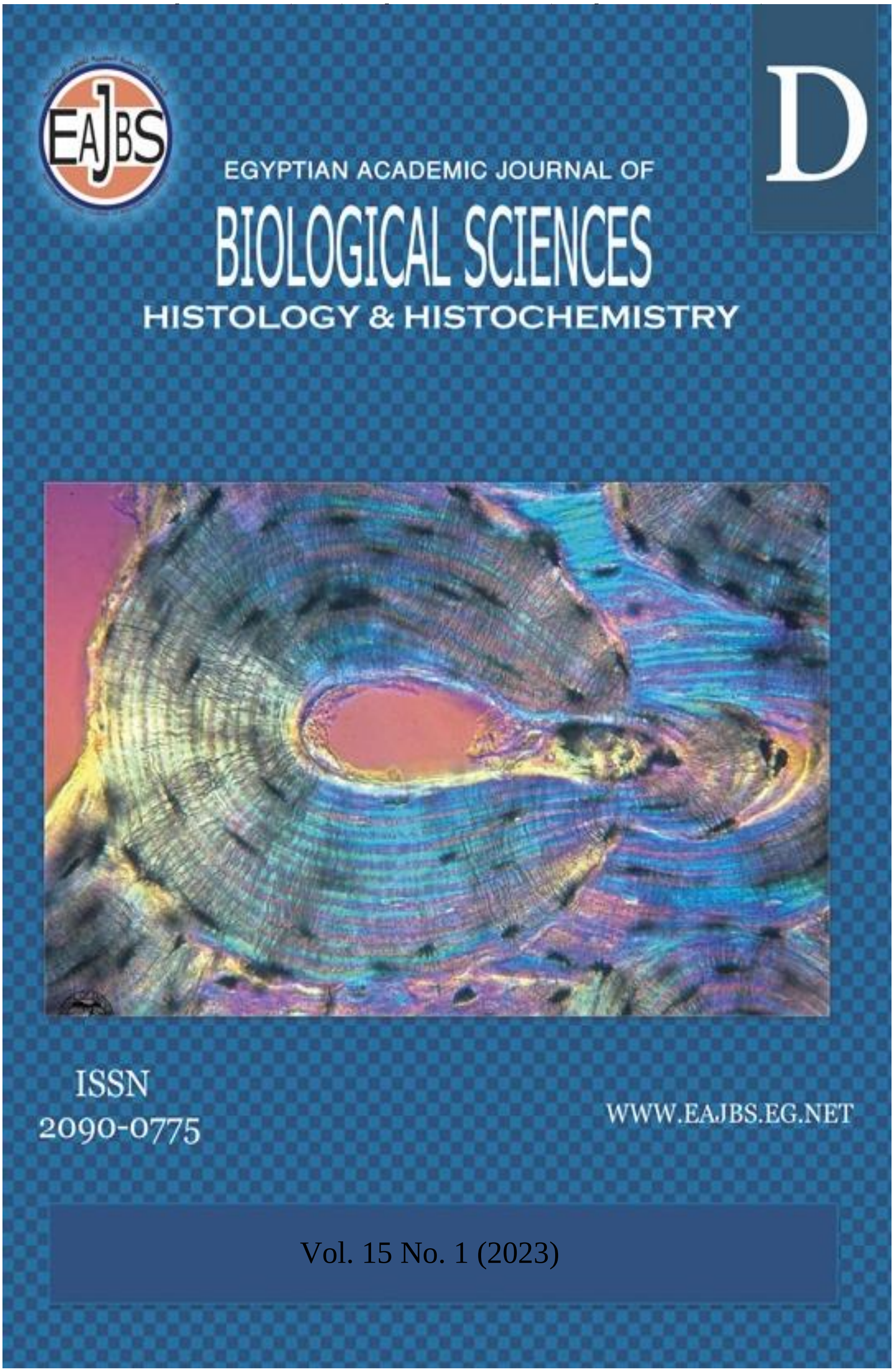

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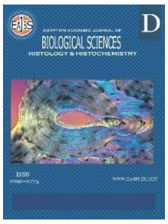

Effect of Roundup on the Suprarenal Cortex in the Adult Male Albino Rat and the Possible Ameliorative Role of Gallic Acid. Histological and Immunohistochemical study

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ABSTRACT

Background: Roundup is one of the economically effective herbicides used in agriculture. It possesses many health hazards to many organs including the adrenal gland. Gallic acid exhibited a high antioxidant potential among phenolic compounds.**Aim of the work:** To evaluate the histological changes of the suprarenal cortex following exposure to Roundup and the possible ameliorative role of gallic acid in these changes. **Material and methods:** Twenty-four adult male rats (weighing 250–300 g) were used and equally divided into four groups: **Group I** (control group) received nothing, **Group II** (gallic acid-treated group) received 20 mg/kg body weight gallic acid daily by gastric gavage, **Group III** (Roundup-treated group) received 0.25 ml/kg body weight Roundup daily by gastric gavage and **Group IV** (Roundup and gallic acid-treated group) received Roundup and gallic acid at the same previous doses. Treatments were administered once daily for 14 days. The adrenal glands were dissected and processed for light and electron microscopic studies. **Results:** The cells of the suprarenal cortex in the Roundup-treated group were disorganized. Many cells showed darkly stained nuclei and highly vacuolated cytoplasm. The thickness of the adrenal capsule showed a significant increase in addition to significantly increased immunoexpression of caspase-3. Ultrastructurally abundant lipid droplets with degenerated mitochondria within the cells were observed. Coadministration of gallic acid led to the amelioration of these changes. **Conclusion:** Roundup has destructive structural effects on the adrenal cortex and should be used cautiously. The concomitant gallic acid treatment ameliorated the Roundup-induced alterations.

INTRODUCTION

The suprarenal cortex consists of three distinctive zones, each having an imperative function. The zona glomerulosa secretes aldosterone, which modulates body electrolyte balance, the zona fasciculata, secretes glucocorticoids which modulate immune and metabolic functions and the zona reticularis that produce androgens (Merlo, *et al.*, 2016)

The herbicide, Roundup consists of glyphosate, as an active ingredient, and the surfactant, polyoxyethyleneamine, (Olah, *et al.*,2022). Owing to its high-water solubility, it is considered a broad-spectrum herbicide that is broadly used. It controls weeds, emerged grasses and cultures for example soy, corn and rice (Williams *et al.*,2000).

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The quantity of glyphosate in the formulation of Roundup differs corresponding to the country in which it is promoted. In Egypt, Roundup is available as 55.9 % polyoxyethyleneamine (the surfactant) and 44.1% isopropylamine salt of glyphosate (El-Shenawy, 2009)

The mechanism of action of glyphosate or Roundup depends on the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase enzyme in plants. This enzyme is crucial for essential amino acids synthesis (Yalçin & Çavuşoğlu, 2022) and accordingly, it was considered nontoxic to animals. However, it can accumulate in soil, plants, water and food. Several toxic effects were suggested by many researchers such as carcinogenicity (Thongprakaisang *et al.,* 2013), and teratogenicity (Dallegrave, *et al.,* 2007). One of the important side effects of Roundup exposure is endocrine disruption (Oláh, *et al.,* 2022). Endocrine-disrupting chemicals are a broad group of substances that impede hormone biosynthesis and impact the endocrine system (Pandey & Rudraiah, 2015)

Gallic acid (3, 4, 5-trihydroxy benzoic acid) is considered a natural antioxidant that contains polyphenolic compounds. It can be found in some fruits like mango and banana and in herbs such as green tea, sumac and oak bark (Dehghani *et al.,* 2020). It has drawn the attention of scientists as it has anti-inflammatory (Bensaad et al., 2017), antioxidant (Ojeaburu & Oriakhi, 2021), antimutagenic (Kaur *et al.,* 2015) and anticarcinogenic properties (Subramanian *et al.,* 2014)

Detailed morphological changes in the suprarenal cortex triggered by Roundup were not yet fully established. Thus this work was conducted to investigate the histopathological changes in the suprarenal cortex after Roundup exposure and whether the gallic acid has a possible ameliorative effect against these changes in adult male albino rats.

MATERIALS AND METHODS

Chemicals:

1-Roundup (glyphosate 44.1 WSC Monsanto Co.) was gained from the Central Agricultural Pesticide Laboratory in Giza, Egypt.

2-Gallic acid was obtained from El Gomhorya company in Assiut, Egypt. It was offered in the form of white powder.

Animals:

In this study, twenty-four adult male albino rats (weighing 250–300 g) gained from the Animal House of the Faculty of Medicine, Assiut University, Assiut, Egypt, were used. The rats were housed in cages and kept at room temperature with a normal 12h light/12h dark cycle. Animals were allowed free access to feeding with standard commercial pellets and tap water ad libitum. All the experimental animal procedures were approved by the Institutional Ethics Committee of the Faculty of Medicine, Assiut University, Assiut, Egypt. Egypt (IRB 17300847).

Experimental Protocol:

The rats were assigned randomly into four groups, each comprising six animals. Each group received drugs as follows:

Group I (Control group): received nothing.

Group II (Gallic acid-treated group): received gallic acid at a dose of 20 mg/kg body weight daily. It was dissolved in distilled water and was received orally through gastric gavage (Ola-Davies & Olukole, 2018).

Group III (Roundup- treated group): received Roundup at a dose of 0.25 ml/kg body weight daily. Concentrations are calculated according to the final active ingredient glyphosate concentration in Roundup which is (44.1%). The dose was calculated as each rat received 100 mg/kg body weight of glyphosate. Dilution of Roundup was done with distilled water and it was orally received through gastric gavage (Saleh *et al.,* 2018). This dose was well below LD50 of Roundup which is 4900 mg/kg body weight (Pandey & Rudraiah, 2015)

Group IV (Roundup and gallic acid - treated group): received Roundup and gallic acid at the same doses as the previous groups.

The treatments were once daily administered for 14 days.

At the end of the experiment, the rats were anaesthetized by ether. The adrenal glands were dissected in all groups.

I- Histological Study:

A.Light Microscopic Study:

The right adrenal glands were fixed in 10% neutral buffered formalin. Then, the specimens were processed for the preparation of paraffin blocks. Sections 5 µm thick were obtained. Next, slides were stained with Hematoxylin and Eosin stain to study the structure of the three zones of the suprarenal cortex. As well as with Masson's trichrome stain to verify the distribution of collagen fibers in the suprarenal cortex (Bancroft & Layton, 2012).

B-Immunohistochemical Study:

Sections of 5 µm thickness were cut on coated slides. The slides were deparaffinized and rehydrated. Caspase-3 expression was revealed using the technique of labeled streptavidin-biotin immunoperoxidase. The anti-caspase-3 antibody (Cell signaling Technology, Ipswich, MA) was used in a dilution of 1:200. The reaction was visualized by the use of Ultravision Detection System, Anti polyvalent, HR/DAB, Thermo Fisher Scientific). The slides were counter-stained by Mayer's hematoxylin, then dehydrated, covered and examined (Sanii *et al.,* 2012)

C-Electron Microscopic Study:

The left adrenal gland was cut into pieces of (1 × 1 mm). They were fixed for 24 hours in 4% buffered glutaraldehyde. The specimens were postfixed in 1% osmium tetroxide and then buffered for 2 hours in phosphate, followed by dehydration by alcohol. Embedding was done in an epoxy resin mixture. Then semithin sections of 1µm thickness were prepared and stained with 1% toluidine blue. Slides were examined with a light microscope and photographed. Ultrathin sections (80–90 nm) were obtained using an LKB ultratome and placed on copper grids. They were stained by uranyl acetate and lead citrate (Woods & Stirling, 2019). The examination was performed using a transmission electron microscope (Jeol-JEM- 100 CXII; Jeol, Tokyo, Japan) in the Electron Microscopic Unit, at Assiut University, Assiut, Egypt.

Morphometric Study:

The Following Parameters Were Evaluated:

a-The thickness of the adrenal capsule was measured in six randomly chosen fields in six sections obtained from each rat in each experimental group using Masson's trichrome stained slides. Measurements were obtained through Olympus cellSense Standard 1.16 software on a computer connected to an Olympus microscope (CX41 Japan) with a digital camera (Olympus DP27) in the Anatomy department, Faculty of Medicine, Assiut University. Pixels were calibrated for actual measurements in a micrometer. The magnification used was X400 (Bekheet & Shokry, 2020).

b-Area percentage for the caspase-3 immunoreactivity was assessed using immunostained slides. The sections were photographed by an Olympus digital camera installed on a microscope, using a 20x objective lens. The images were analyzed by the software “ImageJ” (version 1.53t National Institute of Health, USA). First, the image analyzer was calibrated to convert the measurement units into micrometer units. Each field was enclosed inside the standard frame for measurement, and the areas of the positive reaction of caspase-3 immunoexpression were measured by masking it with a blue binary color. The area percentage was measured in six fields from six serial sections from each animal in each group (Khalaf *et al.,* 2017).

Statistical Analysis:

Data obtained was analyzed using SPSS 20.0 (SPSS Inc., USA). ANOVA test (analysis of variance) was applied for the comparison between all the experimental groups followed by

Tukey's post-hoc analysis**.** Data were represented in the form of mean ± standard error of the mean. The P value <0.05 was considered to be of statistical significance.

RESULTS

I-Histological Results:

Histological examination of the sections of group I (control group) and group II (Gallic acid-treated group) showed the same normal structure. Figures of the control group were instructive for both groups.

A-Light Microscopic Examination Results:

1- Hematoxylin and Eosin Staining:

Examination of the control group showed normal architecture with distinctly visible three regions of the suprarenal cortex; zona glomerulosa, zona fasciculata and zona reticularis. A thin capsule formed of regularly arranged fibers was observed (Fig. 1a). The zona glomerulosa was seen to consist of arched or ovoid clusters of columnar to pyramidal cells with rounded vesicular nuclei and pale cytoplasm. The zona fasciculata was formed of parallel cords of polyhedral cells with rounded vesicular nuclei that had prominent nucleoli and pale vacuolated cytoplasm (Fig. 1b). The Zona reticularis cells were small, polyhedral, closely packed, deeply stained and arranged in a network of interlacing cords between which blood sinusoids were observed (Fig.1c).

Examination of the Roundup-treated group showed a disturbed arrangement of the three zones (Fig. 2a). The zona glomerulosa cells appeared irregular in arrangement, and most of the cells showed darkly stained nuclei and markedly vacuolated cytoplasm. Some cells were ballooned. The presence of an irregular thickened covering capsule that exhibited lamellar separation of its fibers was observed (Fig. 2b). The cells of zona fasciculata appeared losing their regular arrangement with highly vacuolated cytoplasm and the nuclei were deeply stained (Fig.2c). The cells of zona reticularis appeared in the form of interlacing cords of cells, most of which appeared with vacuolated cytoplasm (Fig. 2d).

In the Roundup and gallic acid treated group the suprarenal cortex was surrounded by a capsule with a nearly normal thickness and regularly arranged fibers. It had an apparent normal arrangement of the three zones (Fig. 3a). In the zona glomerulosa the cells appeared arranged in clusters, and most of the cells appeared with rounded vesicular nuclei and vacuolated cytoplasm with few cells appeared highly vacuolated. The zona fasciculata cells exhibited pale cytoplasm and rounded vesicular nucleus and were arranged in columns. (Fig, 3b). The cells in the zona reticularis were in interlacing cords with blood sinusoids in between. Few cells appeared with vacuolated cytoplasm and darkly stained nuclei (Fig. 3c)

2-Masson's Trichrome Staining:

In the suprarenal cortex of the control group, the surrounding capsule appeared with regularly arranged fibers and fine collagen trabeculae among cells of the zona glomerulosa and zona fasciculata (Fig. 4a). A markedly thickened capsule was observed in the Roundup-treated group with irregularly arranged collagen fibers and extending thickened trabeculae between the zona glomerulosa cells (Fig. 4b). A nearly regular capsule with fine collagen fibers was seen extending as trabeculae between the zona glomerulosa cells in the suprarenal cortex of the Roundup and gallic acid treated group (Fig. 4c).

3-Toluidine Blue Staining:

Examination of semithin sections in the suprarenal gland of the control group showed that the surrounding capsule exhibited regularly arranged fibers and fibroblasts. The cells in the zona glomerulosa were arranged in clusters separated by blood sinusoids. The cells had well-defined cell boundaries, rounded vesicular nuclei with prominent nucleoli in addition to numerous lipid droplets (Fig. 5a). The zona fasciculata showed normal polyhedral cells arranged in cords separated by blood sinusoids with well-defined cell boundaries. The cells had rounded nuclei with prominent nucleoli and the cytoplasm appeared to contain lipid droplets (Fig. 5b). The zona reticularis cells were organized in the form of anastomosing cords between which blood sinusoids were observed. the cells showed small distinct lipid droplets (Fig. 5c).

In the Roundup-treated group, examination of the semithin sections showed an apparent increase in the thickness of the covering capsule with irregularly arranged fibers and fibroblasts. The cells of zona glomerulosa were disorganized with markedly accumulated cytoplasmic lipid droplets and most of them exhibit irregular darkly stained nuclei and ill-defined cell boundaries (Fig. 6a). The zona fasciculata cells lost their regular arrangement in columns and showed deeply stained irregular nuclei with the cytoplasm packed with lipid droplets (Fig. 6b). In the zona reticularis the cells appeared in the form of interlacing cords separated by large, congested sinusoids and showed an apparent increase in lipid droplets (Fig. 6c).

In the Roundup and gallic acid treated group, the suprarenal cortex showed a relatively thin capsule with regularly arranged fibers and the cells in the zona glomerulosa (Fig. 7a), the zona fasciculata (Fig. 7b) and the zona reticularis (Fig. 7c) appeared similar to those in the suprarenal cortex of the control rats.

B-Immunohistochemical Results:

The immunohistochemical study revealed that the cells of the three zones of the control group showed a negative to mild immunoexpression of caspase-3 (Figs. 8a and 8b). In the Roundup-treated rats, overexpression of caspase-3 was noted both in the zona glomerulosa, along sinusoids and cells in the zona fasciculata and in the cells of the zona reticularis (Figs. 8c and 8d). The cytoplasm of the suprarenal cortex cells showed a moderate expression of caspase-3 in the Roundup and gallic acid-treated group (Figs. 8e and 8f).

C-Electron Microscopic Examination Results:

Ultrastructurally examination of the suprarenal cortical cells in the control group showed that the cells of the zona glomerulosa showed a rounded euchromatic nucleus, numerous mitochondria with lamellar or leaf-like cristae, smooth endoplasmic reticulum and lipid droplets (Fig. 9a). The cells of the zona fasciculata appeared with a rounded euchromatic nucleus, mitochondria with their cristae were of vesicular type in addition to the abundant smooth endoplasmic reticulum and lipid droplets (Fig.9b). The cells in the zona reticularis showed a rounded nucleus with a regular nuclear envelope and finely dispersed chromatin, abundant mitochondria with tubulovesicular cristae, and few lipid droplets (Fig. 9c).

In the Roundup-treated group, the cell of the zona glomerulosa exhibited an irregular outline. The nucleus had an irregular nuclear membrane with condensed chromatin was observed. Accumulated lipid droplets of various sizes, some of which were fused, occupied most of the cytoplasm and displaced the nucleus eccentrically and indented it. Mitochondria showed disrupted cristae were noted (Fig. 10a). The cells in zona fasciculata exhibited irregular nuclear membrane with condensed chromatin and dilatation of perinuclear cisterna. There were abundant lipid droplets, degenerated mitochondria with disrupted cristae in addition to numerous lysosomes were observed. A disrupted and dilated smooth endoplasmic reticulum was detected (Fig. 10b). Some cells showed shrunken irregular nuclei, large cytoplasmic vacuolations and abundant lipid droplets with a flocculent matrix. Mitochondria showed swelling with a condensed core. Deposition of lipids in the blood sinusoids between the zona fasciculata cells was observed (Fig.10c). In the zona reticularis, the cells showed an irregular condensed nucleus. The cytoplasm exhibited that the lipid droplets were more numerous than in the control group in which central extracted cores and a dense peripheral halo were noticed. Large lysosomes were observed in addition to cytoplasmic vacuolations (Fig. 10d).

Examination of the Roundup and gallic acid treated group revealed a nearly normal structure of the zona glomerulosa cells with a rounded regular nucleus, few lipid droplets, mitochondria, and smooth endoplasmic reticulum (Fig. 11a). The zona fasciculata cells appeared with an euchromatic nucleus and prominent nucleolus, smooth endoplasmic reticulum, some large lipid droplets, and mitochondria with densely packed vesicular cristae (Fig. 11b). The cells of the zona reticularis appeared with rounded nuclei, normal mitochondria, lipid droplets and lysosomes (Fig. 11c).

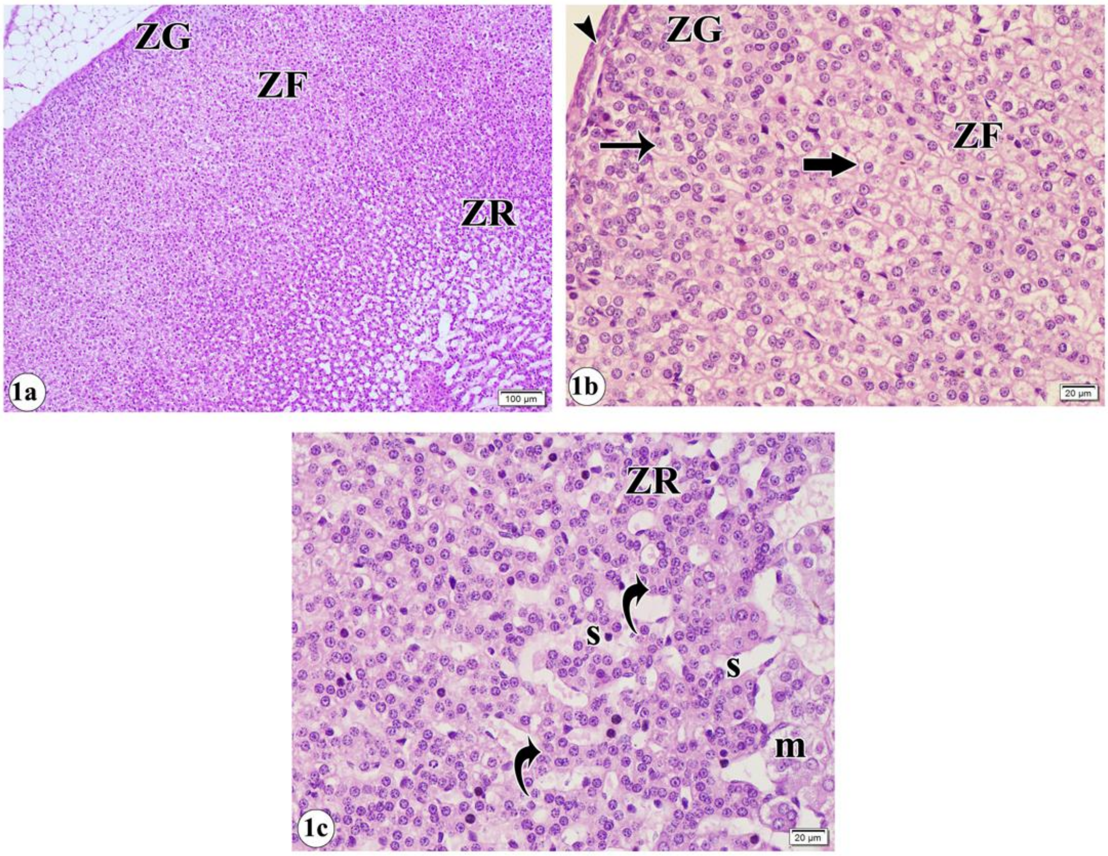


Fig. 1: Photomicrographs of the suprarenal cortex of the control group showing: **Fig. 1a:** Normal architecture of the three zones, The zona glomerulosa (ZG), the zona fasciculata (ZF) and the zona reticularis (ZR). (H&E X100). **Fig. 1b:** A thin connective tissue capsule (arrowhead) is observed. The zona glomerulosa (ZG) appeared as arched or ovoid clusters of columnar to pyramidal cells with pale cytoplasm and rounded vesicular nuclei (thin arrow). The zona fasciculata cells are arranged in parallel cords of polyhedral cells (ZF) with rounded vesicular nuclei having prominent nucleoli and pale vacuolated cytoplasm (thick arrow). (H&E X400) **Fig. 1c:** The zona reticularis showing interlacing cords of small polyhedral deeply stained cells (curved arrow) separated by blood sinusoids (s). Note the adrenal medulla (m) (H&E X400).

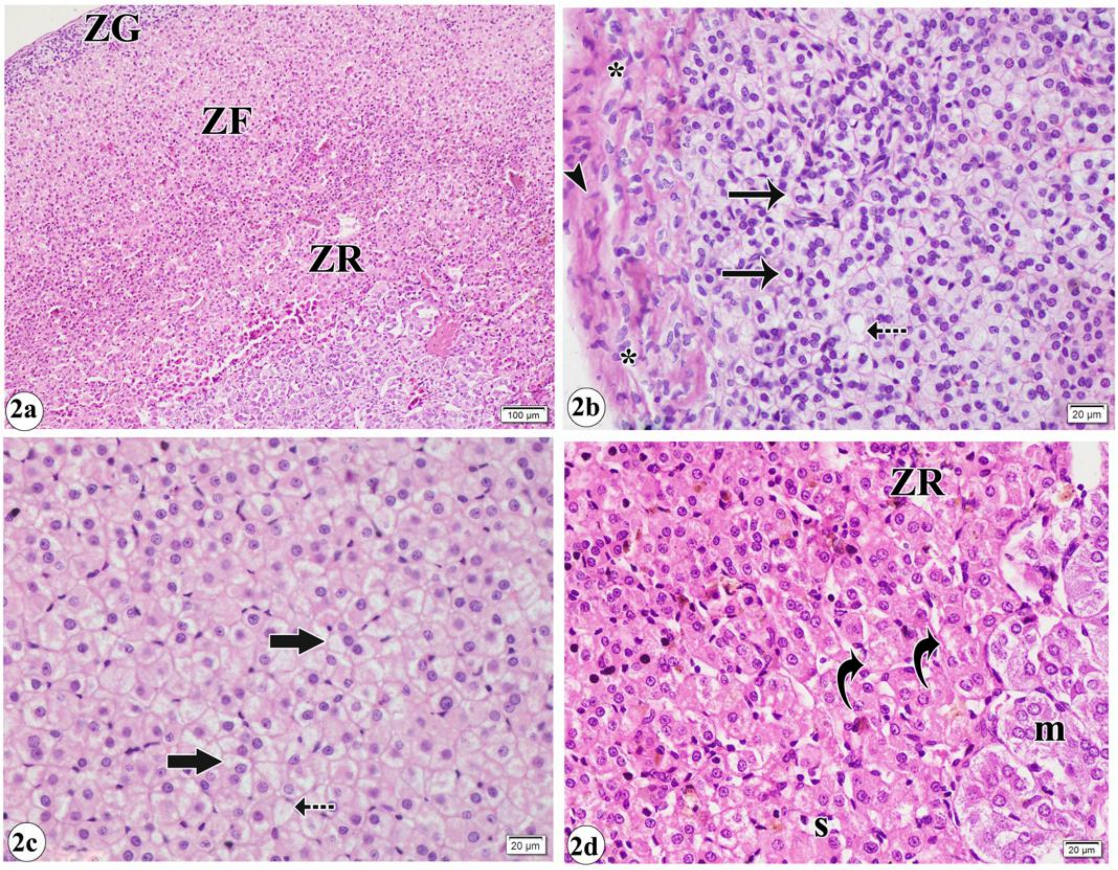


Fig. 2: Photomicrographs of the suprarenal cortex of the Roundup-treated group showing: **Fig. 2a:** Disturbed normal architecture of the three zones; zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) is noticed (H&E X100).**Fig. 2b**: Thick surrounding capsule with irregularly arranged fibers (arrowhead) is observed with lamellar separation (*). The zona glomerulosa appeared with irregularly arranged cells with dense nuclei and markedly vacuolated cytoplasm (thin arrow) some cells appear ballooned (dotted arrow). (H&E X400) **Fig. 2c:** The zona fasciculata cells are irregularly arranged and showed a highly vacuolated cytoplasm and deeply stained nuclei (thick arrow). Some cells appear ballooned (dotted arrow). (H&E X400) **Fig. 2d:** In the zona reticulata the cells are arranged in interlacing cords (ZR) and most of them appear with a vacuolated cytoplasm (curved arrow). The adrenal medulla is observed (m). (H&E X400).

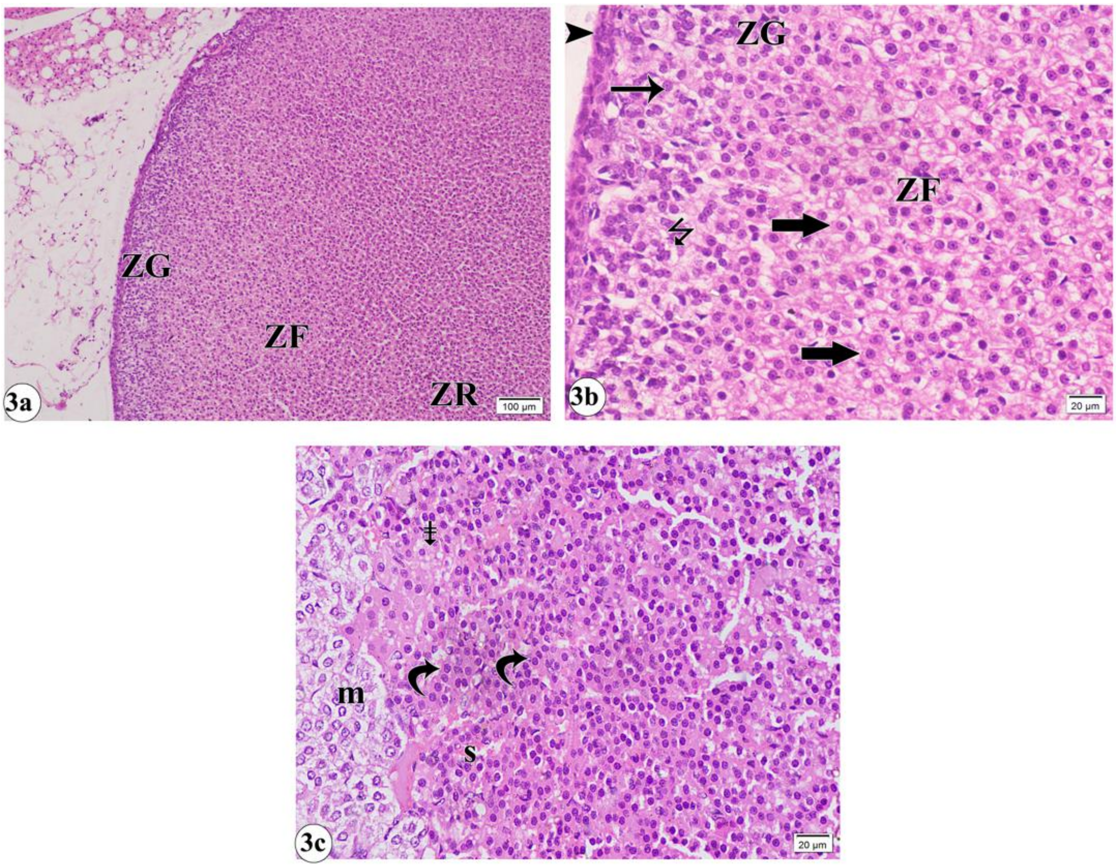


Fig. 3: Photomicrographs of the suprarenal cortex of the Roundup and gallic acid treated group showing: **Fig. 3a**: Apparent normal architecture of the three cortical zones; zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). (H&E X100). **Fig. 3b**: The zona glomerulosa appeared as clusters of cells with pale cytoplasm and vesicular nuclei (thin arrow). Few cells appeared highly vacuolated (zigzag arrow). The zona fasciculata cells are seen arranged in parallel cords of cells (ZF) with vesicular nuclei and pale cytoplasm (thick arrow). Note a nearly thin connective tissue capsule with regularly arranged fibers (arrowhead). (H&E X400) **Fig. 3c:** The zona reticularis cells are arranged in interlacing cords (curved arrow) separated by blood sinusoids some of which are congested(s) with some cells appearing with darkly stained nuclei and vacuolated cytoplasm (arrow with double stroke) (H&E X400).

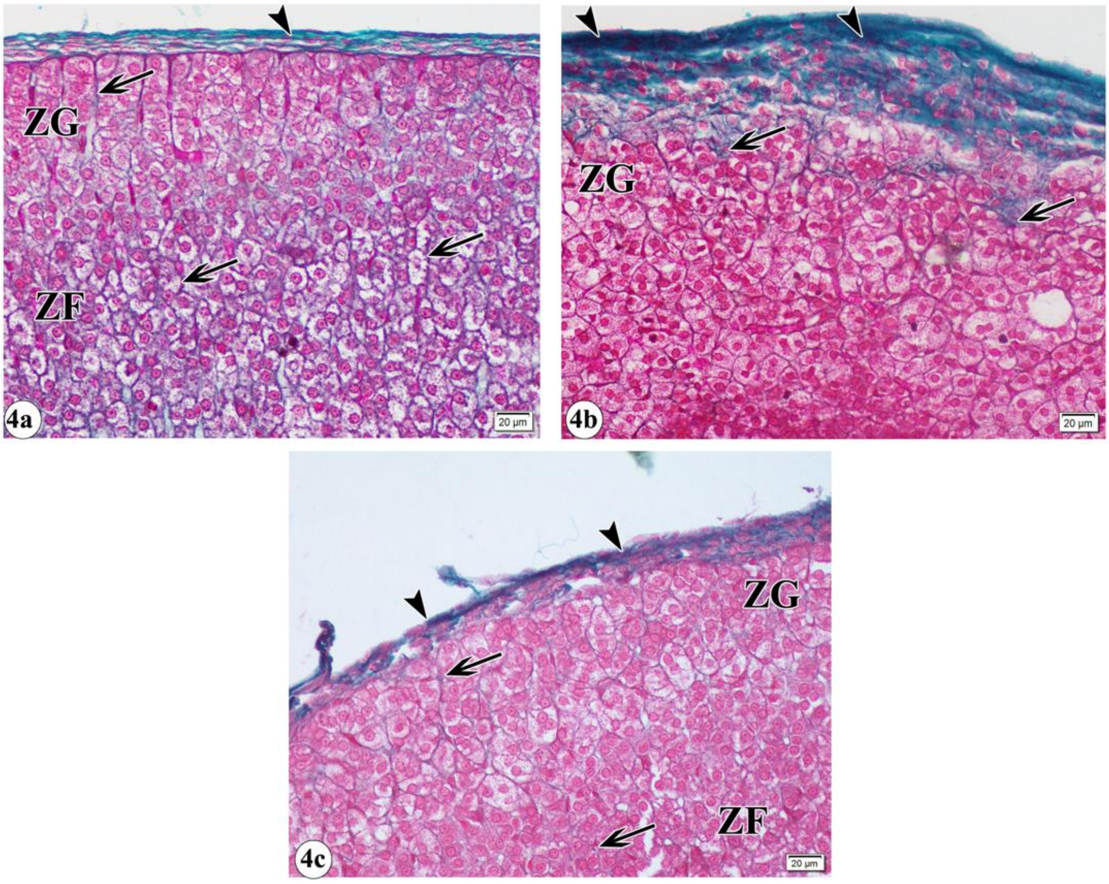


Fig. 4: Photomicrographs of the suprarenal cortex of the control group (a), Roundup-treated group (b) and Roundup and gallic acid-treated group(c), showing: **Fig. 4a:** The surrounding capsule (arrowhead) that is formed of regular collagen fibers (arrowhead) with extending thin trabeculae (arrow) between clusters of cells in the zona glomerulosa (ZG) and columns of cells in zona fasciculata (ZF) cell. (Masson's trichrome X400) **Fig. 4b**: A thickened capsule (arrowhead) that is formed of irregular collagen fibers (arrowhead) appears with extending thickened trabeculae between zona glomerulosa cells (ZG) (arrow). (Masson's trichrome X400) **Fig. 4c:** An apparently thin capsule that is formed of regularly arranged collagen fibers (arrowhead) with extending fine fibers between the zona glomerulosa cells ZG cells and columns of cells in the zona fasciculata (ZF) (arrow). (Masson's trichrome X400).

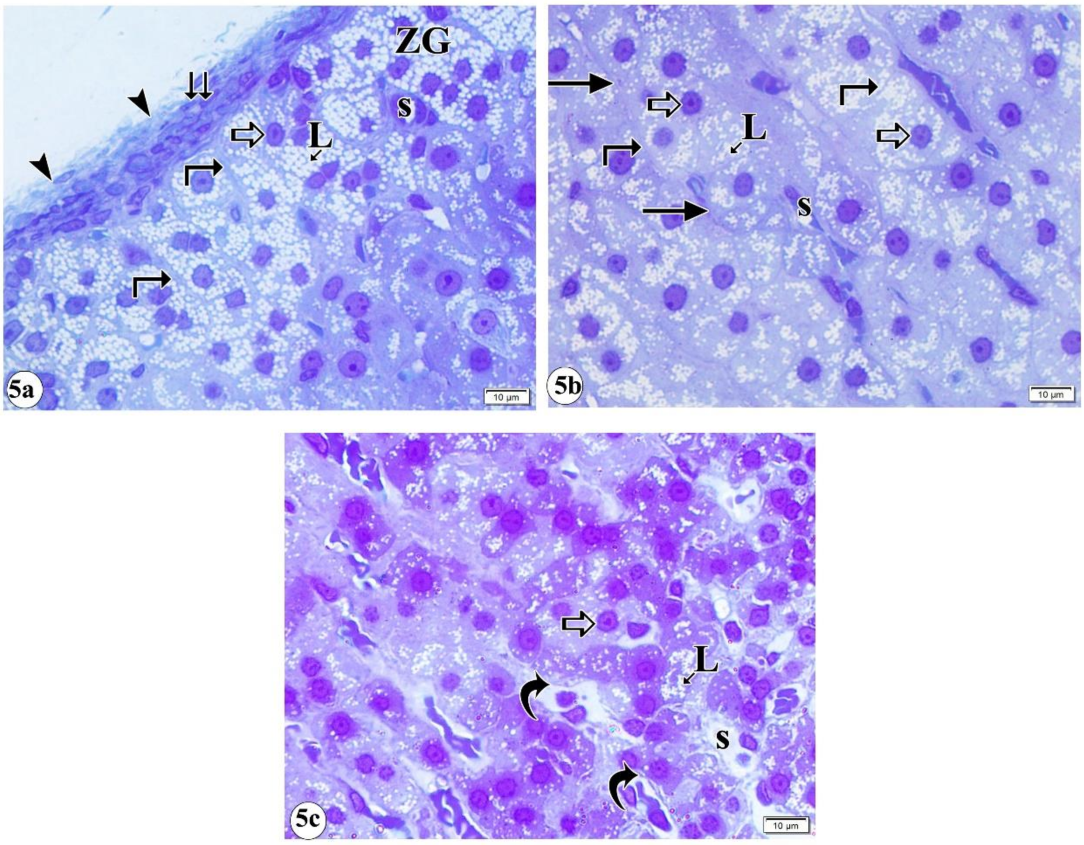


Fig. 5: Photomicrographs of semithin sections of the suprarenal cortex of the control group showing: **Fig. 5a:** The surrounding capsule consisting of regularly arranged fibers (arrowhead) and multiple fibroblasts (double arrow). The zona glomerulosa cells (ZG) appeared in clusters with rounded vesicular nuclei (thick arrow) and lipid droplets (L) and well-defined cell boundaries (kinked arrow) separated by blood sinusoids (s). (Toluidine blue X1000) **Fig. 5b**: The zona fasciculata cells are polyhedral and arranged in cords (thin arrows) with well-defined cell boundaries (kinked arrow), lipid droplets (L) and rounded vesicular nuclei with prominent nucleoli (thick arrow). Blood sinusoids between the cords of cells (s) are observed. (Toluidine blue X1000) **Fig. 5c:** The zona reticularis cells are arranged in irregular anastomosing cords of cells (curved arrow) with blood sinusoids (s). The cells have rounded nuclei with prominent nucleoli (thick arrow) and small distinct lipid droplets (L) (Toluidine blue X1000).

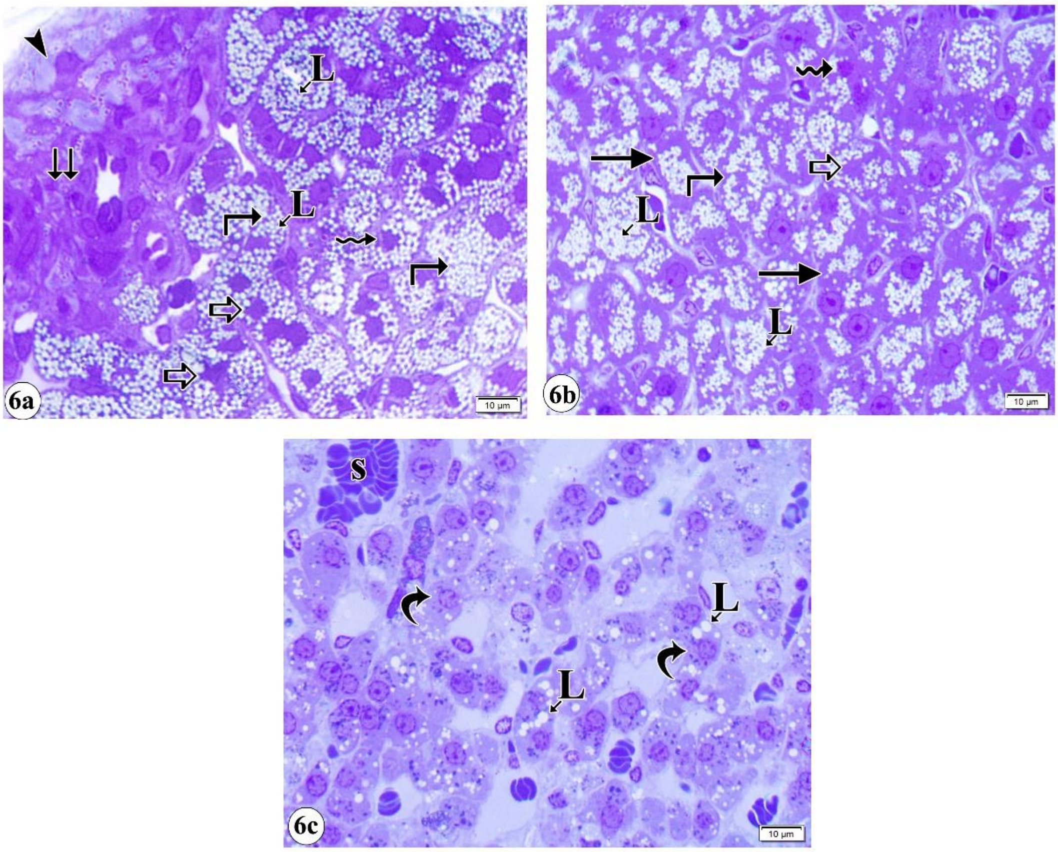


Fig. 6: Photomicrographs of semithin sections of the suprarenal cortex of the Roundup-treated group showing: **Fig. 6a:** Thickened capsule with irregularly arranged fibers (arrowhead) and fibroblasts (double arrow). Disorganized zona glomerulosa cells that appear with irregular darkly stained nuclei (thick arrow), and ill-defined boundaries (kinked arrow) with marked accumulation of lipid droplets (L). Some nuclei are indented with lipid droplets (wavy arrow). (Toluidine blue X1000) **Fig. 6b**: The cells in the zona fasciculata are irregularly arranged (thin arrow). The cells appeared packed with lipid droplets (L). Some nuclei are indented with lipid droplets (wavy arrow), and some are darkly stained (thick arrow). Some cells have ill-defined boundaries (kinked arrow). (Toluidine blue X1000) **Fig. 6c:** The zona reticularis cells are arranged in irregular anastomosing cords of cells (curved arrow) separated by large, congested blood sinusoids (s). Some cells show an apparent increase in lipid droplets(L). (Toluidine blue X1000).

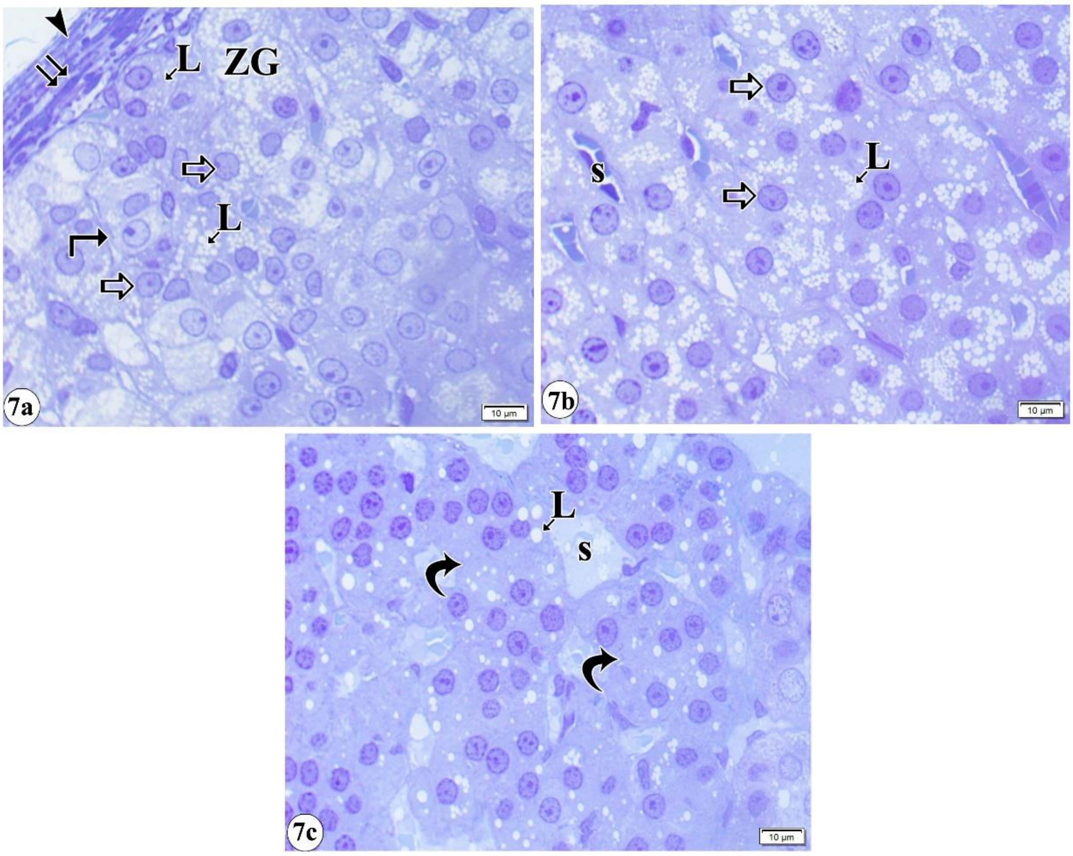


Fig. 7: Photomicrographs of semithin sections of the suprarenal cortex of the Roundup and gallic acid treated group showing: **Fig.7a:** The surrounding capsule shows regularly arranged fibers (arrowhead) and fibroblasts (double arrow). The cells in the zona glomerulosa (ZG) appear in clusters with rounded vesicular nuclei with prominent nucleoli (thick arrow), lipid droplets (L) and well-defined borders (kinked arrow). (Toluidine blue X1000) **Fig. 7b:** The zona fasciculata cells appear similar to those in the control group in regularly arranged columns with rounded nuclei and prominent nucleoli (thick arrow), and lipid droplets (L) and separated by blood sinusoids (s). (Toluidine blue X1000) **Fig. 7c:** The zona reticularis cells are arranged in anastomosing cords (curved arrow) with blood sinusoids (s). The cells have few lipid droplets (Toluidine blue X1000).

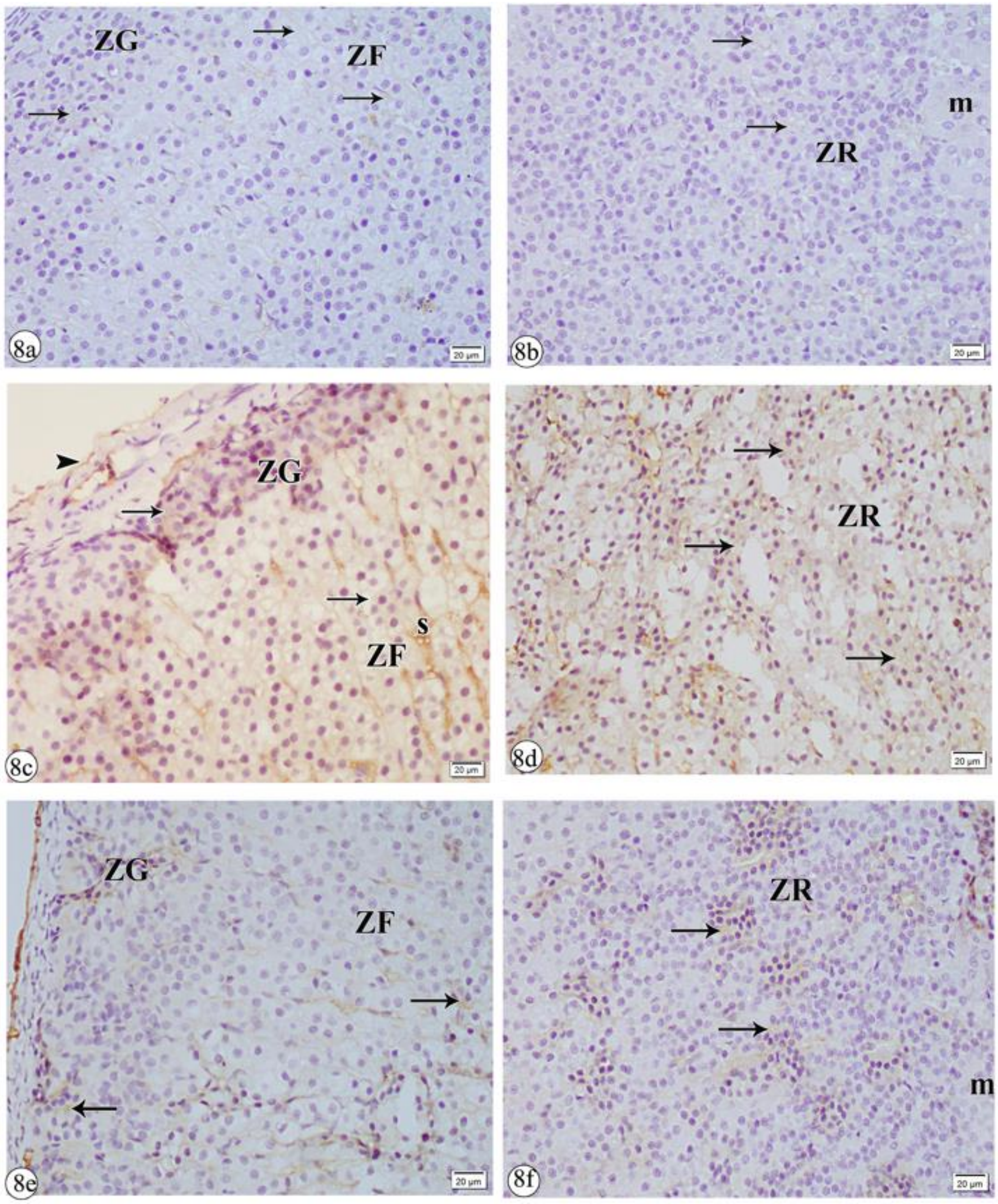


Fig. 8: Photomicrographs of the suprarenal cortex of the control group (a-b), the Roundup-treated group (c-d) and the Roundup and gallic acid-treated group (e-f). **Fig. 8a**: Showing a negative to mild immunoexpression of caspase-3 (arrow) within the cells of the zona glomerulosa (ZG) and fasciculata (ZF). (Caspase-3 immunostaining X400) **Fig. 8b:** Showing a negative to a mild immunoexpression of caspase-3 (arrow) in the cells of zona reticularis mild (ZR). The adrenal medulla (m) is noticed. (Caspase-3 immunostaining X400) **Fig. 8c:** A strong positive immunoexpression for caspase-3 in the capsule (arrowhead), within the cytoplasm in the cells (arrow) of zona glomerulosa (ZG) and zona fasciculata (ZF) and along the blood sinusoids (s). (Caspase-3 immunostaining X400) **Fig. 8d:** Showing a strong positive immunoexpression for caspase-3 within the cells (arrow) of zona reticularis (ZR). (Caspase-3 immunostaining X400) **Fig. 8e:** A moderate immunoexpression for caspase-3 within the cells (arrow) of zona glomerulosa (ZG) and zona fasciculata (ZF)(arrow) (Caspase-3 immunostaining X400) **Fig. 8f:** Moderate immunoexpression for caspase-3 within the cells (arrow) of the zona reticularis (ZR). Adrenal medulla(m) is noticed (Caspase-3 immunostaining X400).

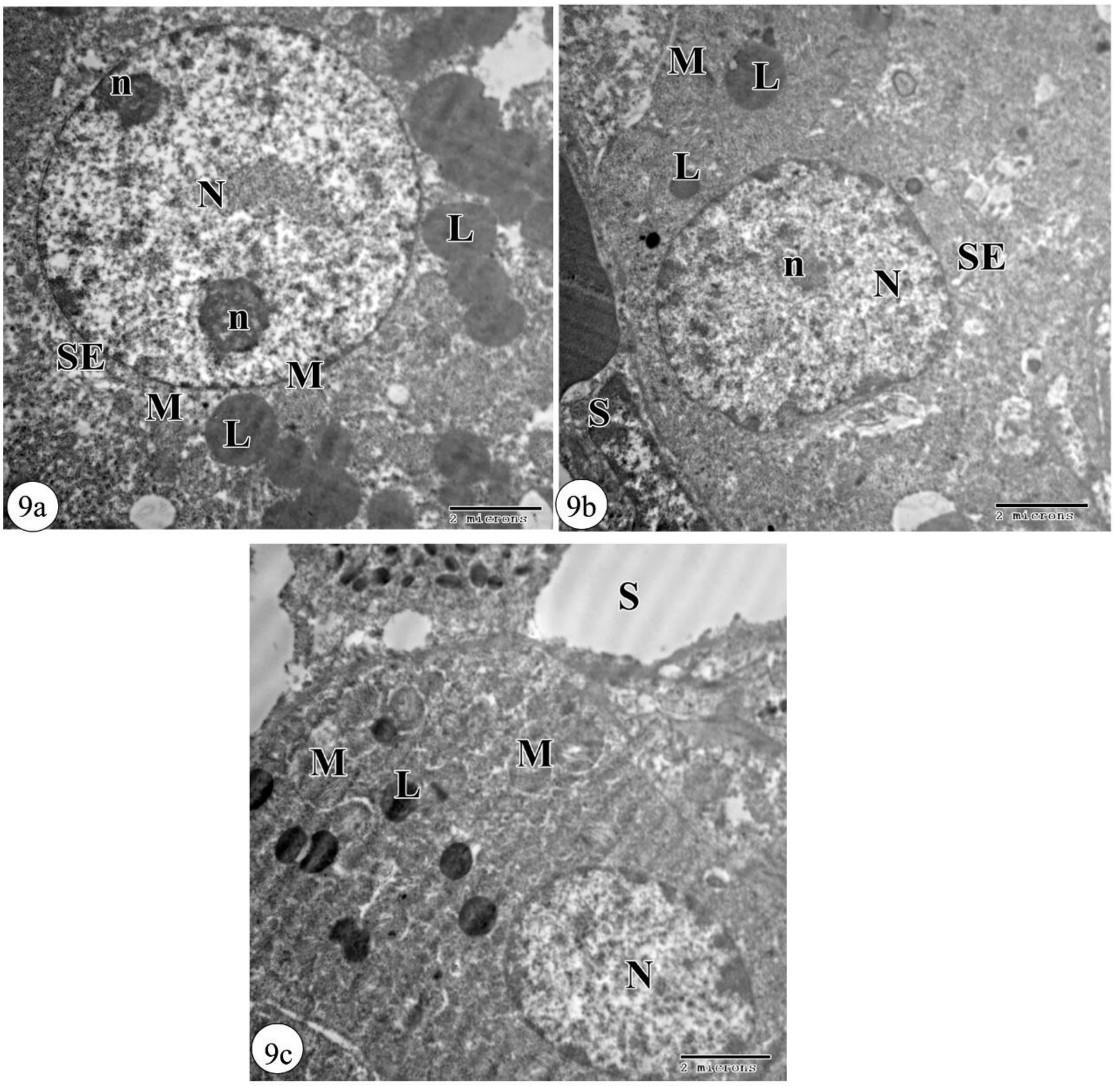


Fig. 9: Electron photomicrographs of the suprarenal cortical cells in the control group showing: **Fig. 9a:** A zona glomerulosa cell with rounded euchromatic nucleus (N) with double nucleoli (n). Mitochondria with lamellar cristae (M), smooth endoplasmic reticulum (SE) and lipid droplets (L) are observed in the cytoplasm. (TEM X7200) **Fig. 9b:** A zona fasciculata cell with a rounded euchromatic nucleus(N) having well-defined nucleolus (n), mitochondria that exhibit vesicular cristae(M), lipid droplets(L) and abundant smooth endoplasmic reticulum (SE). Notice the nearby endothelial lining of blood sinusoids(S). (TEM X7200) **Fig. 9c:** A zona reticularis cell with a rounded nucleus (N), the cytoplasm shows the presence of abundant mitochondria (M) with tubulovesicular cristae and few lipid droplets (L). Notice the nearby blood sinusoid (S) (TEM X7200).

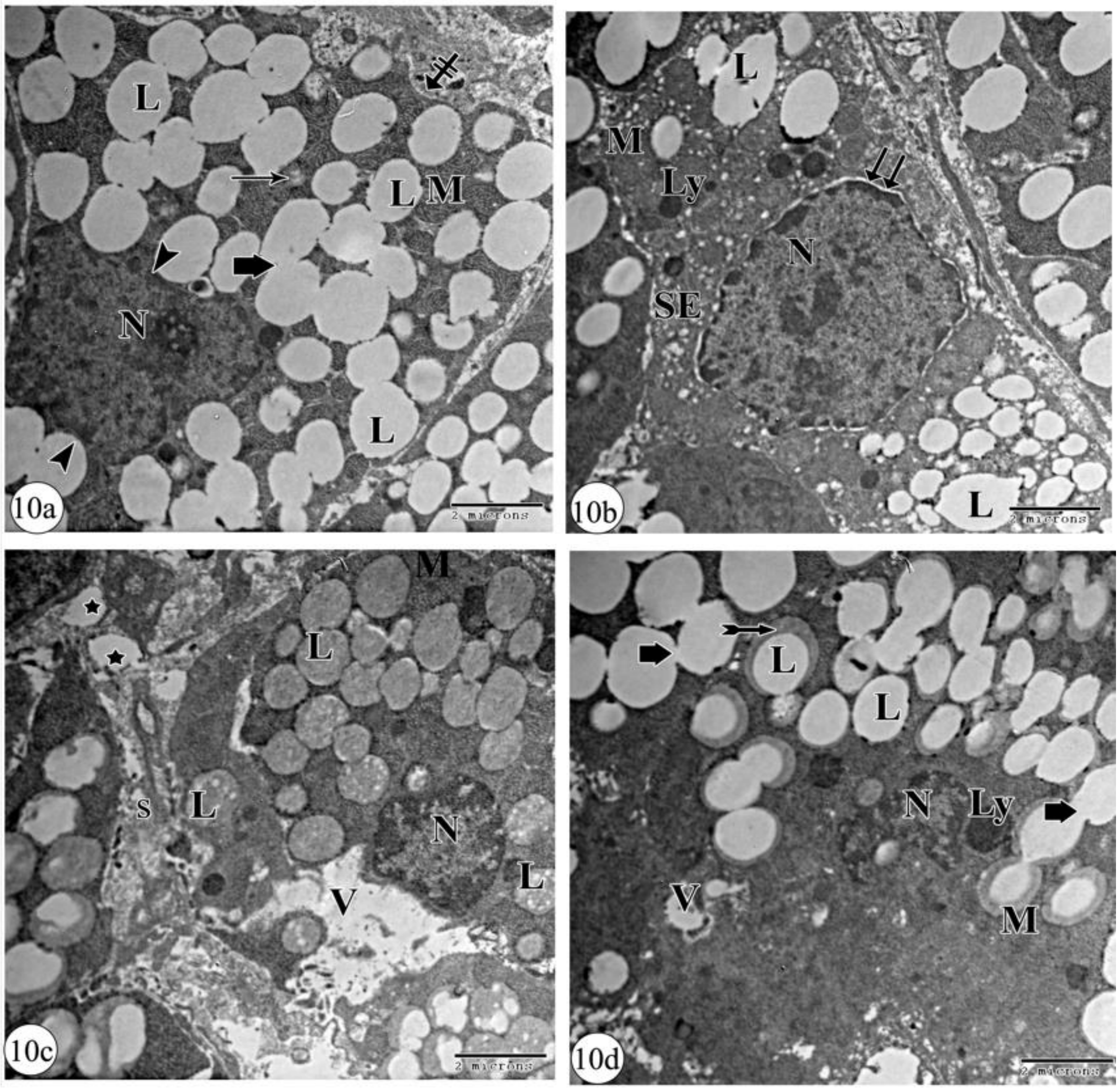


Fig. 10: Electron photomicrographs of the suprarenal cortical cells in the Roundup-treated group showing: **Fig. 10a:** Showing a zona glomerulosa cell having an indented irregular nucleus with peripherally condensed chromatin (N) and mitochondria with disrupted cristae (M) and degenerated core (thin arrow). The cytoplasm is packed with lipid droplets (L), some of which are fused (thick arrow) and some indent the nucleus (arrowhead). Notice the irregular outline of the cell (arrow with double stroke) (X7200) **Fig. 10b:** A zona fasciculata cell with the nucleus shows peripherally condensed chromatin (N) and dilated perinuclear cisterna (double arrow). Destructed mitochondria (M), abundant lipid droplets (L), distorted dilated smooth endoplasmic reticulum (SE) and numerous lysosomes (Ly) are observed. (TEM X7200) **Fig. 10c:** Another zona fasciculata cell with shrunken irregular nucleus (N), abundant lipid droplets with flocculent contents (L). Mitochondria show swelling with condensed core (M). Notice the presence of large vacuol**es** in the cytoplasm (V) and deposition of lipids (star) in the blood sinusoids (s) between the zona fasciculata cells**.** (TEM X7200) **Fig. 10d**: A zona reticularis cell shows an irregular nucleus with an irregular nuclear membrane and chromatin condensation (N), mitochondria with dense core (M), large lysosomes (Ly) and cytoplasmic vacuolations (V). Note numerous lipid droplets (L) with a central extracted core and a dense peripheral halo (tailed arrow), some of them are fused (thick arrow) (TEM X7200).

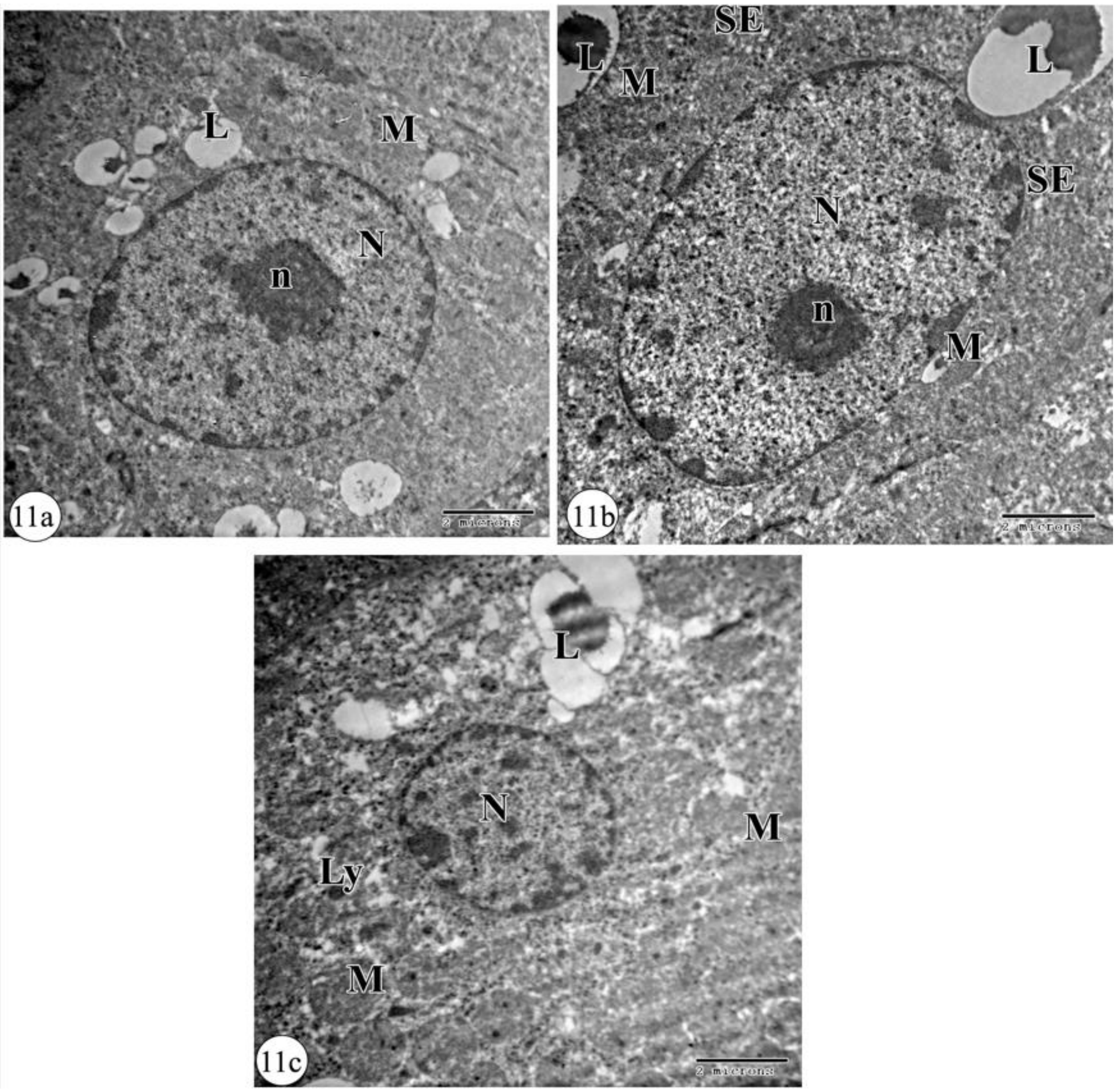


Fig. 11: Electron photomicrographs of the suprarenal cortical cells in the Roundup and gallic acid-treated group showing: **Fig. 11a:** A zona glomerulosa cell has a rounded euchromatic nucleus (N) with well-defined nucleolus(n), mitochondria (M) with lamellar cristae and some lipid droplets (L). (TEM X7200) **Fig. 11b:** A zona fasciculata cell has an euchromatic nucleus (N) with prominent nucleolus (n)and abundant smooth endoplasmic reticulum (SE). Mitochondria with vesicular cristae (M) and some large lipid droplets (L) are observed. (TEM X7200) **Fig. 11c:** A zona reticularis cell with euchromatic nucleus (N), mitochondria (M), some lipid droplets (L) and lysosomes (Ly) (TEM X7200).

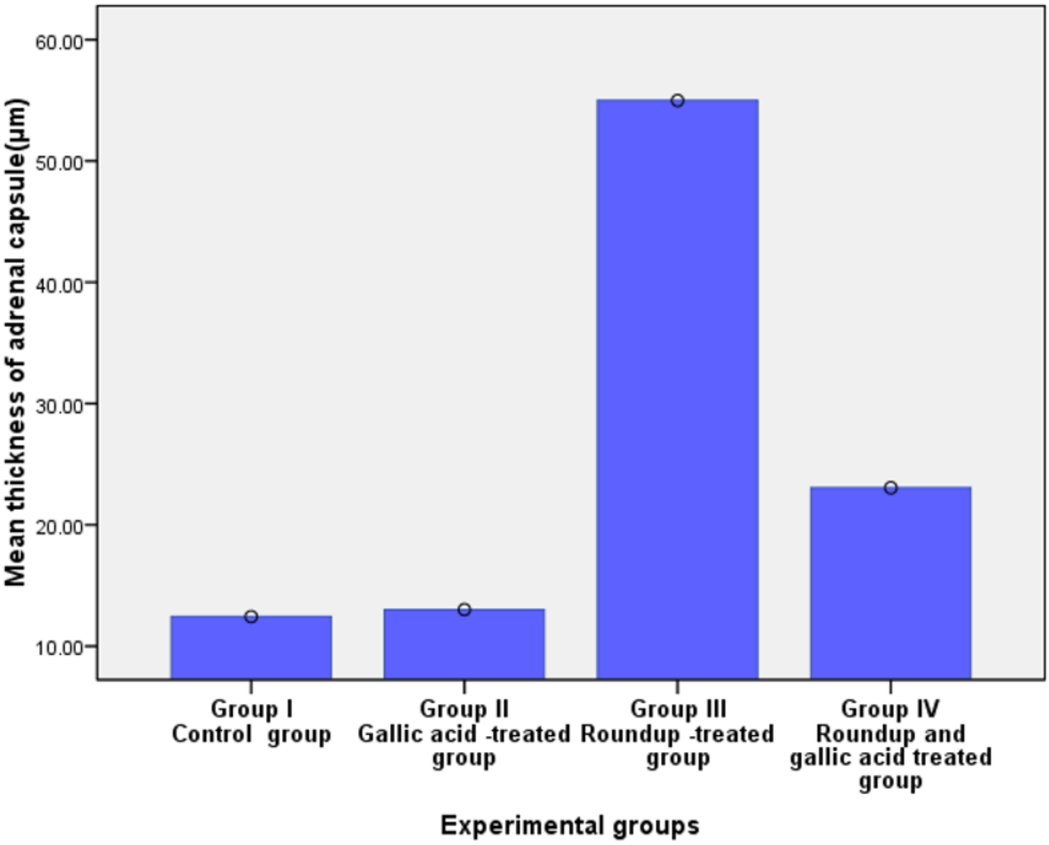
II-Morphometric Results:

Statistical analysis revealed a statistically significant increase for both the thickness of the adrenal capsule and the area percentage of caspase-3 immuno expression in the suprarenal cortex in the Roundup-treated group as compared to the control group with the P-value is <0.05. However, a significant decrease for both measures was detected in the Roundup and gallic acid-treated group when it was compared to the Roundup-treated group with a P-value is < 0.05. (Table 1, Graph 1,2).

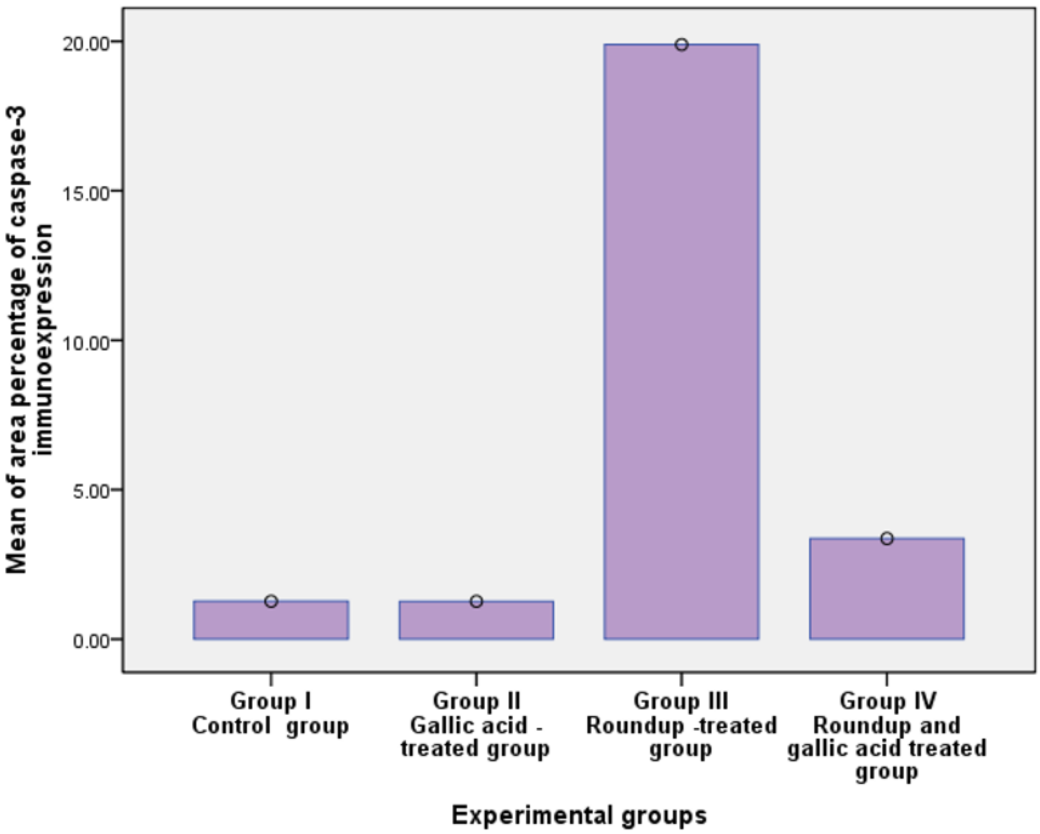
Table 1: A comparison between the experimental groups concerning the adrenal capsule thickness (µm) and the area percentage of caspase-3 immunoexpression (Data are shown as mean ± SEM).

	Control group Group I	**Gallic acid-treated group. Group II**	**Roundup-treated group. Group III**	**Roundup and gallic acid treated group. Group VI**
The mean thickness of the adrenal capsule (µm)	12.44 ± 0.20	13.01 ± 0.32	54.98 ± 0.97*	23.05 ± 0.60**
The mean Area percentage for the caspase-3 immunoexpression	1.26 ± 0.06	1.27± 0.10	19.89 ± 0.83*	3.37 ± 0.17**

* Significant versus the control group.
** Significant versus the Roundup-treated group.



Graph 1: A comparison between the experimental groups concerning the thickness of the adrenal capsule (µm)



Graph 2: A comparison between the experimental groups concerning the area percentage of caspase-3 immunoexpression

DISCUSSION

Roundup is considered the most famous commercial formulation of all herbicides containing glyphosate. It is used worldwide, and this represents a risk for the general population, not just for farmers, as contamination by glyphosate involves water and food consumption. Increasing concerns about their possible toxicity to endocrine, gastrointestinal, reproductive and nervous systems have been raised (Nerozzi *et al.,* 2020). In the present work, the histological effect of Roundup on the suprarenal cortex was investigated in addition to the evaluation of the protective role of gallic acid.

The present work revealed that H&E staining showed disturbed arrangements of cells in the zona glomerulosa and the zona fasciculata with many of them had darkly stained nuclei. Similar observations were confirmed in the suprarenal cortex of rats exposed to the endocrine disruptor tributyltin (Solaiman and Sawires 2019). El-Shenawy (2009) studied the oxidative stress response of Roundup and glyphosate and assumed that Roundup, by reducing the level of GSH, led to an oxidative imbalance, resulting in cell death. Owagboriaye *et al.,* (2017) reported that interstitial cells and seminiferous tubules degenerative changes were signs of the toxicity of Roundup to the rat testes. They explained that it disturbed the body metabolism by diminishing the antioxidant defense that initiates lipid peroxidation and changes in biochemical and hematological profiles.

In the present work, many cells appeared with marked vacuolations and some were ballooned in the Roundup-treated group. This was confirmed by semithin and ultrathin sections examination by the observation of marked accumulation of lipid droplets with variable sizes and fused outlines. This was in agreement with Pandey & Rudraiah (2015) who indicated that adrenal cortical cells showed a greater lipid droplet number in Roundup-treated rats. The accumulation of lipids in the adrenal cortical cells was linked with decreased utilization of cholesterol (Kraemer, 2007). The accumulated lipid droplets contained much of the cholesterol used in steroid synthesis. These stores of cholesterol are known to be in the form of ester that represent the required cholesterol substrate for maintained steroidogenesis (Ahmad *et al.,* 2022). Impaired steroidogenesis occurs by the downregulation of biosynthesis or metabolism of cholesterol and the cytochrome P-450 enzymes disruption. These lead to cytoplasmic lipid droplet accumulation (Rosol *et al.,* 2013). This also was consistent with previous researchers who reported that after the suppression of steroidogenesis, lipid droplets accumulation was observed in the zona fasciculata cells and the cells of zona reticularis following dexamethasone treatment (Thomas et al., 2004) and after nicotine administration in the zona fasciculata cells (Khalaf *et al.,* 2017).

Similar observations were noted by Solaiman and Sawires (2019) who studied the impact of tributyltin on the suprarenal cortex, and they owed these results to the endocrine-disrupting effect of it that caused impaired steroidogenesis.

Furthermore, Mutsuga *et al.* (2017) reported that cytoplasmic cholesterol accumulation was produced by the inhibition of cholesterol conversion to pregnenolone by CYP11A1, which is a steroidogenesis enzyme in the inner mitochondrial membrane. Impaired steroidogenesis was considered to play a significant role in suprarenal cortex toxicity (Khalaf *et al.,* 2017)

In addition, loss of cell boundaries was observed especially in the zona glomerulosa. A similar observation was noted by Abdel Malak & Amin (2018) on rat exposure to recurrent acute restraint stress episodes and this was explained as a part of the impairment of steroidogenesis.

The suprarenal gland presents some biochemical and structural characteristics that render it an ideal target for endocrine disrupting chemicals (EDCs), for example, the high cell membrane content of polyunsaturated fatty acids. Glucocorticoids biosynthesis is a target for endocrine-disrupting chemicals as they alter the biosynthesis, secretion, metabolism, and transport of glucocorticoids. These toxic effects influence reproduction, behavior, and development (Ahmad *et al.,* 2022).

In this work, an increase in the deposition of collagen fibers with a significant increase in thickness was observed in the adrenal gland capsule in Roundup-treated rats. This was in agreement with Merlo *et al.* (2016) who reported that the adrenal cortex displayed an increase in collagen deposition in a time-dependent manner in tributyltin chloride-treated rats. Glyphosate by its endocrine-disrupting effect can increase TNF-∝ production by monocytes (Buoso *et al.,* 2020). The cytokines such as TNF-∝ stimulate collagen deposition (Pratt & Kaplan, 2001). Thickening of the adrenal capsule was demonstrated by many researchers in stressed rats (Sadek *et al.,* 2021 and Zaki *et al.,* 2018). The suprarenal capsule is a signaling center that controls cell zonation and renewal by the capsular enzymes that imprint the fate of glomerulosa cells (Vidal *et al.,* 2016).

In this study, Roundup-treated rats exhibited a significant increase in the caspase-3 immunoexpression in the suprarenal cortical cells in comparison to the control rats. These observations were in agreement with Pandey & Rudraiah, (2015) who demonstrated a higher incidence of apoptotic cells in the adrenal cortex in the Roundup-treated rats in comparison to the control ones. Zhang *et al.* (2023) reported that glyphosate increased levels of mRNA of the apoptotic genes, caspase 3 and Bax.

Ultrastructurally, the present results indicated that the cells of the three zones showed destructive changes that include irregular nuclear membrane, condensed chromatin and cytoplasmic vacuolations, especially in the cells of the zona fasciculata. This was in accordance with Saleh *et al.* (2018) who demonstrated degenerated nucleus, necrosis, rarified cytoplasm, and disorganized cell organelles in the liver cells of Roundup-treated rats. Similar observations also were detected by Abd El-Gawad *et al.* (2016) and Altayeb & Salem (2017) who noticed similar changes in adrenal cortical cells after exposure to stress.

In the same context, Benachour & Séralini (2009) demonstrated that glyphosate causes human embryonic, placental and umbilical cells necrosis. Besides, Cattani *et al.* (2014) established that glyphosate in its commercial formulation Roundup elicits oxidative stress in cerebral cortex, substantia nigra and hippocampus.

Distorted mitochondria and smooth endoplasmic reticulum were observed on ultrastructural examination, especially in the cells of zona fasciculata. This could be explained by that they are known to be the keystone in steroidogenesis in the suprarenal cortical cells and are the main organelles affected by the steroidogenesis-inhibiting drugs (Khalil, 2015). Swelling that was identified in the mitochondria of the cells of zona fasciculata probably resulted from the inhibition of cholesterol to pregnenolone conversion and its accumulation in the mitochondria. This in turn results in mitochondrial hypertrophy and destruction (Elshennawy & Aboelwafa, 2015; Khalaf *et al.,* 2017). These findings were also in accordance with (Merke *et al.*, 2000) who noticed the presence of large mitochondria and dilated smooth endoplasmic reticulum in zona glomerulosa cells in 21- hydroxlase deficiency patients.

In the present study, gallic acid was found to be effective in protecting the adrenal cortical cells from the damaging effect of Roundup as most of the structural changes induced by roundup were improved. Hematoxylin and Eosin-stained sections of Roundup and gallic acid-treated rats showed relatively preserved adrenal cortical structure.

Apparent normal capsule thickness was noted, and this was confirmed by the morphometric results that revealed a significant decrease of the adrenal gland capsule thickness in Roundup and gallic acid-treated rats in comparison to Roundup-treated rats. This could be attributed to the antifibrotic activity of gallic acid. Similar observations were reported by (Hussein *et al.*, 2020) who found that gallic acid stopped the increase in TGF-β1 and Smad3 expression levels, and protected the liver from thioacetamide-induced fibrosis. Gallic acid is known to decrease oxidative stress markers and the pro-inflammatory cytokines, such as TNF-α expression (Ekinci Akdemir *et al.,* 2021)

A decrease in the immunostaining of caspase -3 was detected in the suprarenal cortex of the roundup and gallic acid-treated rats. This was in according with the observations of Silva *et al.* (2023) who detected that gallic acid plus doxorubicin reduced the percentage of caspase-3 in the ovarian follicles and increase the proliferation of follicular cells compared to the doxorubicin-treated group. The anti-apoptotic effect of gallic acid was demonstrated by Ekundayo *et al.,* (2022) who stated that it significantly protects against caspase-3-mediated apoptosis in rats treated with aluminum chloride.

A marked reduction in the cellular lipid droplet accumulation was detected in the cells of the suprarenal cortex in the Roundup and gallic acid-treated group. This was shown both by semithin and ultrastructural examination. This could be justified by the enhancement of mRNA expression levels of CYP11a, and CYP19a1, enzymes that are involved in steroidogenesis, by gallic acid (Ul Haq Shah *et al.,* 2022)

Electron microscopic examination of the adrenal cortex cells in the Roundup and gallic acid-treated rats showed improvement of the changes in the cell organelles induced by Roundup. Dutta & Paul (2019) reported that gallic acid protects against bisphenol-induced damage of the mitochondria isolated from the liver of the rat by the inhibition of oxidative stress-mediated damage. These results could be clarified by that gallic acid has anti-inflammatory and antioxidant effects as supported by Ojeaburu & Oriakhi (2021) who noticed that it increased antioxidant enzyme activities and decreased the expression of pro-inflammatory markers.

Conclusion

Roundup has obvious histopathological effects on the cells of the suprarenal cortex. Gallic acid has an ameliorative role in these effects possibly through its antioxidant effect. Based on the present results, it is recommended to limit the use of Roundup.

Conflicts of interest: There are no conflicts of interest.

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