Original Article

Mitochondrial Complex I Inhibitor Class A Rotenone Induced Toxicity In Rats, Adrenal Gland And The Ameliorative Effect Of Ferulic Acid Via Regulation Of Apoptosis Signaling

Hend M. Hassan[1*], Dina Salem[2], Alaa M. Badawy[1], Ahmed A. Shaaban[3, 4], Heba M. ElHessy[1*]

1. *Human Anatomy and Embryology Department, Faculty of Medicine, Mansoura University, Egypt.*
2. *Pharmacology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt*
3. *Pharmacology and Biochemistry Department, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa 11152, Egypt*
4. *Pharmacology and Toxicology Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt*

Email:Heba1.elhessy@mans.edu.eg

DOI: 10.47750/pnr.2023.14.S02.88

Abstract

Introduction Adrenal gland is a crucial part of our body. It is part of the Hipothalamo-Pituitary-Adrenal axis and the sympatho-adrenomedullary system. So, by turn, it acts as an essential stress regulator. Rotenone is a widely used organic pesticide providing a trustworthy model of Parkinson disease and causes neurotoxicity and systemic damage. Ferulic acid is a natural polyphenol that is widely present in vegetables and fruits with powerful antioxidant, and antiapoptotic properties **Materials and methods** Rats (Twenty -four) were randomly allocated into the following four equal groups: Ferulic acid was given orally to the control and Sham groups (50 mg/kg/d), Rotenone group received a daily dose of 2.5 mg/kg/d dissolved in 1ml of sunflower oil i.p, group 4 Rotenone + Ferulic acid Group. After 8 weeks, Serum samples were taken and Paraffin sections of the adrenal glands were stained with Hematoxylin and eosin, Luxol fast blue, and Bax and BCL2 immune staining. **Results** Rotenone has glucocorticoid-like action and disruptive histopathological effect in form of congested sinusoids, hemorrhage, and vacuolations. It also increases the BAX/BCL2 ratio indicating stimulation of the apoptosis process. Ferulic acid was proved to have an ameliorative effect in enhancing Rotenone induced adrenal gland toxicity. **Conclusions** Rotenone caused noticeable deterioration of rat adrenal gland via enhancing apoptosis. Co-administration of ferulic acid alleviated its destructive effects via regulation of apoptosis signals.

Key words Rotenone-Ferulic Acid-Adrenal gland- Histopathological Ranking-Apoptosis Regulators.

INTRODUCTION

Adrenal gland is vital part of our body. It is part of the Hypothalamo-Pituitary-Adrenal axis and the sympatho-adrenomedullary system. So, it acts as essential stress regulator **(Berger et al., 2019)**. Adrenal gland is formed of cortex and medulla. The cortex consists of three different zones, the outer zona glomerulosa (ZG), the zona fasciculata (ZF), and the zona reticularis (ZR). Each one of them has particular morphological and functional properties. Aldosterone is produced by the zona glomerulosa, whereas Zona fasciculata/ reticularis synthesize cortisol in humans and cattle, and corticosterone in rodents **(Miller & White, 2022).** Adrenal gland is characterized by their high vascularization and blood supply making them specifically susceptible to both endothelial dysfunction and hemorrhage and consequently apoptosis **(Kanczkowski et al., 2022)**.

Rotenone (ROT) is widely used organic pesticide. Rotenone provides a trustworthy model of Parkinson disease (PD). In addition, rotenone systemic therapy can cause neurotoxicity and systemic damage **(Lapointe et al., 2004).** Rotenone exhibits oxidative stress by increasing the generation of reactive oxygen species (ROS) in the mitochondria, which causes DNA fragmentation, cytochrome c release, and caspase-3 activation indicating activation of apoptosis process **(Li et al., 2003)** and the underlying mechanism of that through suppression of mitochondrial complex I **(Najafi et al., 2022)**. It is also known as a unique endocrine disruptor of glucocorticoids as It increases serum cortisone levels by stimulating the adrenal glands through a receptor-mediated mechanism **(Youssef et al., 2003).** Further studies investigating histopathological effects of rotenone on the adrenal gland need to be done.

Apoptosis is known to be the most prevalent form of programmed cell death. BCL2 (B-cell lymphoma 2) family and BAX (BCL2-Associated X Protein) are considered to be key regulators of apoptosis process **(Danial & Korsmeyer, 2004).** In one hand BCL2 family act as suppressor of apoptosis while BAX and other BAX-like subgroups act as promotor of this process **(Youle & Strasser, 2008).** These apoptosis proteins present in the endoplasmic reticulum, the nuclear envelope, and the outer mitochondrial membrane**(Wang et al., 2022).** As rotenone is one of known toxins enhancing apoptosis, so will study effect of rotenone on apoptosis regulators (BAX and BCL2) in adrenal gland as a possible underlying mechanism.

Ferulic acid (FA) is a Polyphenol that has been widely used in the pharmaceutical, food, and cosmetics industries **(Zduńska et al., 2018).** It is abundant in fruits and vegetables, including bananas, citrus fruits, eggplant and cabbage, as well as in seeds and leaves **(Kikugawa et al., 2017)**. Ferulic acid has been acknowledged as a crucial chemical structure supporting a number of biological functions, including antioxidant, anti-inflammatory, antiviral, antiallergic, antibacterial, antithrombotic, anticarcinogenic, and hepatoprotective properties, either directly or indirectly **(Kim & Park, 2019)**. Numerous preclinical investigations conducted in recent years have referred the FA cytoprotective properties to both its ability as a free radical scavenger and as enhancer to the cellular stress **(Sgarbossa et al., 2015).** Additionally, **Liu et al. (2021)** stated that FA acts as a protective factor against apoptosis through prevention of mitochondrial malfunction and ROS production**.** To our knowledge, there are no published research on the possible protective effect of FA in adrenal glands rotenone toxicity.

The goal of the current investigation was to assess the toxic effect of rotenone on the adrenal gland. Histopathological grading of the effect and correlation between these histopathological changes and BAX/BCL2 ratio as a possible underlying cause of those changes. We also evaluated whether FA may potentially prevent the destructive effects caused by rotenone in rats' adrenal glands targeting BAX/BCL2 as apoptotic markers.

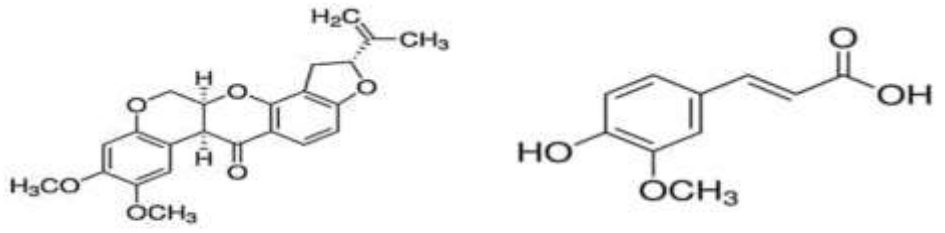
Materials and Methods

Ethical approval

Following NIH and EU norms for animal care, this study received approval from the Animals' Experimentation Committee at Delta University (Code number: FPDU7). The experiment was conducted at Pharmacology and Biochemistry department, Faculty of pharmacy, Delta University. Rats were housed under veterinary care. The number of rats used, and animal discomfort were both minimized as far as possible.

Chemicals and drugs

Rotenone (Cat. No. R 8875) and ferulic acid (Cat.No.12,870-8) were obtained from Sigma Aldrich, Germany **(Fig.1)**. Rabbit monoclonal Anti-Bax antibody, Rabbit polyclonal anti-BCL2 antibody and Anti-Mouse / anti-Rabbit HRP-DAB IHC kit were purchased from Abcam, Cambridge, United Kingdom **(**Cat. No. 32503, 194583, 64264 respectively). The commercial enzyme-linked immunosorbent assay (ELISA) kit (DRG Co., Marburg, Germany) was used to assess serum cortisol levels.



Rotenone **Ferulic acid**

Figure (1) Chemical structure of Rotenone and Ferulic acid.

Animals used

Twenty-four adult male albino rats weighing (200-250) grams were used in this study. They were selected because it has been shown that rotenone is more toxic to the females and that the mortality rate is high in the females than males **(Gupta et al., 2019).** The rats were housed in metal cages with soft wood chips for bedding and fed with regular rodent chow and had free access to water for 2 weeks for acclimatization and to ensure normal growth and behavior before the start of the experiment.

Experimental Design

Rats were randomly divided into four groups of 6 rats each; **Control group:** received 1 ml of sunflower oil daily via intraperitoneal injection (i.p). While in the **Sham group** 50 mg/kg of ferulic acid were prepared in 1 ml distilled water and administered orally through gastric gavage daily **(Ojha et al., 2015)**. **Rotenone group:** received a daily dose of 2.5 mg/kg of rotenone dissolved in 1ml of sunflower oil i.p **(Sharma et al., 2016). Rotenone + Ferulic acid group**: received a daily dose of 2.5 mg/kg of rotenone dissolved in 1ml of sunflower oil i.p and ferulic acid (50 mg/kg/d) prepared in 1 ml distilled water orally via gastric gavage **described by (Ojha et al., 2015)**. All drugs and chemicals were administered for 8 weeks.

Specimens Collection

Blood samples

At the assigned time, Rats were anaesthetized with chloral hydrate (300 mg/kg, I.P) **(Feng & Yuan, 2015),** blood samples were collected via cardiac puncture. Subsequently, serum samples were separated by centrifugation (6000 rpm, 20 min) and stored at –80 °C until assessing cortisol levels using the ELIZA kits.

Tissue collection

Rats were sacrificed and Adrenal glands were dissected out carefully and processed for histological and immunohistochemical studies.

Histopathological examination

Hematoxylin and Eosin stain For routine histopathological examination **(Mahar et al., 2021)**. In the present study, we evaluated the histopathological changes represented in vacuolations, dilated sinusoids and hemorrhage in all studied groups. A semiquantitative score was given based on the absence (= 0) or presence (= 1) of each of the histopathological finding (vacuolations, dilated sinusoids and hemorrhage) and we gave them a total score for each section.

Immunohistochemical detection of apoptotic markers (BAX and BCL2)

On positive slides, 5µm thick paraffin sections were deparaffinized in xylene and rehydrated. 0.03% hydrogen peroxide was added to block endogenous peroxidase activity (H_2O_2). Sections were boiled in 0.01 M citrate buffer (pH 6) for 20 min to reveal the antigenic location. To prevent nonspecific binding, the samples were incubated with 5% normal goat serum in PBS for 20 min. Monoclonal antibodies for BCL2 (1:50) and BAX (1:250) were used to incubate the primary antibody on the sections for an extended period of time at 4 °C. Utilizing a DAB substrate and an avidin-biotinylated peroxidase complex (ABC-kit), positive reactions were detected using immunocytochemistry. Sections were then mounted after being dehydrated in alcohols, cleaned in xylene, and counterstained with hematoxylin **(Shen et al., 2021).**

Image analysis

Using a light microscope (Olympus model BX53, Tokyo, Japan) attached to a digital camera (Toucan model BX53, Japan) connected to a computer, images were captured. The cortex of the adrenal gland was examined in five randomly spaced, 5µm thick sections for each zone of each rat in each group at a 400x magnification with a 40x lens (area: 0.071mm2). Calculated measure was the area fraction of BAX and BCL2 immune expression. The computerized image analysis was done using Image-j (Fiji). In the cytoplasm and nuclei of cortical cells from all three layers, immune expression was visible as a brownish pigmentation. Color deconvolution plug in was used and H-DAB-vector was selected to yield three differently colored images: green, brown, and blue. Calibration of the DAB images (brown-colored) was performed by measuring area fraction **(Heddleston et al., 2021).**

Statistical analysis

The data was depicted as the mean ± standard deviation (SD). One-way ANOVA for multiple comparisons between

groups was used to analyze the data, followed by the post-hoc (Tukey test) using SPSS software (SPSS Inc., Chicago, Illinois, USA). Statistical significance was defined as a P value 0.05. The Pearson's Correlations among Histopathological score, BAX/BCL2 ratio were plotted, and the Pearson's correlation coefficients were labeled.

Results

There were no statistically significant differences in between data of both control and sham group so they were treated as one group named control group. **Biochemical results**

There was a significant increase ($P < 0.001$) in the serum cortisol level in the ROT-treated group as compared with control group. In contrary, ROT+FA treated group showed a significant decrease ($P < 0.001$) in serum cortisol level as compared to ROT-treated group **(Table 1)**.

Table (1) The mean blood cortisol levels ± SD in all groups.

	Control group	**Rotenone group**	**Rotenone + ferulic acid group**
Variant	**Mean±SD**	**Mean±SD**	**Mean±SD**
Serum cortisol level (ug/dl)	0.2±0.03	0.77±0.15 *	0.21±0.12 #

*Significantly different from control, # significantly different from Rotenone group. P value < 0.001.

Histopathological results

Hematoxylin and Eosin staining (Figs. 2 & 3)

Hematoxylin and Eosin-stained sections of **control** group revealed that adrenal gland was formed of an inner pale central **medulla** and outer dark **cortex** surrounded by a connective tissue capsule. The cortex was organized into 3 zones: zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR). **ZG** was the outermost zone lying beneath the capsule. Its cells were arranged in small nests **(Fig. 2B)**. The cells of this zone were columnar with deeply stained rounded nuclei and acidophilic cytoplasm. **ZF** was the middle and the broadest zone **(Fig. 2C)**. Cells of this zone were large and polyhedral containing central rounded vesicular nuclei and faint acidophilic cytoplasm. They were observed as parallel cords disjoined by blood sinusoids. **ZR** was the innermost zone which lying just near to the medulla. Cells were organized in anastomosing cords separated by blood sinusoids **(Fig. 2D)**. The cells appeared small, rounded, and dark. They had hyper chromatic nuclei and dense cytoplasm. **The medulla** was formed of follicles of chromaffin cells that contain basophilic catecholamines granules and vesicular nuclei **(Fig. 3A)**. **In ROT-treated group**, Sections showed dilated and congested blood sinusoids, hemorrhage and vacuolations in zona glomerulosa **(Fig. 2F)**, zona fasciculata **(Fig. 2G)** and zona reticularis **(Fig.2H)**. There was also increased thickness of zona fasciculata**.** More marked changes including hemorrhage and vacuolations were found in medulla **(Fig. 3B). In ROT+FA treated group,** sections of the adrenal gland showed more preservation of the architecture with less vacuolation, hemorrhage and congested sinusoids **(Figs. 2I, 2J, 2K, 2l and 3C)**.

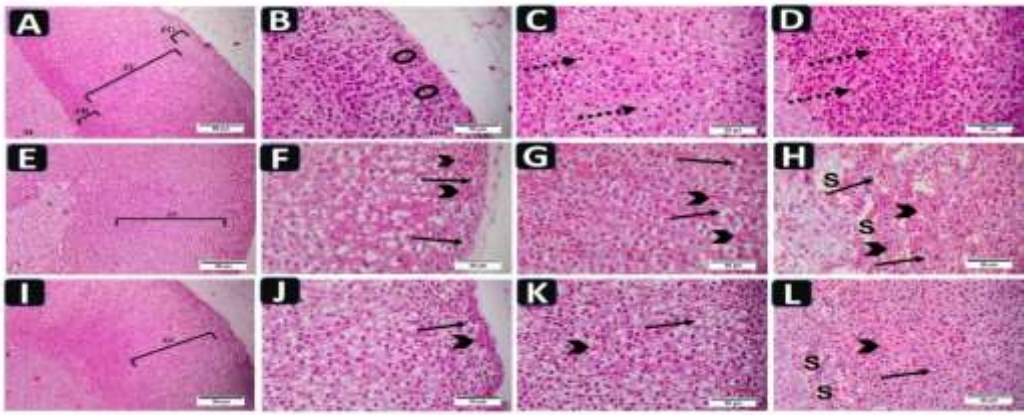


Fig. 2: Hematoxylin and eosin-stained sections of adrenal gland cortex; in **control group** as shown in **(A)** adrenal gland was formed of medulla (M) and cortex which was subdivided into 3 zones; zona glomerulosa (ZG), zona Fasciculata (ZF) and Zona reticularis (ZR). Zona glomerulosa the outermost layer showing tight clusters of epithelial cells (circles) separated by trabeculae as showen in **(B)**, Zona Fasciculata (ZF) the middle layer cells appear large polyhedral containing large rounded nuclei and cytoplasm stained faintly acidophilic **(Dotted arrows)** arranged in parallel cords **(C)** and zona Reticularis (ZR) cells are rounded dark cells arranged in anastomosing cords and have hyper chromatic nuclei and dense cytoplasm **(Dotted arrows) (D)**. In **ROT group (E, F, G and H)** showing increase thickness of zona fasciculata **(ZF),** increased number and congestion of blood sinusoids **(S)**, Hemorrhage **(arrows head)** and vacuolations **(arrow)** in Zona glomerulosa **(F),** Zona fasciculata **(G)** and zona Reticularis **(H).** In **FA-treated group (I, J, K and L)** showed less prominent hemorrhage **(arrows head)**, dilated congested blood sinusoids **(S)** and vacuolations **(arrows)** than the rotenone group.

Histopathological scoring

A descriptive analysis of different histopathological changes indicating the destructive effect of rotenone and the ameliorative effect of ferulic acid is shown in **Table 2**. The scores of the histopathological changes (= sum of scores in the 5 examined fields) were significantly higher in Rotenone group ($p < .05$) as compared to control group in contrary the rotenone treated with ferulic group showed significant decrease in the histopathological score corelated to rotenone group.

Table (2) Histopathological score of the cortex and medulla in all groups:

	Control	**Rotenone**	**Rotenone + Ferulic acid**	**P value**
Cortex				
Mean ± SD	0.57±0.57	2.48±0.51 *	0.81±0.7 #	0.001
Medulla				
Mean ± SD	0.6±0.5	2.48±0.57 *	1.23±1.02 #	0.001

*Significantly different from control and # significantly different from Rotenone group.

Results of immunohistochemical assessment of apoptotic markers (BAX and BCL2)

Cellular stress induced by the rotenone was assessed by measuring the immune expression of apoptotic markers (BAX and BCL2), known to modulate the apoptotic pathway. Thus, to determine the anti-apoptotic activity produced by ferulic acid **(Figs. 4 & 5). Proapoptotic BAX** immune-expression increased significantly ($P < 0.001$) in ROT-treated group as compared with the control group. The immune reaction was detected in both nuclei and cytoplasm of cells of the three cortical zones. There was a significant decrease in BAX expression in ROT+FA treated group as compared to ROT- treated group **(Fig. 6A).** As regards **BCL2 expression,** ROT-treated group showed a significant decrease ($P < 0.001$) as compared to control group. While ROT+FA treated group showed a significant increase ($P < 0.001$) as compared to ROT-treated group **(Fig. 6B).**

Data of BAX and BCL2 immune expression were used to attain the BAX/BCL2 ratio **(Fig. 6C)** to emphasize the adrenal gland ability to adapt or overcome, the action of ROT, and the effectiveness of FA to ameliorate its disruptive effect. This study found that ROT significantly increased the ratio ensuring its destructive effect on the adrenal gland. Meanwhile the FA significantly decreased this ratio emphasizing its efficacy in alleviating ROT toxic effects.

Correlation between the histopathological scoring of ZR and BAX/BCL2 ratio

There was a a statistical significance (p value = 0.03) with positive correlation (r = 0.997) of strong association between the histopathological scoring and BAX/BCL2 ratio in the adrenal cortex as shown in **fig. 6D**.

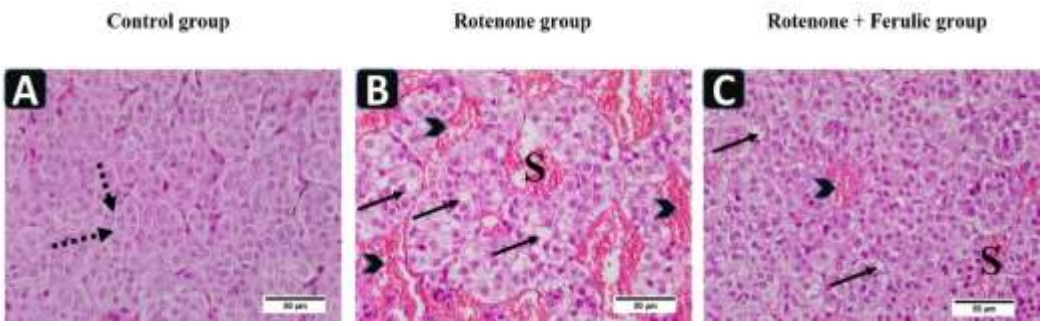


Fig. 3: Hematoxylin and eosin-stained sections of adrenal gland medulla; Control group (A) The medulla was formed of follicles of chromaffin cells **(Dotted arrows)** that contained basophilic catecholamines granules and vesicular nuclei. Connective tissue trabeculae were seen extending between the medullary follicles. In **rotenone-treated group (B),** there were dilated blood sinusoids **(S)**, hemorrhage **(arrows head)** in between chromaffin follicles and vacuolations **(arrows). FA-treated group (C)** showed less prominent hemorrhage **(arrows head)**, congested blood sinusoids **(S)** and vacuolations **(arrows)** than ROT-treated group.

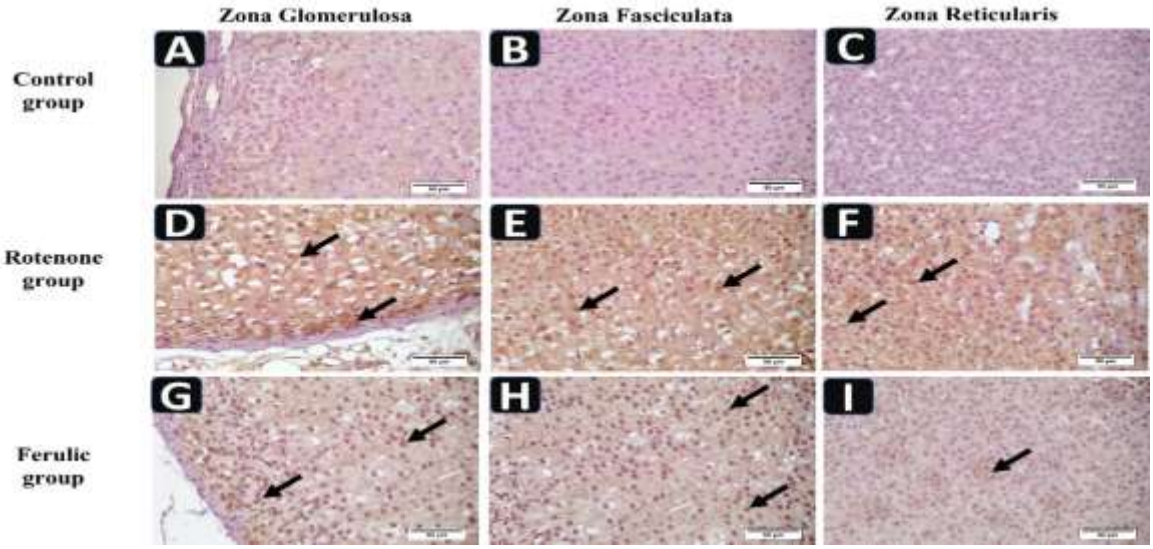


Fig. 4: BAX immunohistochemical staining of rats adrenal gland cortex; BAX was expressed in both nuclei and cytoplasm of cortical cells of all three layers. **Rotenone-treatd group** (D, E and F) showed prominent BAX expression in all zones of cortex (A, B and C) while **ferulic acid-treated group** (G, H and I) showed a siginficant decrease in BAX expression as compared to rotenone-treated group.

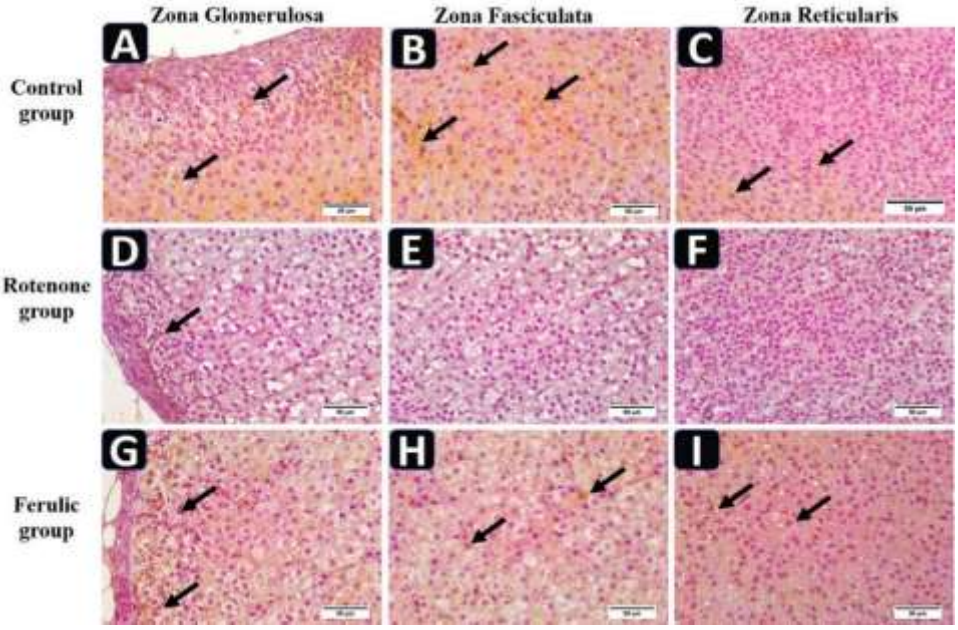


Fig. 5: BCL2 immunohistochemical stain of rats adrenal gland cortex; BCL2 was expressed in both nuclei and cytoplasm of cortical cells. **Rotenone-treated group** (D, E and F) showed no or minmal BCL2 expression in all zones of cortex as compared to control group (A, B and C) while **ferulic acid-treated group** (G, H and I) showed a siginficant increase in BCL2 expression as compared to rotenone-treated group.

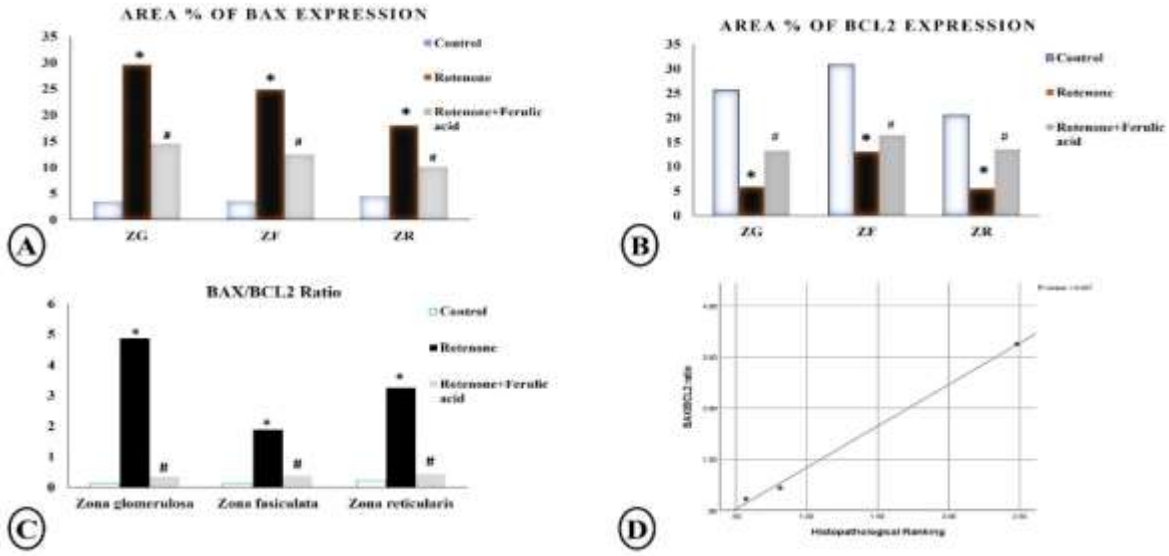


Fig. 6: Histograms Illustrating data analysis; (A) Rats' adrenal cortex's mean area fraction of BAX immune expression, (B) Rats' adrenal cortex's mean area fraction of BAX immune expression, (C) BAX/BCL2 ratio, (D) Pearson correlation curve among BAX/BCL2 ratio and histopathological grading

Discussion

The goal of the current investigation was to assess the toxic effect of Rotenone on the adrenal gland. Histopathological grading of the effect and correlation between these histopathological changes and BAX/BCL2 ratio and examine if FA could be able to stop the predicted ROT disruptive effect by targeting the apoptotic markers BAX/BCL2.

Rotenone is frequently used as a natural insecticide, pesticide, and to eradicate fish from lakes. Being a lipophilic substance, it is easily able to pass the blood-brain barrier **(Bové et al., 2005).** Chronic low-dose rotenone exposure resulted in uniform complex I mitochondrial inhibition across the brain, and it has been demonstrated to cause nigrostriatal circuit degeneration **(Betarbet et al., 2000).** Following prolonged intraperitoneal injection of rotenone**, (Cannon et al., 2009)** consistently replicated many characteristics of Parkinson's disease in rats. Researchers declared that ROT therapy in mice led to not only generalized central nervous system but also cause systemic toxicity **(Lapointe et al., 2004).** Rotenone is toxic through a number of pathways, including oxidative damage, inflammation, apoptosis, microtubule malfunction, and also autophagic modification**(Chou et al., 2010).**

According to **Johnson et al. (2019),** ROT poisoning results in chronic stress, which in turn stimulates the adrenal glands to release more catecholamines. The adrenal gland plays a significant part in the body's reaction to physiological stresses and is able to adjust to these needs because it is the primary creator of stress hormones. Proper adaptation is especially crucial because stress system dysregulation is the root cause of many human disorders, such as obesity, depression, and Parkinson's disease **(Berger et al., 2019).** Ferulic acid (FA) is a natural substance known by its anti-inflammatory, antioxidant, and anti-apoptotic properties **(Ren et al., 2017).** It can be good suggestion for ameliorating Rotenone toxicity.
Results of the present study revealed that there was a significant increase in serum cortisol level in ROT-treated group as compared with control group ($P<0.001$) and they were significantly decline in the FA treated group ($P<0.001$).These results were in agreement with those of **Youssef et al. (2003)** who suggested that rotenone may have effects similar to those of glucocorticoids as a result of raising serum corticosterone levels. Parkinson patients also had greater cortisol levels in both blood as well as urine compared to controls (**(Dodiya et al., 2020).** According to **Lorigooini et al. (2021),** FA has anxiolytic-like effects in mice via inhibiting the NMDA receptor pathway. Additionally, **Sborgi et al. (2021)** hypothesized that ferulic acid might induce an anti-anxiety effect via acting on the GABAA receptor's

benzodiazepine binding site. As a result, it can lower cortisol levels.

Histopathological studies were conducted on the rats' adrenal glands to assess morphological changes that confirmed rotenone toxicity. ROT administration resulted in deteriorative changes in the form of dilated and congested blood sinusoids and vacuolations in the three cortical zones. There was also increased thickness of zona fasciculata**.** More marked changes were observed in the adrenal medulla.
Vacuolations and pyknosis can be referred to as degenerative changes in the adrenal cells result from Rotenone toxicity**.** This is attributed to Rotenone's capacity to prevent the transfer of electrons from complex I to ubiquinone, which prevented oxidative phosphorylation and allowed for the controlled creation of cellular chemical energy **(Hasan et al., 2020)**. Reactive oxygen species (ROS) are produced as a result, and these ROS further promote mitochondrial malfunction and oxidative stress, which in turn cause cellular degeneration as described by **(Fato et al., 2009; Swarnkar et al., 2010).**

Moreover, the adrenals had hemodynamic changes that affected all layers of the adrenal cortex, where the sinusoids and capillaries were dilated and congested, as well as morphological abnormalities **(Koldysheva et al., 2005).** Along with vacuolar degeneration, **Yu et al. (2012)** found that stress-related adrenocortical damage frequently includes steatosis and bleeding which can explain presence of dilated congested sinusoids and presence of hemorrhage.

Results of the present study highlighted the effect of FA administration on restoration of adrenal gland architecture. There were less vacuolations and congested sinusoids. The enhancing effect of ferulic on the vacuolation can be explained by its anti-apoptotic and antioxidant effects as described by**(Elhessy et al., 2020)**. Ameliorative effect of ferulic on blood sinusoids congestion and hemorrhage could be attributed to its ability to restore endothelial function through enhancing the bioavailability of basal and stimulated NO as declared by **(Suzuki et al., 2007)**. FA's antioxidant and anti-inflammatory properties most likely function as mediators for its protective benefits against ROT toxicity **(Ojha et al., 2015)**.

The present study chose to focus on apoptosis since it is essential for embryonic development, preservation of tissue homeostasis, and defense against cancerous transformation. Numerous intracellular signals can trigger intrinsic apoptosis such as the gateway proteins BCL2 and BAX **(Wei et al., 2001).** The oncoprotein BCL2 is believed to control programmed cell death and promote cell survival. Its primary duties include maintaining mitochondrial integrity and controlling the release of mitochondrial proteins implicated in the apoptotic pathway into the cytoplasm **(Cory & Adams, 2002).** However, BAX is probably a significant regulatory gatekeeper for apoptosis. It is necessary for the apoptotic process to be completed **(Cartron et al., 2003).**

The present study revealed that the adrenal cortex of ROT-treated group showed a significant increase in BAX and decrease in BCL2 immune-expression ($P < 0.001$) as compared with the control group. Co-administration of FA resulted in a significant increase in BCL2 and decrease in BAX immune-expression ($P < 0.001$) as compared with ROT-treated group. These results were confirmed by quantitative measurement of the area % of positive BAX and BCL2 immune reaction in the adrenal cortical sections. The results of this investigation supported the hypothesis set by **Li et al. (2003)** that ROT can cause apoptosis by increasing the amount of mitochondrial ROS produced. Additionally, **Pan et al. (2009)** came to the conclusion that rotenone is the cause of mitochondrial complex I electron transport chain blockage, which results in cellular death. According to **Ahmadi et al. (2003)**, ROT generated significant neuronal apoptosis as a method of cell death.

BAX/BCL2 ratio was found to be significantly increased in ROT-treated group as compared to the control group ($P<0.001$). The ratio decreased significantly in ROT+FA treated group as compared with ROT-treated group ($P<0.001$).These findings supported **Teijido and Dejean (2010)** explanation that BAX/BCL2 ratio controls apoptosis, functionally regulating the mitochondria. A high level of the anti-apoptotic Bcl2 protein ensures cell survival. Increased levels of the proapoptotic protein BAX ensure cell death by inactivating Bcl2 and assures FA's potential to inhibit apoptosis in coincidence with those of **(Das et al., 2016)**, who assured that FA cause decline of the BAX/BCL2 ratio in case of ionizing radiation exposure and this is the main cause of enhancing apoptosis. **Li et al. (2018)** also linked FA's anti-apoptotic effects to its function in the ROS-mediated signaling system.

A semiquantitative score for the histopathological findings in the adrenal gland sections of all studied groups like hemorrhage, dilated sinusoids and vacuolations was performed. We correlate BAX/BCL2 ratio with the histopathological scoring in the adrenal cortex to. In dead, our results declared that more increase in BAX/BCL2 ratio is strongly associated with more deteriorative changes in the adrenal cortex enhancing the theory that BAX/BCL2 is

one of the main responsible pathways of Rotenone toxicity and histopathological changes were enhanced using ferulic acid acting mainly on the apoptosis regulators BAX and BCL2.

Conclusion

Rotenone caused noticeable deterioration of rat adrenal gland via enhancing apoptosis. Co-administration of ferulic acid alleviated its destructive effects via regulation of apoptosis-related proteins (BAX and BCL2). Thus, the current article is evidence of qualifying FA as a sound protector against Rotenone toxicity.

Conflict of Interest

The authors have no conflict of interest.

Funding

No specific grant was given to this research by funding organizations in the public, private, or not-for-profit sectors.

Authors Contributions

Hend M. Hassan and Heba M. Elhessy designed the study, examined the adrenal gland tissue specimens and interpreted the histological and immunohistochemical results. **All authors** contributed to the study conception, revised the manuscript and approved the submitted manuscript.

Data availability

The datasets analyzed during the current study are available from the corresponding authors on reasonable request.

References

1. Ahmadi, F. A., Linseman, D. A., Grammatopoulos, T. N., Jones, S. M., Bouchard, R. J., Freed, C. R., . . . Zawada, W. M. (2003). The pesticide rotenone induces caspase-3-mediated apoptosis in ventral mesencephalic dopaminergic neurons. *Journal of neurochemistry, 87*(4), 914-921.
2. Berger, I., Werdermann, M., Bornstein, S. R., & Steenblock, C. (2019). The adrenal gland in stress–Adaptation on a cellular level. *The journal of steroid biochemistry and molecular biology, 190*, 198-206.
3. Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., & Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature neuroscience, 3*(12), 1301-1306.
4. Bové, J., Prou, D., Perier, C., & Przedborski, S. (2005). Toxin-induced models of Parkinson's disease. *NeuroRx, 2*(3), 484-494.
5. Cannon, J. R., Tapias, V., Na, H. M., Honick, A. S., Drolet, R. E., & Greenamyre, J. T. (2009). A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of disease, 34*(2), 279-290.
6. Cartron, P.-F., Juin, P., Oliver, L., Meflah, K., & Vallette, F. M. (2003). Impact of proapoptotic proteins Bax and Bak in tumor progression and response to treatment. *Expert review of anticancer therapy, 3*(4), 563-570.
7. Chou, A. P., Li, S., Fitzmaurice, A. G., & Bronstein, J. M. (2010). Mechanisms of rotenone-induced proteasome inhibition. *Neurotoxicology, 31*(4), 367-372.
8. Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer, 2*(9), 647-656.
9. Danial, N. N., & Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell, 116*(2), 205-219.
10. Das, U., Biswas, S., Sengupta, A., Manna, K., Chakraborty, A., & Dey, S. (2016). Ferulic acid (FA) abrogates ionizing radiation-induced oxidative damage in murine spleen. *International journal of radiation biology, 92*(12), 806-818.
11. Dodiya, H. B., Forsyth, C. B., Voigt, R. M., Engen, P. A., Patel, J., Shaikh, M., . . . Kordower, J. H. (2020). Chronic stress-induced gut dysfunction exacerbates Parkinson's disease phenotype and pathology in a rotenone-induced mouse model of Parkinson's disease. *Neurobiology of disease, 135*, 104352.
12. Elhessy, H. M., Eltahry, H., Erfan, O. S., Mahdi, M. R., Hazem, N. M., & El-Shahat, M. A. (2020). Evaluation of the modulation of nitric oxide synthase expression in the cerebellum of diabetic albino rats and the possible protective effect of ferulic acid. *Acta Histochemica, 122*(8), 151633.
13. Fato, R., Bergamini, C., Bortolus, M., Maniero, A. L., Leoni, S., Ohnishi, T., & Lenaz, G. (2009). Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen species. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1787*(5), 384-392.
14. Feng, X., & Yuan, W. (2015). Dexamethasone enhanced functional recovery after sciatic nerve crush injury in rats. *BioMed research international, 2015*.
15. Gupta, R. C., Mukherjee, I. R. M., Malik, J. K., Doss, R. B., Dettbarn, W.-D., & Milatovic, D. (2019). Insecticides *Biomarkers in toxicology* (pp. 455-475): Elsevier.
16. Hasan, W., Kori, R. K., Jain, J., Yadav, R. S., & Jat, D. (2020). Neuroprotective effects of mitochondria-targeted curcumin against rotenone-induced oxidative damage in cerebellum of mice. *Journal of biochemical and molecular toxicology, 34*(1), e22416.
17. Heddleston, J. M., Aaron, J. S., Khuon, S., & Chew, T.-L. (2021). A guide to accurate reporting in digital image acquisition–can anyone replicate your microscopy data? *Journal of cell science, 134*(6), jcs254144.
18. Johnson, M. E., Zhou, X.-F., & Bobrovskaya, L. (2019). The effects of rotenone on TH, BDNF and BDNF-related proteins in the brain and periphery: relevance to early Parkinson's disease. *Journal of chemical neuroanatomy, 97*, 23-32.
19. Kanczkowski, W., Evert, K., Stadtmüller, M., Haberecker, M., Laks, L., Chen, L.-S., . . . Beuschlein, F. (2022). COVID-19 targets human adrenal glands. *The Lancet Diabetes & Endocrinology, 10*(1), 13-16.

20. Kikugawa, M., Ida, T., Ihara, H., & Sakamoto, T. (2017). Ferulic acid and its water-soluble derivatives inhibit nitric oxide production and inducible nitric oxide synthase expression in rat primary astrocytes. *Bioscience, biotechnology, and biochemistry, 81*(8), 1607-1611.
21. Kim, J. K., & Park, S. U. (2019). A recent overview on the biological and pharmacological activities of ferulic acid. *Excli Journal, 18*, 132-138.
22. Koldysheva, E., Lushnikova, E., Nepomnyashchikh, L., & Tornuev, Y. V. (2005). Morphogenesis of adaptation and compensatory reactions in mouse adrenals during restitution after thermal exposure. *Bulletin of Experimental Biology and Medicine, 140*(4), 464-467.
23. Lapointe, N., St-Hilaire, M., Martinoli, M. G., Blanchet, J., Gould, P., Rouillard, C., & Cicchetti, F. (2004). Rotenone induces non-specific central nervous system and systemic toxicity. *The FASEB journal, 18*(6), 717-719.
24. Li, L., Zhong, Y., Ma, Z., Yang, C., Wei, H., Chen, L., . . . Li, Y. (2018). Methyl ferulic acid exerts anti-apoptotic effects on L-02 cells via the ROS-mediated signaling pathway. *International journal of oncology, 53*(1), 225-236.
25. Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A., & Robinson, J. P. (2003). Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *Journal of Biological Chemistry, 278*(10), 8516-8525.
26. Liu, M., Zhang, C., Xu, X., Zhao, X., Han, Z., Liu, D., . . . Liu, Z. (2021). Ferulic acid inhibits LPS-induced apoptosis in bovine mammary epithelial cells by regulating the NF-κB and Nrf2 signalling pathways to restore mitochondrial dynamics and ROS generation. *Veterinary Research, 52*(1), 1-11.
27. Lorigooini, Z., Nouri, A., Balali-Dehkordi, S., Bijad, E., Dehkordi, S. H., Soltani, A., & Amini-Khoei, H. (2021). Ferulic acid through mitigation of NMDA receptor pathway exerts anxiolytic-like effect in mouse model of maternal separation stress. *Journal of Basic and Clinical Physiology and Pharmacology, 32*(1).
28. Mahar, Y., Qamar, A., Hidayat, M., Salman, S., Chaudhry, S., & Saeeduddin, M. F. (2021). Effect Of L-Arginine And Insulin On Adrenal Gland Damaged By Streptozotocin In Albino Rats. *Journal of Ayub Medical College, Abbottabad: JAMC, 33*(1), 134-138.
29. Miller, W. L., & White, P. C. (2022). History of adrenal research: from ancient anatomy to contemporary molecular biology. *Endocrine Reviews*.
30. Najafi, N., Ghasemzadeh Rahbardar, M., Hosseinzadeh, H., Hayes, A. W., & Karimi, G. (2022). Chemical agents protective against rotenone-induced neurotoxicity. *Toxicological & Environmental Chemistry, 104*(1), 149-175.
31. Ojha, S., Javed, H., Azimullah, S., Khair, S. B. A., & Haque, M. E. (2015). Neuroprotective potential of ferulic acid in the rotenone model of Parkinson's disease. *Drug design, development and therapy, 9*, 5499.
32. Pan, T., Rawal, P., Wu, Y., Xie, W., Jankovic, J., & Le, W. (2009). Rapamycin protects against rotenone-induced apoptosis through autophagy induction. *Neuroscience, 164*(2), 541-551.
33. Ren, Z., Zhang, R., Li, Y., Li, Y., Yang, Z., & Yang, H. (2017). Ferulic acid exerts neuroprotective effects against cerebral ischemia/reperfusion-induced injury via antioxidant and anti-apoptotic mechanisms in vitro and in vivo. *International journal of molecular medicine, 40*(5), 1444-1456.
34. Sborgi, S. M. S., Fernandes, L. C., Santos, A. G., Ferro, M. M., & Miyoshi, E. (2021). Anxiolytic activity of ferulic acid in the light-dark test in zebrafish. *Research, Society and Development, 10*(11), e582101119894-e582101119894.
35. Sharma, N., Jamwal, S., & Kumar, P. (2016). Beneficial effect of antidepressants against rotenone induced Parkinsonism like symptoms in rats. *Pathophysiology, 23*(2), 123-134.
36. Shen, Z., Liu, P., Sun, Q., Li, Y., Acharya, R., Li, X., & Sun, C. (2021). FTO inhibits UPRmt-induced apoptosis by activating JAK2/STAT3 pathway and reducing m6A level in adipocytes. *Apoptosis, 26*(7), 474-487.
37. Suzuki, A., Yamamoto, M., Jokura, H., Fujii, A., Tokimitsu, I., Hase, T., & Saito, I. (2007). Ferulic acid restores endothelium-dependent vasodilation in aortas of spontaneously hypertensive rats. *American journal of hypertension, 20*(5), 508-513.
38. Swarnkar, S., Singh, S., Mathur, R., Patro, I., & Nath, C. (2010). A study to correlate rotenone induced biochemical changes and cerebral damage in brain areas with neuromuscular coordination in rats. *Toxicology, 272*(1-3), 17-22.
39. Teijido, O., & Dejean, L. (2010). Upregulation of Bcl2 inhibits apoptosis-driven BAX insertion but favors BAX relocalization in mitochondria. *FEBS letters, 584*(15), 3305-3310.
40. Wang, Q., Chen, W., Zhang, B., Gao, Z., Zhang, Q., Deng, H., . . . Shen, X. L. (2022). Perfluorooctanoic acid induces hepatocellular endoplasmic reticulum stress and mitochondrial-mediated apoptosis in vitro via endoplasmic reticulum-mitochondria communication. *Chemico-Biological Interactions, 354*, 109844.
41. Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews Molecular cell biology, 9*(1), 47-59.
42. Youssef, J., Elbi, C., Warren, B., Yourtee, D., Nagarur, R., Molteni, A., . . . Badr, M. (2003). Glucocorticoid-like effects of antihepatocarcinogen Rotenone are mediated via enhanced serum corticosterone levels: Molecular Fitting and Receptor Activation Studies. *Journal of Carcinogenesis, 2*, 2.
43. Yu, J., Xu, S., Wang, W.-X., Deng, W.-H., Jin, H., Chen, X.-Y., . . . Sun, H.-T. (2012). Changes of inflammation and apoptosis in adrenal gland after experimental injury in rats with acute necrotizing pancreatitis. *Inflammation, 35*(1), 11-22.
44. Zduńska, K., Dana, A., Kolodziejczak, A., & Rotsztejn, H. (2018). Antioxidant properties of ferulic acid and its possible application. *Skin pharmacology and physiology, 31*(6), 332-336.