance vs group V, f: Significance vs group VI



Histogram (1) shows the mean area % of caspase-3 immune reactivity for experimental animals in different groups and comparison between all groups (Post Hoc test).

Results of biochemical studies:

Mean values of biochemical markers in all groups Table (2):

The cadmium group (Group II) and Group VI (withdrawal group) showed significant increase of Malondialdehyde (MDA) levels compared with the control group, group III (Omega-3 fatty acids + cadmium treated group), group IV (Ascorbic acid + cadmium treated group) and group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group).

The cadmium group (Group II) and Group VI (withdrawal group) showed significant reduce of GSH (Glutathione) and SOD (Superoxide Dismutase) levels compared with the control group, group III (Omega-3 fatty acids + cadmium treated group) , group IV (Ascorbic acid + cadmium treated group) and group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) **(Table 2).**

Mean values of hormones in all groups, Table (3):

The aldosterone levels of the cadmium group (II) and group VI (withdrawal group) were significantly lower than those of the control group ($p \leq 0.05$). In comparison to Group II (cadmium treated group), group III (Omega-3 fatty acids + cadmium treated group), group IV (Ascorbic acid + cadmium treated group), and group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) significantly restored aldosterone levels.

Group II (cadmium treated group) and Group VI (withdrawal group) showed significantly increased ACTH (Adrenocorticotropic hormone) compared to control group. Group III (Omega-3 fatty acids + cadmium treated group), group IV (Ascorbic acid + cadmium treated group) and group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) showed significant reduce of Adrenocorticotropic hormone level.

Corticosterone and DHEA (Dehydroepiandrosterone) levels were significantly reduced in group II (cadmium treated group) compared to the control group. Group III (Omega-3 fatty acids + cadmium treated group), group IV (Ascorbic acid + cadmium treated group) and group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group) showed significant increase of corticosterone and DHEA levels. Group VI (withdrawal group) again reflected a similar pattern to Group II (cadmium treated group) (**Table 3)**

Table (2) show the mean values of biochemical markers in all groups. and Comparison between them (Post Hoc LSD test).

	Control	**Group II (cadmium treated group)**	**Group III (Omega-3 fatty acids + cadmium treated group)**	**Group IV (Ascorbic acid + cadmium treated group)**	**Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group)**	**Group VI (withdrawal group)**
MDA (Malondialdehyde) (nmol/ mg protein)	21.2 ± 6.8	52.4 ± 8.9 [a, c, d&e]	31.3 ± 9.8 [b]	26.6 ± 8 [b&f]	23.6±7.4 [b&f]	44.4±10.2 [a, d&e]
GSH(Glutathione) (nmol/mg protein)	19.1 ± 2.9	7.2 ± 1.5 [a, c, d&e]	15.2 ± 0.9 [b&f]	18.4 ±1.3 [b&f]	18.7±3.1 [b&f]	8.2±1.5 [a, c, d&e]
SOD (Superoxide Dismutase) (U/ mg protein)	5.1± 1.7	0.53±0.15 [a, c, d&e]	3.2 ± 0.97 [b&f]	4.2 ± 1.5 [b&f]	4.7 ±1.6 [b&f]	0.97±0.18 [a, c, d&e]

Data expressed as mean±SD, *: significance ≤ 0.05, a: Significance vs Control, b: Significance vs group II, c: Significance vs group III, d: Significance vs group IV, e: Significance vs group V, f: Significance vs group VI

Table (3) show the mean values of hormones in all groups. and Comparison between them (Post Hoc LSD test).

	Control	**Group II (cadmium treated group)**	**Group III (Omega-3 fatty acids + cadmium treated group)**	**Group IV (Ascorbic acid + cadmium treated group)**	**Group V (Omega-3 fatty acids + Ascorbic acid + cadmium treated group)**	**Group VI (withdrawal group)**
Aldosterone (pg/ ml)	342 ± 17	67.3 ± 3.5 [a, c, d&e]	334.3 ±16.6 [b&f]	336.3 ± 17.5 [b&f]	339.3±17 [b&f]	74.3 ± 5.1 [a, c, d&e]
ACTH (pg /ml)	10.95 ± 2.5	38.03 ± 3.3 [a, c, d&e]	13.6 ± 3.9 [b&f]	12.9 ±3.4 [b&f]	12.1± 2.9 [b&f]	36.3 ± 3.7 [a, c, d&e]
Corticosterone (ng/ ml)	54.4± 7.6	8.2 ± 2.9 [a, c, d&e]	49.7 ± 3.1 [b&f]	52 ± 7.4 [b&f]	53.4 ±7 [b&f]	9.6±2.2 [a, c, d&e]
DHEA (ng / dl)	26.1±2.4	7.9 ± 2.5 [a, c, d&e]	21.01±1.3 [b&f]	23.8 ± 2.6 [b&f]	25.3 ±3 [b&f]	9.3 ± 2.2 [a, c, d&e]

Data expressed as mean±SD, *: significance ≤ 0.05
a: Significance vs Control, b: Significance vs group II, c: Significance vs group III, d: Significance vs group IV, e: Significance vs group V, f: Significance vs group VI

Discussion:

Subcutaneous administered $CdCl_2$ was assessed for its cytotoxic effects on the adrenal gland of adult male rats in the present study. Cadmium is a hazardous heavy metal that is readily absorbed and accumulates in tissues for long periods, leading to persistent biochemical and functional disturbances in vital organs. Reactive oxygen species (ROS), including superoxide radical, hydroxyl ion, and hydrogen peroxide, are the primary mechanisms by which it induces toxicity. These ROS interact with cellular biomolecules, resulting in DNA damage, protein denaturation, and lipid peroxidation [4].

Examination of Hematoxylin and Eosin-stained sections of cadmium-treated rats revealed marked pathological alterations in the adrenal cortex and medulla, including loss of normal architecture, cytoplasmic vacuolation, pyknotic nuclei, karyolysis, and vascular congestion. These findings were consistent with previous studies reporting similar degenerative changes such as autophagy, apoptosis, karyolysis and nuclear shrinkage in cadmium-treated group [4]; [18]. Comparable alterations were also observed with other toxic exposures, including diazepam and exposure to stress [17]; [9].

Ultrastructural assessment confirmed these results, showing degenerated mitochondria with disrupted cristae, irregular nuclear outlines, cytoplasmic vacuolation, dilated smooth endoplasmic reticulum, enlarged lysosomes containing digested organelles, and a reduction in lipid droplets. These mitochondrial alterations are typical consequences of oxidative stress. Our findings agreed with [4], who reported that loss of mitochondrial cristae and increased lysosomal activity in the cells of the zona glomerulosa, zona fasciculata and zona reticularis, and were also in line with the results observed after exposure to acrylamide or tributyltin [20]; [21]. They noted that the cells of the zona reticularis, zona fasciculata, and zona glomerulosa showed irregular nuclear outlines, degraded mitochondria with damaged cristae, and cytoplasmic vacuolation.

Immunohistochemical staining showed strong cytoplasmic caspase-3 expression in the adrenal cortex and medulla of cadmium-treated rats, confirming apoptotic activation. These results were consistent with [4], who reported strong cytoplasmic caspase-3 expression in the adrenal cortex, frequent apoptotic cells, following cadmium exposure and also with findings after exposure to acrylamide [20]; who reported that the acrylamide-treated group exhibited significantly higher B-cell lymphoma-2-associated X (BAX) expression in the cortex, while the Bcl-2 expression in the cells of the adrenal cortex was significantly reduced. This was in contrast to the control group and was consistent with the results obtained after exposure to tributyltin, [21], who found that the group treated with tributyltin had a higher mean number of CD44-positive cells in their adrenal cortex cells than the control group.

Administration of omega-3 fatty acids to cadmium-treated rats demonstrated partial restoration of adrenal structure. Only a few pyknotic nuclei were observed, and caspase-3 immunoreactivity was markedly reduced. Ultrastructural findings showed more preserved nuclei, mitochondria, and lipid droplets. These findings supported [22], who suggested that omega-3 fatty acids possess antioxidant and cytoprotective properties against cadmium toxicity and few pyknotic nuclei were observed. Similar ameliorating effects were reported with diazepam- and stress-induced adrenal damage, preserved more nuclei, mitochondria, and lipid droplets. [17]; [9] Furthermore, [2]; [7] emphasized the importance of omega-3 supplementation in preventing neurodegenerative disorders associated with cadmium exposure.

Ascorbic acid administration produced similar protective outcomes. Histological examination showed improved adrenal architecture with fewer vacuolated cells,

however, caspase-3 expression was only mildly positive. Ultrastructural evaluation revealed preservation of nuclei and mitochondria with abundant lipid droplets. These results were in agreement with [23], who reported an antioxidant properties of vitamin C against cadmium toxicity, preservation of nuclei and mitochondria with abundant lipid droplets and with [9], who demonstrated its ameliorative effects against $CdCl_2$-induced neurotoxicity. Cadmium toxicity leading to astrogliosis, this astrogliosis was reversed in the groups treated with ascorbic acid. Vitamin C, as a potent antioxidant, scavenges ROS and reduces oxidative damage [24].

The combined administration of omega-3 fatty acids and ascorbic acid provided the greatest protective effect, showing nearly normal adrenal architecture, minimal caspase-3 expression, and well-preserved cellular ultrastructure. This suggested a synergistic antioxidant action, leading to enhanced cytoprotection compared with either treatment alone.

In contrast, the cadmium withdrawal group exhibited persistent pathological changes, vacuolated cells, frequent karyolysis, and degenerated mitochondria. Caspase-3 remained moderately expressed, indicating ongoing apoptosis. These results differed from [25], who observed almost normal adrenal architecture in a tramadol withdrawal group, ultrastructural findings showing more preserved nuclei, mitochondria, and lipid droplets similar to control group. but were consistent with [26], who reported that the extremely long biological half-life of cadmium (16–30 years), explaining its persistence and poor recovery after exposure cessation.

Biochemical assays confirmed that cadmium exposure induced oxidative stress, as shown by elevated MDA and decreased GSH and SOD levels. These results were agreed with [4] who found that cadmium exposure causes oxidative stress, increased MDA level, and decreased SOD level. These results were agreed with [27] who found that significant increase radiofrequency waves exposure causes oxidative stress, increased MDA level, and decreased SOD level. Even when compared to controls, vitamin C increased the enzyme by 45–50%. The fact that vitamin C increased SOD by 50% while decreasing MDA by 46% suggests that vitamin C may mitigate the oxidative stress that radiofrequency waves may cause in the adrenal glands.

These alterations were significantly ameliorated by omega-3, ascorbic acid, and their combination, but not by withdrawal. Similarly, hormonal analysis revealed a marked reduction in adrenal hormones after cadmium exposure, with restoration observed in the treated groups but not in the withdrawal group. These results supported previous findings that cadmium disrupts adrenal function through oxidative stress and apoptosis [28]. These results differed from [25], who observed a highly significant increase in the levels of ACTH, aldosterone, corticosterone and DHEAS in a tramadol withdrawal group the recovery group. These results were in consistent with [29]. They found that the group using mobile phones exposed to 900 MHz mobile phone radiation had a significantly higher serum ACTH level.

These results were in consistent with [30] they noted a significant rise in the levels of oxidative stress indicators, such as malondialdehyde (MDA), which is a byproduct of lipid peroxidation. Meanwhile, it causes the concentration of endogenous antioxidant defense enzymes, such as superoxide dismutase (SOD), to significantly drop in the tartrazine group. Additionally, it recorded a highly significant decrease in serum corticosterone, cortisol, aldosterone, and DHEA-S levels in the tartrazine group.

Conclusion:

Taken together, these results confirmed that cadmium exerted severe cytotoxic effects on the adrenal gland through oxidative stress, mitochondrial damage, and apoptosis. Both omega-3 fatty acids

and ascorbic acid provided significant protection, with the combined treatment showing superior efficacy. Withdrawal alone was ineffective, highlighting cadmium's long-lasting bioaccumulation. These findings emphasized the potential therapeutic role of antioxidants in mitigating cadmium-induced endocrine and cellular toxicity. Cadmium is a highly toxic to adrenal tissue, while Omega-3 and ascorbic acid, especially in combination, can significantly ameliorate its damaging effects.

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