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ORIGINAL ARTICLE

Effect of Energy Drink (Red Bull) on The Pituitary-Adrenal Axis of Adult Male Albino Rats: A Histological and Immunohistochemical Study

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ABSTRACT

Background: Energy drinks, such as Red Bull (RB), have become very popular, especially among young adults, who believe that they provide more energy and improve performance levels. Endocrine glands such as the pituitary and adrenal glands (which are functionally connected through the pituitary-adrenal axis), are among the organs susceptible to the cytotoxic effects of Red Bull. Therefore, the goal of the present study was to assess the effects of RB chronic intake on these two glands in adult male albino rats as well as the consequences of its withdrawal.

Methods: Thirty-nine adult male albino rats were equally divided into 3 groups: control group (I), Red Bull group (II), and withdrawal group (III). Analysis of serum levels of ACTH and corticosterone for all groups was done. Specimens from both glands were processed for light and electron microscopic examination. Morphometric and statistical analyses were also done.

Results: The Red Bull-given group displayed significantly lower serum levels of ACTH and corticosterone in comparison to the control group. Both the anterior lobe of the pituitary and the adrenal cortex showed marked degenerative changes. The pars distalis showed many cells with dark nuclei and vacuolated cytoplasm. Blood sinusoids were congested. Decreased number of PAS stained basophils was also detected. In the adrenal gland, increased zona fasiculata (ZF) thickness with marked fibrosis were detected. Immunohistochemical staining for anti- iNOS and anti-Caspase 3 showed statistical significant increase compared to the control group. Cessation of RB intake showed partial improvement in the histopathological and biochemical changes in both glands.

Conclusions: Chronic intake of energy drinks alters the pituitary-adrenal axis structure and function, which cannot be completely ameliorated by cessation of its administration.

Keywords: Energy drink; Pituitary-adrenal axis; Red Bull; Histology

INTRODUCTION

The energy drink; Red Bull contains many ingredients like calcium pantothenate (vitamin B5), niacinamide (vitamin B3), citric acid, taurine, sodium bicarbonate, magnesium carbonate, carbonated water, sucrose, glucose, pyridoxine HCl, vitamin B12, and both natural and artificial flavors. There are 80 mg of caffeine in a 250 ml can of Red Bull [1].

Energy drinks are used because of their CNS-stimulating effect from caffeine [2]. Additionally, CNS stimulation can activate the hypothalamo-pituitary-adrenal (HPA) axis, leading to ACTH-mediated secretion of cortisol (in humans) and corticosterone (in rats). Caffeine can also act directly on the adrenal gland by antagonizing adenosine receptors on adrenal cells, resulting in increased catecholamine and glucocorticoid synthesis. Taurine has also been reported to increase glucocorticoids [3]. Energy drinks do not exceed the 400 mg acceptable maximum limit for caffeine intake in healthy

persons. However, they are usually consumed alongside, rather than as a replacement for, caffeine from other sources, so adverse effects can occur in cases of drinking energy drinks if warnings are not heeded [4].

White et al. [5] reported that EDs produce neurodegenerative changes in the hippocampus, which could disturb the regulatory circuit that connects the hippocampus and the hypothalamus, leading to unregulated chronic glucocorticoid signalling. This could produce many health hazards, of which, cytokine levels elevation, emerging of metabolic disorders, including insulin resistance, and acceleration of cellular aging [6].

Being under the continuous stimulatory effect of consuming RB for two months could induce oxidative stress [7]. Oxidative stress is known to be a mean contributing factor in the emerging of many diseases and causes cellular damage by overproducing reactive oxygen species (ROS), that damage DNA, oxidise protein, and produce lipid peroxides [8]. Thus, prolonged consumption of RB without cessation could result in excessive tissue damage. For these reasons, we aimed to clarify the possible degenerative changes that could occur in both the anterior lobe of the pituitary gland and the adrenal gland cortex following chronic ingestion of RB, as these two glands are functionally connected by the pituitary-adrenal axis. Moreover, we also investigated the possibility of amelioration at both the biochemical and structural levels of both glands after a period of cessation of RB administrati

METHODS

Chemicals

Energy drink (Red Bull)

Red Bull cans (250 ml) were bought from a market in Egypt. Taurine (400 mg), 32 mg of caffeine, glucose and sucrose (11.3 g), 240 mg of gluconolactone, niacin (7.2 mg), vitamins B12 (0.4 mg), B2 (0.64 mg), B6 (0.8 mg) , 2.4 mg panthenol, inositol (20 mg), artificial flavour, and sparkling water are all included in 100 millilitres of Red Bull.

IgG type rabbit anti-iNOS polyclonal antibody: (catalog no. GB11119), dilution 1:500, Servicebio, Wuhan, China.

IgG type rabbit anti-Caspase 3 polyclonal antibody: (catalog no. A11953), diluted 1:100, ABclonal, Wuhan, China.

Animals

This study was done on thirty-nine healthy adult (14-18 weeks) male Wistar albino rats, their weight ranged from 180-200 grams. They were brought from Zagazig University Faculty of Medicine animal house. Ethical approval and care of experimental animals

Suitable environment of twelve-hour dark-light cycle and a comfortable temperature of 25±1°C was maintained for all rats. All procedures were carried out in compliance with IACUC rules for the care and use of laboratory animals in Zagazig University. This adherence to guidelines underscores the commitment to animal welfare and the integrity of the research process (**Approval: ZU-IACUC/3/F/96/2023).**

Experimental protocol

Three equal groups of 13 rats in each were included in the experiment as follows:

Group I (Control group): Rats received no treatment for 8 weeks.

Group II (Red Bull group): Rats received Red Bull at a dose of 1.5 ml/100 g. body weight once daily [9] for 8 weeks [10] by gastric tube.

Group III (Withdrawal group): Rats received Red Bull at a dose of 1.5 ml/100 g. body weight once daily [9] for 8 weeks, then rats were left for another 8 weeks without any treatments [10].

During the experimental period, the food consumption of the rats was followed. Rat's mortalities were recorded. At the end of the experiment, according to the corresponding duration for each group (8 weeks for group I, II and 16 weeks for group III), rats were fasted overnight. On the following day, at 9:00 am, the rats were administered intraperitoneal (IP) injection of a ketamine-xylazine mixture (80 mg/kg for ketamine & 8 mg/kg for xylazine) to induce anesthesia [11]. A capillary tube was then used to draw blood samples from each rat's retro-orbital venous

plexus and place them in a separator tube. After 30 minutes of letting the blood coagulate at room temperature, the serum was separated by centrifuging it at 3000 rounds per minute (rpm) at 4°C for 10 to 15 minutes . The serum was then kept at -20°C until it was time to measure the levels of corticosterone and ACTH hormones.

After blood collection, a mid-line incision was carried out in the rats' skulls, another incision was done in the meninges. Then, the brain was exposed and dissected out from the skulls. The pituitary glands were dissected carefully from meninges after addition of formaline fixative on it to prevent its damage.The pituitary glands were subsequently preserved in buffered formalin 10% (pH 7) and prepared using paraffin technique [12] to be examined by light microscopy. The adrenal glands were gently removed after making another incision in the upper abdomen. All rats' right adrenal glands were cleared of connective tissue and fat. They were then weighed to the nearest 0.001 g with an electronic scale. they were preserved in buffered formalin 10% (pH 7) and prepared to be examined by light microscopy. The left adrenal glands from all animals were fixed in glutaraldehyde in cacodylate buffer (2.5%) and processed for electron microscope examination. Morphometric measurements and statistical analyses were done for the examined sections.

Methods

Biochemical studies

Using the enzyme-linked immunosorbent assay (ELISA) technique [13]. The samples were examined in the Clinical Pathology Department, Faculty of Medicine- Zagazig University, for detection of the serum levels of ACTH and corticosterone. An ELISA Kit (Catalog Number: CSB-E06875r) was used for measuring serum ACTH levels, and an ELISA Kit (Catalog # K7430-100, 100 assays) was used for measuring serum corticosterone levels.

Histological studies

Light Microscope Techniques: Paraffin technique [12]

Each animal's pituitary and right adrenal gland specimens were preserved in formaline saline (10%). Specimens were then dehydrated, cleared, and embedded in wax of paraffin. Five µm thick sections were cut and stained.

The pituitary glands were stained for Haematoxylin and Eosin (H&E) stains, Periodic acid Schiff (PAS) reaction and immunohistochemical stain using anti-iNOS antibodies.

The right adrenal glands were stained for Haematoxylin and Eosin (H&E) staining, Mallory's trichrome stain, and immunohistochemical stain using anti-caspase 3 antibodies.

For immunohistochemistry, sections embedded in paraffin were dewaxed, rehydrated, and left in an incubator at 4°C for an entire night with the primary antibody . The sections were incubated with the matching biotinylated secondary antibody for an hour at room temperature following three phosphate buffer saline (PBS) rinses. After being incubated with streptavidin peroxidase for ten minutes, the samples were subjected to three more PBS washes. As a chromogen, 3,3′-diaminobenzidine-hydrogen peroxide was added to observe immunoreactivity. A counterstain of mayer's haematoxylin was used. When creating the negative control sections, PBS was used in place of primary antibodies [14]. A Canon PowerShot A620 (UK, England) was used to take pictures of the sections after they had been inspected using an Olympus microscope (JAPAN, C5060-AUD, 5H01155) at the Unit of Image Analysis present in the Department of Medical Histology & Cell Biology at Zagazig University Faculty of Medicine. The photomicrographs were enhanced with a scale bar according to calibration. The reaction appeared as a brown cytoplasmic color, which indicates apoptosis in anti-Caspase-3, and oxidative stress indicator in anti-iNOS immunostaining.

Transmission Electron Microscope Technique

Specimens of the left adrenal glands (1mm3) were fixed in 2.5% gluteraldehyde buffered with 0.1 M cacodylate buffer at PH 7.4 and then post-fixed in 1% osmium tetroxide .

Following dehydration in increasing alcohol grades, the tissues were embedded in epoxy resin to create 1 µm semithin sections, which were subsequently stained with toluidine blue (1%) and seen under a light microscope [15]. The Faculty of Medicine at Tanta University in Egypt used a transmission electron microscope (JEOL, JEM-2100, Tokyo, Japan) to inspect and take pictures of ultrathin sections that had been stained with lead citrate and uranyl acetate [16].

Morphometric studies:

Sections were morphometrically analysed at Zagazig University's Faculty of Medicine, Department of Human Anatomy and Embryology. The area percent was examined in randomly selected five separate fields in each slide section at a total magnification of 400, to conduct a quantitative assessment. The following parameters were computed: the anterior pituitary's area percentages of anti-iNOS-positive and PAS-stained cells In the adrenal glands, the area percentages of Mallory trichrome-staining and anti-caspase 3-positive cells were measured. The zona fasciculata thickness in H&E-stained sections at a total magnification of 100 was measured by image analysis program Digimizer 4.3.2 (MedCalc Software bvba, Belgium) [17]. The computer system software (version 1.48v, NIH, Bethesda, Maryland, USA) measured a red binary color that covered all of these locations which were further converted into micrometer2 and expressed as area percentage (the area%).

Statistical analysis

The obtained data from general observation (the adrenal gland weight), biochemical analysis (serum ACTH and corticosterone levels), morphometric analysis (the area percent of PAS- stained cells, iNOS-positive cells, the zona fasiculata thickness, the area percent of Mallory trichrome-staining and anti-caspase 3-positive cells) were statistically analysed. All the data obtained were expressed as means ±SD. Determination of the statistical significance between groups using ANOVA followed by the Least Significant Difference test for comparison between each two groups was done.

P values below 0.05 were regarded as statistically significant, whereas those below 0.001 were regarded as highly significant. The IBM SPSS 18.0 software was used [18].

RESULTS

Biochemical results

Mean values of ACTH (pg/ml) and corticosterone (ng/ml) serum levels were statistically analysed using one way ANOVA test that revealed statistically significant differences among the different groups as P value was < 0.001. LSD test was done to compare between each two groups. The mean values of the two hormones were statistically significantly lower in group II in comparison to group I. The mean values of both hormones were significantly higher in Group III compared to group II, although there was non significant difference between Group I and Group III (Fig. 4J & K).

Histological and morphometric results

Light Microscopic Examination of the Pituitary gland

Haematoxylin and Eosin-stained sections of the pars distalis showed that: the parenchyma of the pars distalis in the control group showed normal appearance as it was composed of anastomosing cords of acidophils, basophils, and chromophobes that were surrounded by blood capillaries lined with endothelial cells. Acidophils had large, eccentric, vesicular nuclei with prominent nucleoli and a homogenous, acidophilic cytoplasm. Basophils appeared with rounded, vesicular, eccentric nuclei surrounded by basophilic, granular cytoplasm. Chromophobes appeared with pale-stained cytoplasm (Fig. 1A). The Red Bull group showed a disorganized normal arrangement of the parenchymal cells. Most of the cells showed dark nuclei, vacuolated cytoplasm, and obvious loss of cytoplasmic granules.Wide spaces between the adjacent cells were seen. The blood capillaries between cell cords were dilated and congested and lined with endothelial cells (Fig. 1B). The withdrawal group showed

restoration of the normal appearance of cell clusters which were composed of acidophils with acidophilic cytoplasm and eccentric vesicular nuclei, basophils with basophilic granular cytoplasm and eccentric vesicular nuclei and chromophobe cells with pale-stained cytoplasm. while some cells still showed vacuolated cytoplasm. Dilated blood capillariesand empty spaces between the cells were still seen (Fig. 1C).

Sections of the control group stained with PAS revealed deeply stained pink cytoplasm of many PAS-positive cells (basophils) (Fig. 2A) while the Red Bull group revealed a decrease in both the staining affinity and number of basophils (PAS-stained cells) (Fig. 2B). The withdrawal group showed a positive reaction in the cytoplasm of many basophil cells (Fig. 2C).

Compared to the control group, group II had a significant statistical decrease in the area % of PAS-stained basophil cells in the anterior pituitary gland's pars distalis. Although it was still much lower than the control group, group III demonstrated amelioration as evidenced by a statistically significant rise in the percentage of PAS-stained basophils as compared to group II (Fig. 3D).

Immuno-histochemical stained sections with anti-iNOS antibodies showed weak reaction in the cells of the control group (Fig. 3A) while a positive immune reaction appeared as a brown color in the cytoplasm of many cells in the Red Bull group (Fig. 3B). The withdrawal group showed positive immune reaction that appeared as a brown color in the cytoplasm of some cells (Fig. 3C).

When group II's pars distalis of the anterior pituitary gland was compared to the control group, there was a highly significant increase in the area percentage of iNOS immunoexpression, indicating severe oxidative stress. While there was a highly significant increase when compared to the control group, group III demonstrated amelioration, as evidenced by a highly significant decrease when compared to group II (Fig. 3D).

Light Microscopic Examination of the adrenal gland

Haematoxylin and Eosin-stained sections showed a regular, thin connective tissue capsule surrounding the adrenal gland of the control group. The adrenal cortex included the zona glomerulosa (ZG), where cells were arranged in arched clusters; the zona fasciculata (ZF), made up of straight cell cords; and the zona reticularis (ZR), which had anastomosing cords of cells. The medulla consisted of large, basophilic cells (Fig. 4A). Higher magnification of the adrenal cortex showed that the zona glomerulosa cells had rounded, basal vesicular nuclei. The polyhedral zona fasciculata cells have granular, lightly stained acidophilic cytoplasm and central, spherical vesicular nuclei. Sinusoidal capillaries coated with endothelial cells ran between the cell cords (Fig. 4B).

The Red Bull administration severely affected the adrenal cortex histological structure in the form of a thickened and irregular connective tissue capsule and disorganized cellular arrangement in both zona glomerulosa and fasciculata. Many cells appeared having darkly stained nuclei and vacuolated cytoplasm, and others showed deeply stained acidophilic cytoplasm. Blood vessels in the capsule and sinusoidal capillaries between the cell cords were dilated and congested. Areas of separation between the adjacent cells were indicative of cell loss (Figs. 4C,D,E&F). The withdrawal group showed normal arrangement of the three zones of the suprarenal cortex. The medulla was composed of large basophilic cells (Fig. 4G). When examined at a higher magnification, the zona glomerulosa (ZG) cells showed basal, rounded vesicular nuclei. The cells of zona fasciculata (ZF) had light acidophilic cytoplasm with central vesicular nuclei, but some of them also displayed small dark nuclei and highly stained acidophilic cytoplasm, and a small number of cells were vacuolated (Fig. 4H).

Regarding the percentage of ZF thickness in relation to the adrenal cortex, the highest values were recorded in group II, which was significantly higher than the control. Group III showed a non-significant difference from group I, but a significant decrease in zona

fasciculata thickness was observed than group II (Fig. 4I).

Mallory trichrome-stained sections revealed few collagen fibers in the control group's sinusoidal capillaries and capsule (Fig. 5A). Excessive deposition of collagen fiber was observed in the capsule, between the cell cords and surrounding the capillaries in the Red Bull group (Figs. 5B, C&D). Collagen fibers were moderately abundant in the capsule and surrounding the sinusoidal capillaries in the withdrawal group (Fig. 5E).

Regarding the percentage of collagen fiber deposition in the adrenal cortex, a highly statistically significant increase was detected in group II when compared to the control, while group III was significantly improved than group II but still significantly higher than the control (Fig. 5F).

Immuno-histochemical stained sections with anti-Caspase 3 antibodies showed weak cytoplasmic immune reaction for anti-caspase 3 antibodies in the control group (Fig. 6A). The Red Bull group's cells displayed excessive apoptotic changes, which in many cases manifested as positive cytoplasmic immunoreactivity (Fig. 6B). Few cells in the withdrawal group displayed positive cytoplasmic immunoreactivity (Fig. 6C).

The significant increase in the area percentage of Caspase 3 immunoexpression in group II in comparison to group I indicated the apoptotic changes in group II. Although group III's apoptosis was much lower than group II's, it was still significantly higher than group I (Fig.6D).

Toludine blue-stained sections of the control group showed cells of the adrenal cortex with vesicular nuclei and prominent nucleoli. Zona fasciculata cells had numerous lipid droplets of uniform shape and sinusoidal capillaries appeared between cell cords (Fig. 6E). The Red Bull group showed zona fasciculata cells with irregular dark nuclei and variable-sized lipid droplets. (Fig. 6F). The withdrawal group showed ZF had central, rounded pale-stained nuclei with prominent nucleoli, and many lipid droplets of uniform size and shape. Few cells had irregular dark nuclei (Fig. 6G).

Electron microscope results of the adrenal gland

Zona Fasciculata cells of the control group had euchromatic nuclei surrounded by regular nuclear envelopes. Their cytoplasm showed many lipid droplets of uniform sizes, and normal-shaped mitochondria with tubular cristae (Fig. 7A). The chronic intake of Red Bull severely altered the cellular ultrastructure as wide spaces containing excess collagen fibers were noticed between the adjacent cells. The nuclei appeared shrunken and heterochromatic. The cytoplasm had variable sized mitochondria and few lipid droplets. Extensively dilated SER and cytoplasmic vacuoles were also detected (Fig. 7B&C). Severely affected section of the same group showed zona fasciculata cells with many ballooned mitochondria that lost cristae. Moreover, areas of cytoplasmic loss indicating degenerated organelles were detected (Fig. 7D). Cells of the withdrawal group showed partially ameliorated ultrastructure. They had euchromatic nuclei but with irregular envelopes. Lipid droplets were increased in number and mitochondria were variable-sized with tubular cristae. Normally appearing supranuclear Golgi apparatus and dilated SER were evident in addition to wide intercellular spaces (Figs. 7E&F).

FIGURE LEGENDS

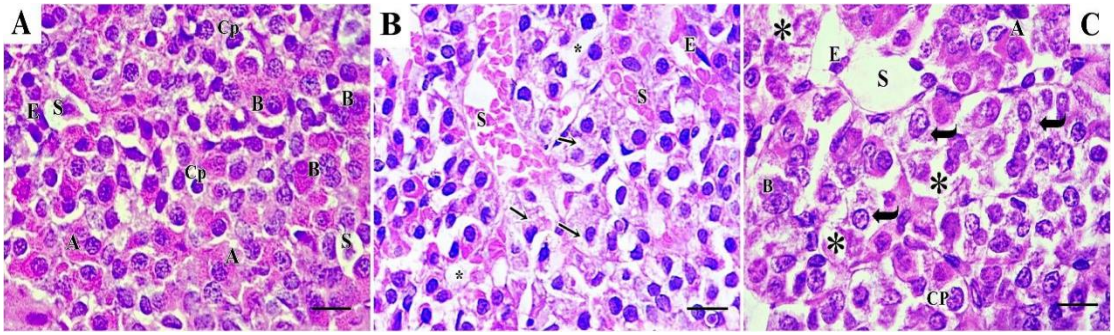

Figure 1: Photomicrographs of H&E-stained sections of pars distalis of the pituitary gland showing A: The control group shows clusters of cells; Chromophil cells are composed of acidophils (A) with acidophilic cytoplasm and eccentric vesicular nuclei and basophils (B) with basophilic granular cytoplasm and eccentric vesicular nuclei. Chromophobe cells (Cp) with pale-stained cytoplasm. Blood capillaries (S) lined with endothelial cells (E) are noticed between cell clusters. B: The Red Bull group shows most cells with small dark nuclei and cytoplasmic vacuolation with loss of cytoplasmic granules (arrows). Wide spacing between adjacent cells (asterisk) and congested, dilated blood capillaries (S) lined with endothelial cells (E) are seen. C: The recovery group shows the cell clusters composed of acidophils (A) with acidophilic cytoplasm and eccentric vesicular nuclei and basophils (B) with basophilic granular cytoplasm and eccentric vesicular nuclei. Chromophobe cells (Cp) with pale-stained cytoplasm. Some cells appear with vacuolated cytoplasm (curved arrow). Dilated blood capillaries (S) lined with endothelial cells (E) and small empty spaces (asterisk) are seen between the cell clusters. (H&E X1000, Scale bar 10µm)

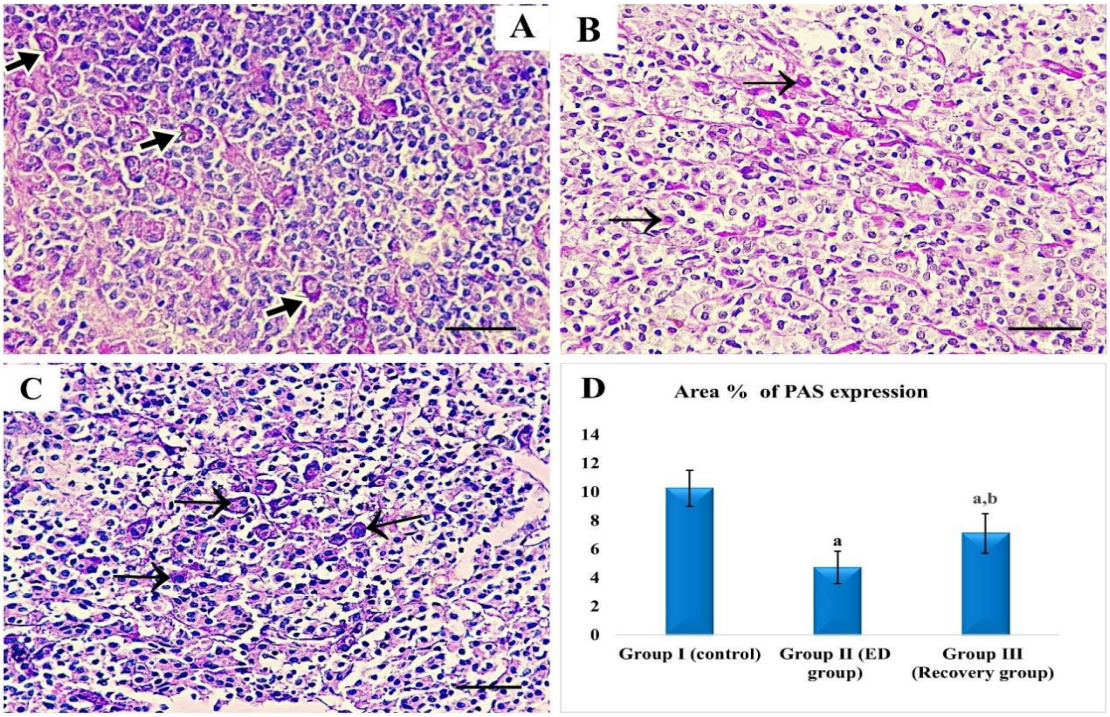

Figure 2: PAS-stained sections of the pars distalis of pituitary gland showing A: The control group shows deeply-stained pink cytoplasm in many basophil cells (arrows). B: The Red Bull group shows a decreased number and staining affinity of PAS-positive basophils (arrows). C: The recovery group shows positive PAS reaction in the cytoplasm of basophil cells (arrows). (PASX400, Scale bar 30µm). D: The area% of PAS expression; a: significant with control group & b: significant with ED group.

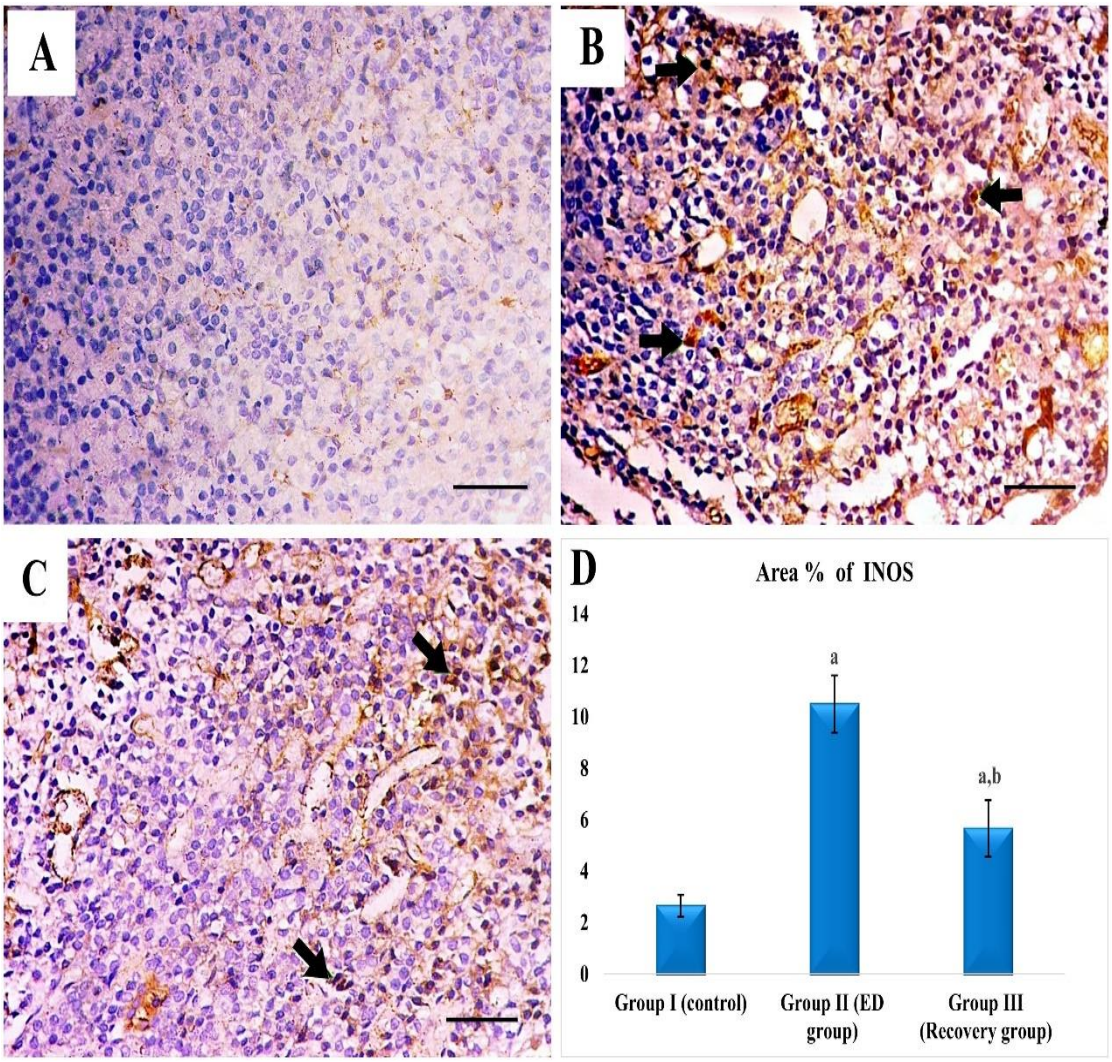

Figure 3: Anti-iNOS stained sections of the pars distalis of pituitary gland showing A: The control group shows weak cytoplasmic iNOS immunoreactivity in the cells. **B:** The Red Bull group shows positive cytoplasmic immune reaction for iNOS in many cells (arrows). **I:** The recovery group shows positive cytoplasmic immune reaction for iNOS in some cells (arrow). **(iNOS x400, Scale bar 30µm). D:** The area% of iNOS expression; a: significant with control group & b: significant with ED group.

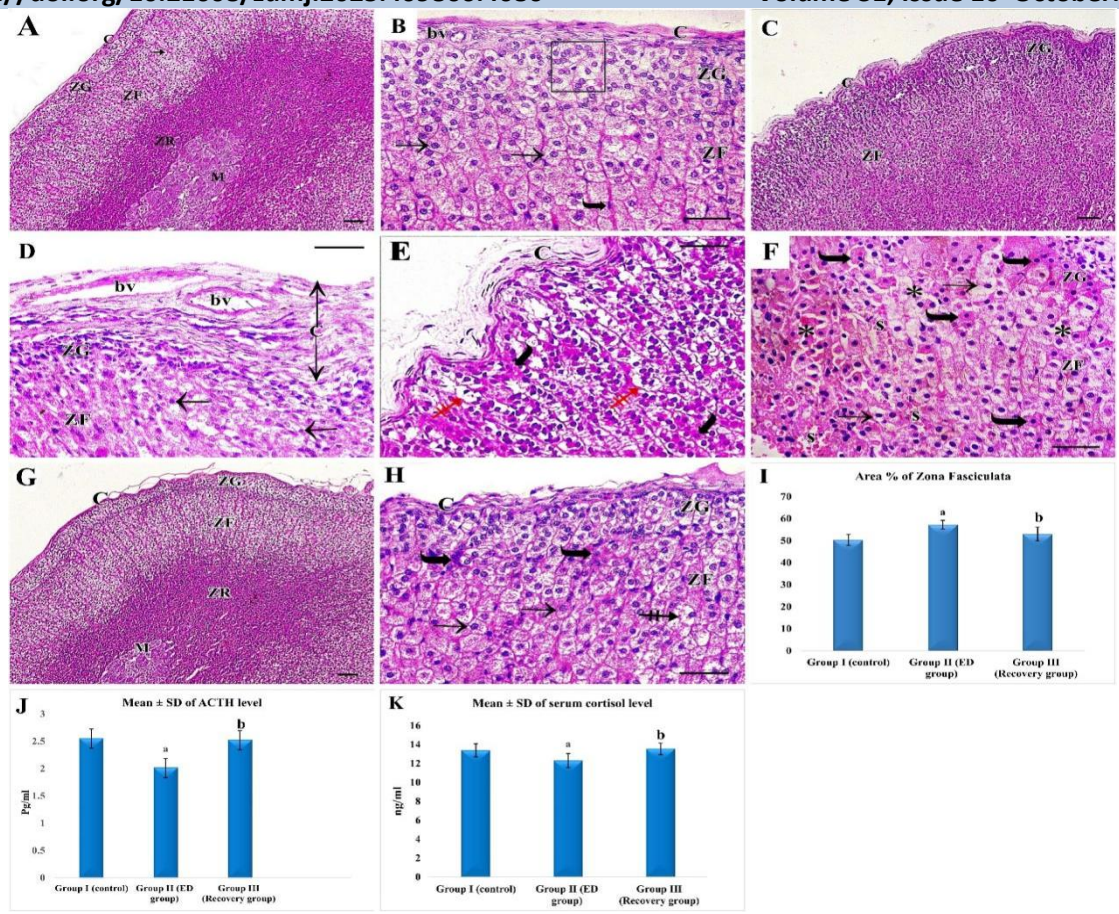

Figure 4: Photomicrographs of H&E-stained sections of the adrenal gland. A: The control group shows the gland is surrounded by a regular thin capsule (C). The adrenal cortex shows zona glomerulosa (ZG) formed of arched clusters of cells, zona fasciculata (ZF) composed of straight cords of cells (arrow) and zona reticularis (ZR) cells are arranged in anastomosing cords. The medulla (M) is formed of large basophilic cells. B: A higher magnification of the control group shows ZG cells with basal, rounded vesicular nuclei (rectangle). The ZF cells with central vesicular nuclei and pale acidophilic cytoplasm (arrows). The cell cords are separated by sinusoidal capillaries lined with endothelial cells (curved arrow). The gland is surrounded by a regular thin capsule (C) that contains blood vessels (bv). C: The Red Bull group shows irregular thick connective tissue capsule (C) and disorganized cellular arrangement in both zona glomerulosa (ZG) and zona fasciculata (ZF). D, E, F: Higher magnifications of the Red Bull group showing: thick and irregular connective tissue capsule (C) that contains dilated blood vessels (bv). Both zona glomerulosa (ZG) and fasciculata (ZF) appear disorganized with loss of normal cellular arrangement. Many cells have dark nuclei (arrows). Many vacuolated cells with dark nuclei (crossed arrows). Some cells appear with deeply stained acidophilic cytoplasm (curved arrows). Congested sinusoidal capillaries (S) and wide spaces between adjacent cells (stars) are also seen.

G: The recovery group (group III) shows a thin connective tissue capsule (C). The zona glomerulosa (ZG) cells are arranged in arched clusters, and the zona fasciculata (ZF) cells are arranged in straight cords with foamy cytoplasm. The zona reticularis (ZR) cells are arranged in anastomosing cords. The medulla (M) is formed of large basophilic cells. H: The recovery group shows a thin and irregular connective tissue capsule (C). Zona glomerulosa (ZG) shows arched clusters of cells with basal, rounded vesicular nuclei. The zona fasciculata (ZF) is composed of straight cords of cells with central vesicul. ar nuclei and pale acidophilic cytoplasm (arrows). vacuolated cells (crossed arrow) and cells with deeply acidophilic cytoplasm and small dark nuclei (curved arrows) can be seen. (A, C, G: X 100, Scale bar 50µm, B, D-F, H X 400, Scale bar 30µm). I: Percentage of zona fasciculata thickness. J&K: Mean ± SD of serum ACTH and cortisol levels respectively (A: Significant with control group, B: Significant with RB group, N: Nonsignificant with control group)

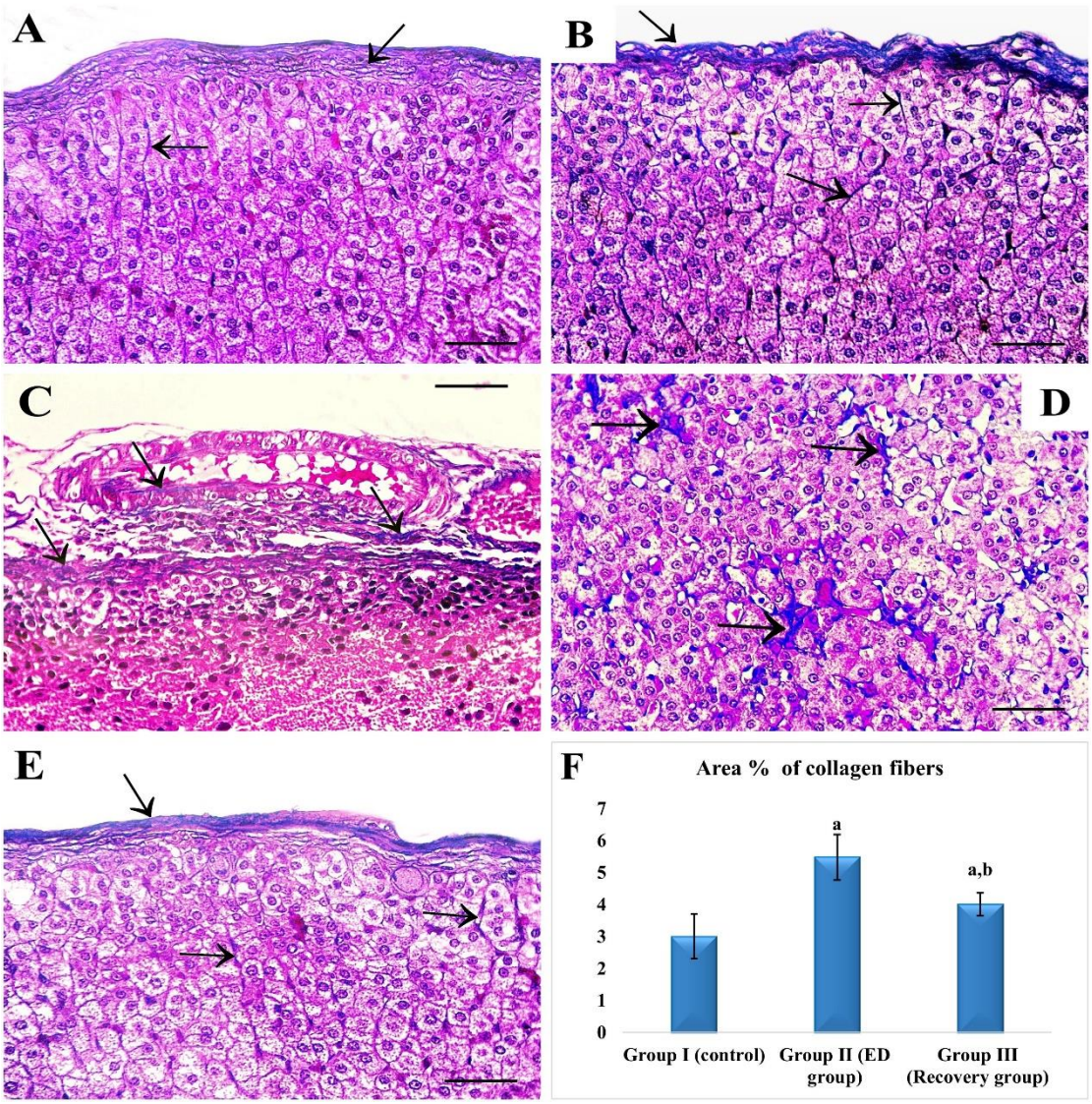

Figure 5: A photomicrograph of Mallory trichrome-stained sections of the adrenal cortex. **A:** The control group shows few collagen fibers in the capsule and around the sinusoidal capillaries (arrow). **B:** The Red Bull group shows excessive collagen deposition in the capsule and between the cell cords (arrows). **C:** Another section of the same group shows excessive collagen deposition in the capsule and around the congested blood vessels (arrows). **D:** The deeper parts of the cortex of the Red Bull group show excessive collagen deposition between cell cords and around the sinusoidal capillaries (arrow). **E:** The recovery group shows moderate collagen deposition in the capsule and around sinusoidal capillaries between the cell cords (arrow). **(Mallory trichrome-stain X400, Scale bar 30µm). F:** The area% of collagen fibers deposition; a: significant with control group & b: significant with ED group.

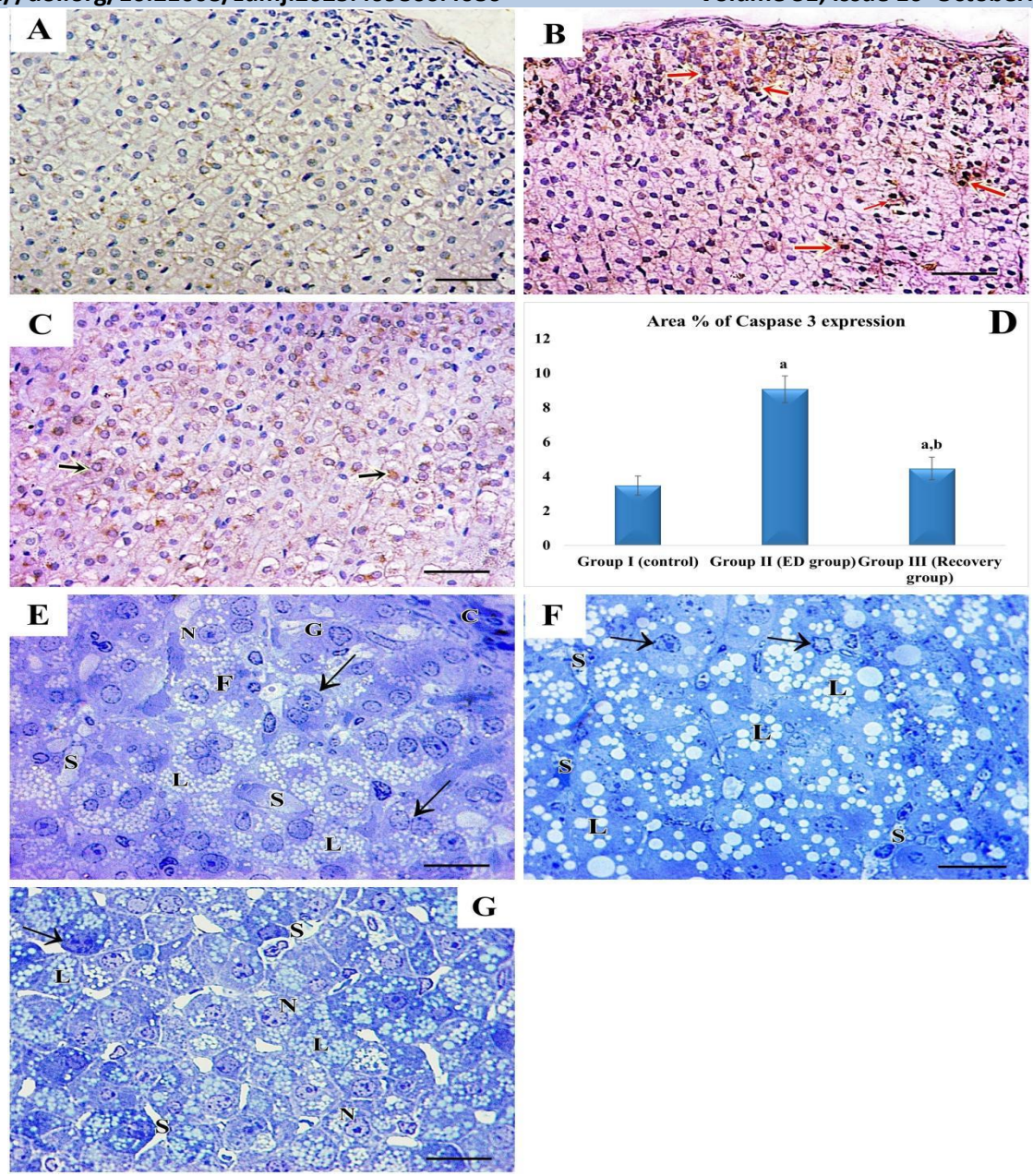

Figure 6: Photomicrographs of anti-Caspase 3 stained sections of the adrenal cortex showing A: The control group shows weak cytoplasmic immunoreactivity in the cells. B: The Red Bull group shows positive cytoplasmic immunoreactivity in many cells (arrows). C: The recovery group shows positive cytoplasmic immunoreactivity in a few cells (arrows). Anti-Caspase 3 X400, Scale bar 30µm). D: Area% of caspase3 immunoexpression; a: significant with control group & b: significant with ED group.
Toluidine blue-stained sections of the adrenal cortex show E: The control group shows thin connective tissue capsule (C), zona glomerulosa cells have basal rounded vesicular nuclei (G) and zona fasciculata (F) cells have central rounded vesicular nuclei with prominent nucleoli (N) and contain many lipid droplets (L) of uniform size and shape. Some cells are binucleated (arrows). Sinusoidal capillaries (S) appear between ZF cells cords. F: The Red Bull group shows zona fasciculata cells with irregular dark nuclei (arrow), and variable-sized lipid droplets (L). Sinusoidal capillaries (S) appear between the cells. G: The recovery group shows ZF cells with large central pale-stained nuclei and prominent nucleoli (N), and many lipid droplets (L) of uniform shape and size. Few cells appear with dark nuclei (arrow). Sinusoidal capillaries can be seen between the cells (S). (Toluidine blue stain X1000, Scale bar 10µm)

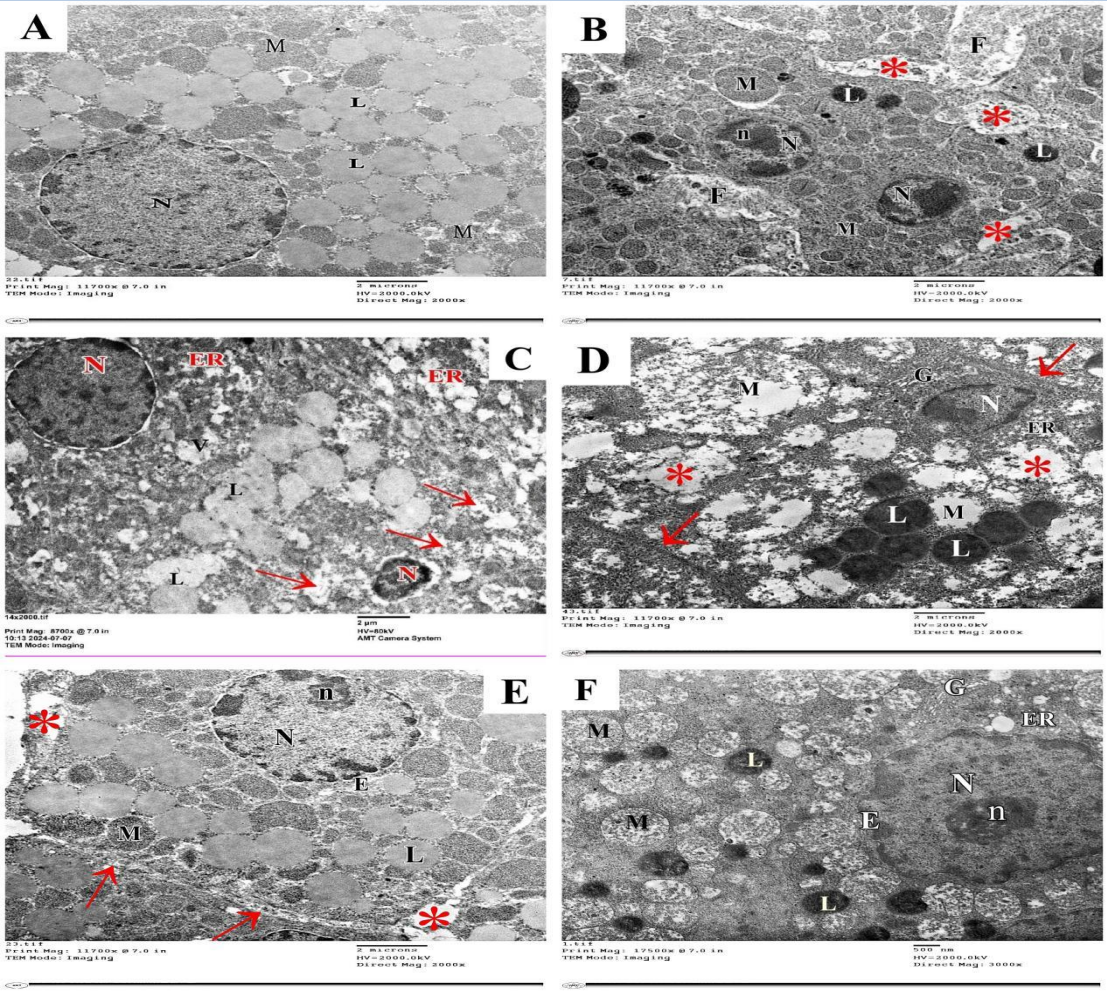

Figure 7: Transmission electron photomicrographs in zona fasciculata cells of the adrenal gland. **A:** The control group shows the cell having a round euchromatic nucleus (N). The cytoplasm shows multiple lipid droplets (L), and multiple normal-shaped mitochondria (M) with tubular cristae. **B :** The Red Bull group shows shrunken heterochromatic nucleus (N) with a prominent nucleolus (n). Wide spaces (asterisk) and excess collagen fibers (F) are present between adjacent cells. The cytoplasm shows variable-sized mitochondria with tubular cristae (M) and few lipid droplets (L). **C :** The same group shows heterochromatic nuclei (N) with a widened nuclear envelope. The cytoplasm shows markedly dilated smooth endoplasmic reticulum (ER), variable-sized lipid droplets (L), areas of cytoplasmic loss (arrows), and vacuolations (V). **D :** The same group shows severely affected cells with mitochondrial ballooning and lost cristae (M), areas of cytoplasmic loss (asterisk), and some lipid droplets (L) can be seen. Dilated smooth endoplasmic reticulum (ER) and supranuclear Golgi apparatus (G) are seen near the shrunken heterochromatic nucleus (N). Intact cell membranes of adjacent cells are also seen (arrows). **E:** The recovery group shows euchromatic nucleus (N) with prominent nucleolus (n) and irregular envelope (E), the cytoplasm has many lipid droplets (L) and mitochondria (M) with tubular cristae. Wide intercellular spaces (asterisks) are seen between adjacent cells (arrows). **F :** Another section of the same group shows nucleus (N) with prominent nucleolus and irregular envelope (E). The cytoplasm shows variable-sized mitochondria with tubular cristae (M), supranuclear Golgi apparatus (G), dilated smooth endoplasmic reticulum (ER), and few lipid droplets (L) **(TEM; A-E: Scale bar 2µm, F: Scale bar 500nm)**.

DISSCUSSION

Long periods of energy drinks (EDs) consumption make the body under the influence of their ingredients constantly, especially caffeine and taurine [19]. Examination of hematoxylin and eosin (H&E)- stained sections of the pars distalis of the pituitary gland of the Red Bull group showed marked cell loss indicated by wide spacing between the cells, which lost their granules and were replaced by cytoplasmic vacuoles. Their nuclei appeared shrunken and darkly stained. The blood capillaries were congested. It was previously reported that excessive consumption of EDs could result in cytoplasmic vacuolation in different tissues as the kidney[20], the hepatocytes [21], and in the cells of the thyroid gland [22]. Ghabrial and Sidhom [23] studied the effect of carbonated soft drinks on the histological structure of the anterior pituitary gland and found marked cytoplasmic vacuoles in the cells. According to Nadhim and Al-derawi [24], these changes were considered as cytotoxic effects. Moreover, Song et al. [25] suggested that vacuoles provide a protective mechanism against cellular damage. Omar and Kamar [25] added that, irregular shrunken darkly stained dense nuclei could present as a sign of apoptosis in the pars distalis.

The basophil cells of RB group were markedly decreased as indicated by PAS staining. We could conclude that the significant depletion of basophils was behind decreased estimated ACTH levels. The PAS positive granules in the pars distalis were reported by Omar and Kamar [26] to represent the stored glycoprotein hormones: ACTH, FSH, LH and TSH present in the basophils. Liu et al. [27] reported that depletion of hormone-secreting cells might be due to the damaging effect of excess ROS induced by EDs which would affect the endocrinal activity of these cells. Enhanced ROS production in the pituitary gland of RB group was proved in the present study by increased expression of iNOS-stained cells. According to Anavi and Tirosh [28], EDs significantly elevated iNOS expression levels in endothelial cells, linking this effect to the synergistic impact of caffeine and taurine on the inflammatory signalling pathways.

The examined adrenal cortex sections of RB group also revealed different levels of tissue damage. Many cells appeared with vacuolated cytoplasm while others showed deeply stained acidophilic cytoplasm. Increased cytoplasmic acidophilia was documented in the research of Sadek et al**.** [29] in which they studied the effect of chronic stress on the adrenal gland. Zarobkiewicz et al. [30] attributed increased acidophilia in ZF cells to decreased lipid droplet (LD) content due to the enhanced LDs consumption in the hormonal synthesis. Hongrui et al. **[31]** reported that, the increased cytoplasmic acidiphilia might represent an ongoing apoptosis. Regarding the cytoplasmic vacuolations observed in many ZF cells, Hakim et al. [32] and Hanna et al. **[33]** also reported similar vacuoles in response to EDs consumption in the cardiomyocytes and the hepatocytes. It was suggested as a sign of cellular damage. Another sign of tissue damage appeared in the form of increased blood congestion in the RB group in both glands of the present study. This finding was also reported by Ayuob and Elbeshbeishy [34] in the the pancreatic tissues due to EDs as a result of the impaired endothelial function due to high sugar content

Toluidine blue staining of the adrenal cortex revealed detailed findings about the cellular cytoplasmic changes in RB group which showed fewer lipid droplets of variable shapes and sizes. Zarobkiewicz et al. [30] found similar results and explained them by increased consumption of LDs during stimulated steroidogenesis. Our findings also revealed that chronic RB consumption, exhausted the cells which appeared in the form of decreased corticosterone levels at the end of the experiment when compared to the control group.

Chronic RB consumption not only affected the cellular structure, but the connective tissue of the capsule and between the cell cords was markedly affected as noticed by enhanced collagen fiber deposition. Abd Elwahab and Mahmoud [35] reported that EDs increase the collagen deposition in the muscular tissue.

They referred that to the ability of ROS generated from EDs to induce transformation of fibroblasts in the connective tissue to more synthetic myofibroblasts.

Enhanced cell apoptosis in the Red Bull group was also detected immunohistochemically by staining with anti-Caspase 3 antibody. Saiki et al. [36] reported increased caspase-3 activity in the liver and the kidney tissues following exposure to high doses of caffeine and attributed it to the mitochondrial damage due to oxidative stress, suggesting the activation of the intrinsic apoptotic pathway. It was noted that, chronic RB consumption in the present study revealed multiple pathological changes in the adrenal cortex, including large vacuolated cells, congested blood vessels, and fibrosis. All these changes can overcome the cell loss due to necrosis and apoptosis, and the net result was an increased percentage of ZF thickness in RB group relative to the control one, as detected by morphometric and statistical results. Another explanation was reported by Zarobkiewicz et al. [30]. They attributed the increased ZF thickness relative to the total cortical thickness in the ED-consuming group to the stimulatory effect of caffeine on ACTH, which has a trophic effect on the adrenal gland.

When examined by electron microscope, the ZF cells of the RB group revealed marked ultrastructural changes, included heterochromatic nuclei, mitochondrial swelling with lost cristae, and disruption of endoplasmic reticulum integrity. Also, large vacuoles that might represent degeneration were found. Hanna et al. [33] reported that increased nuclear heterochromatin may be due to DNA damage by sugar-rich energy drinks. Also**,** Mognato et al. [37] stated that chromatin compaction was reorganized under stress and might represent a protective response that protects DNA from further damage. Demirel et al. [38] reported swollen mitochondria with distortion of their cristae in response to ED consumption. ROS resulted from EDs open the mitochondrial membrane permeability pore (mPTP), leading to excessive swelling that could break the outer mitochondrial membrane [39].

Cessation of RB administration for an additional 8 weeks in the withdrawal group of the present study revealed ameliorated glandular structure in both glands. An obvious decrease in tissue congestion, fibrosis, apoptosis, and pituitary iNOS was detected. Moreover, functional amelioration was also reported by the increased hormonal levels relative to the RB group. This was consistent with the findings of Kassab and Tawfik [10] on studying the submandibular gland recovery after EDs administration. Although some ZF cells still showed deeply stained acidophilic cytoplasm and others were still vacuolated, which means partial gland recovery. This was consistent with the results of Zarobkiewicz et al. [30]. Also, El Desouky et al. [40] reported the same result in the pancreatic tissue.

Further examination of the ultrastructure of the ZF cells supported this improvement regarding the nuclei and the mitochondria. Anavi and Tirosh [28] reported that removing the source of oxidative stress could allow the mitochondria to be repaired. Minimal regression after cessation of the energy drinks was recorded in the renal cortex [41] while reversible effects were reported in the liver [42], and in the submandibular gland [10] in previous studies.

CONCLUSION

In conclusion, chronic consumption of Red Bull, even at low doses of caffeine and moderate doses of taurine, induced noticeable histological damage in both the pituitary and adrenal glands, accompanied by suppressed levels of ACTH and corticosterone. These findings were associated with increased oxidative stress and apoptotic activity, as evidenced by elevated iNOS expression and Caspase 3 immunoreactivity. However, following a two-month withdrawal period, partial recovery was observed at both the structural and functional levels.

These findings highlight the potential reversibility of Red Bull-induced endocrine disruption upon cessation and underscore the need for caution in the long-term use of such stimulants. Further studies are recommended to compare the effects of acute versus chronic exposure and to investigate dose-dependent responses, as well as to study the different

mechanisms mediating tissue damage due to Red Bull administration. We also recommend further research to explore the full scope of energy drinks effects on the neuroendocrine system.

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Data Availability: All raw data is available from corresponding auther upon request

Author contributions

Nabila M. A., Shaimaa A. A.& Samar A. M. created the idea of the research, and analysed the histological and biochemical results. Mona S. F. performed the practical part of the histological, immunohistochemical, and biochemical examination. All authors shared in manuscript writing, reference editing, revised and approved the final manuscript for publication.

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