Original Article

The Possible Protective Role of Folic acid on the Myocardial and Gastrocnemius Muscles in cases of Rotenone-Induced Damage in Adult Male Albino Rat: Histological and Immunohistochemical Study

Omnia I. Ismail, Noha A. Rashed, and Hala Mohamed Hassanin

Department of Human Anatomy and Embryology, Faculty of Medicine, Assiut University, Egypt

ABSTRACT

Background: Rotenone is one of the environmental pollutants and has been applied as a pesticide extensively. For more than 150 years, rotenone has been used commercially in agriculture and fishery management.

Aim of the Work: To detect the possible preventive impacts of folic acid in preventing rotenone cytotoxicity in the heart and skeletal muscles.

Material and Methods: Four groups of forty adult male albino rats were randomly selected, and the rats in group I were used as a normal control group. Group II received folic acid orally in a dose of 20 mg/kg body weight for 35 days, group III subcutaneously injected once a day for 35 days at a dosage of 1.3 mg/kg body weight by Rotenone, group IV rats received both rotenone (as group III) and folic acid (as group II). For light microscopic study using hematoxylin and eosin and picrosirius red stain, ultrastructural study, and immunohistochemical investigation using iNOS and TNFα antibodies, heart and gastrocnemius muscle specimens were prepared.

Results: Rotenone treatment caused degeneration and splitting of the cardiac and muscle fibers with loss of striations, wavy arrangement of many muscle fibers, damaged mitochondria, shrunken nuclei of the cardiomyocytes, many collagen fibres between cardiac and muscle fibers, significant decrease in myofibers thickness as well as strong positive cytoplasmic iNOS and TNFα reactions in heart and gastrocnemius muscle. Folic acid co-administration preserved normal histological structure of the heart and gastrocnemius muscle.

Conclusion: Folic acid can improve degeneration and has a protective influence on cardiac and muscular architecture in rats treated with rotenone most likely via mechanisms dependent on iNOS and TNFα.

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Key Words: Folic acid, heart, iNOS, rotenone, skeletal muscle, TNFα.

Corresponding Author: Omnia Ibrahim Ismail, MD, Department of Human Anatomy and Embryology, Faculty of Medicine, Assiut University, Egypt, **Tel.**: +20 10 0207 0846, **E-mail:** omnia.ismail@med.aun.edu.eg

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INTRODUCTION

Rotenone is one of the environmental pollutants and has been applied as a pesticide extensively. For more than 150 years, rotenone has been used commercially in agriculture and fishery management. As a lipophilic organic substance that selectively inhibits the proliferation of neoplastic cells, rotenone has been used for anticancer treatment[1]. It can use to slow the development of fibrosis in chronic renal dysfunction[2].

Although rotenone degrades slowly in the presence of light and air, temperature variations, and soil composition have all been implicated in this instability. The brown tree snake has been found to contain rotenone residues[3]. Strong epidemiological data suggests that rotenone raise the risk of Parkinson's disease[4]. Most studies have been conducted on effect of rotenone on the central nervous system[5,6].

Among the cell types in the body with the highest concentration of mitochondria are cardiomyocytes. Heart failure, myocardial ischemia-reperfusion injury, cardiac hypertrophy, and other heart illnesses are primarily caused by mitochondrial malfunction, which is marked by bioenergetic reduction and oxidative stress[7]. Rotenone can simulate mitochondrial malfunction in *vitro* through inhibition of mitochondrial respiratory complex I[8].

The skeletal muscle constitutes roughly 40% of the total body weight and is essential for both mobility and metabolism[9].

It has been demonstrated that a wide variety of dietary supplements like folic acid enhance mitochondrial health[10]. Recently, numerous studies have demonstrated that folic acid is a material component necessary for cell growth, and has a primary function in the production of purines and thymine during DNA replication[11]. Folate receptor 1 plays a critical role in controlling muscle development, and through it myoblasts can absorb folic acid by means of endocytosis[12].

Nevertheless, the consequence of rotenone on the rat heart and skeletal muscle are unclear. Moreover, despite

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having anti-inflammatory and antioxidant properties, the folic acid doesn't use much more for cardio-protection and myo-protection against rotenone exposure. The present work aims to investigate rotenone's environmental safety and to detect the potential shielding effect of folic acid against rotenone induced damage of albino rat myocardium and gastrocnemius muscle.

MATERIALS AND METHODS

Chemicals

Rotenone (Catalog number: R8875-1G) and folic acid (Catalog number: S579270) were purchased from Sigma Aldrich, St Louis, MO, USA.

Experimental protocol

Forty adult male albino rats, weighing between 200 and 250 grams, were maintained in a temperature-controlled environment with a light-dark cycle of 12 hours. It was free access for food and drink. The rats were divided into four groups of ten each at random. The Negative control group (I) rats received no treatment. The Positive control group (II) rats received folic acid orally in a dose of 20 mg/kg body weight for 35 days[13]. The Rotenone treated group (III) rats were injected subcutaneously once daily for 35 days in a dose of 1.3 mg/kg body weight by Rotenone which was dissolved in sunflower oil[14]. The Rotenone + folic acid treated group (IV): this group received both rotenone (as group III) and folic acid (as group II). Following anesthesia by intraperitoneal injection of sodium thiopental at a dose of 25 mg/kg, every animal was sacrificed. After being cut apart and preserved in 10% formalin, the heart and gastrocnemius muscle of all rats were examined under a light microscope and electron microscope.

Ethics declaration

The CPCSEA and ARRIVE guidelines for animal handling procedures and experimental methodologies were closely followed in this study[15]. The Ethics Committee at Faculty of Medicine, Assiut University in Egypt gave a permission for study conduction (IRB local approval number: 04-2022-300002).

Light microscopic study

Tissues (heart and gastrocnemius muscle) were fixed in 10% formaldehyde, then dried in increasing ethanol grades, cleaned in xylol, and embedded in paraffin blocks. Then, using a microtome (MICROM HM 340E, WALLDORF, Germany) the paraffin sections about 5 µm thick were prepared. Sections stained with hematoxylin and eosin (H&E) to examine the overall architecture of the myocardium and gastrocnemius muscle and picrosirius red to discover the distribution of the collagen fibers. The stained slides were examined and photographed.

Ultrastructural study

For electron microscopic study, small pieces (1 mm^3) from the heart and gastrocnemius muscle were taken and fixed in 5% glutaraldehyde and then in 1% osmium tetroxide. Then, they were dehydrated, and penetrated with epoxy glue[16]. Toluidine blue staining was applied after the specimens are processed to create semithin sections (1µm). The tissues were placed on grids for electron microscopy, and lead citrate and uranyl acetate were utilized as contrast agents. Grids were examined using an electron microscope (TEM JEOL JEM- 100 SX, Jeol, Tokyo, Japan), electron micrographs were taken.

Immunohistochemical study

Following two days of fixation[17] in 10% neutral-buffered formalin, the myocardium and gastrocnemius tissues from each group were cleaned, dried, and paraffin-embedded. Five µm-cut paraffin sections were fitted on coated slides. After that, each slide underwent several xylene treatments to deparaffinize it, and it was then rehydrated in graded alcohols. For ten minutes, the slides were exposed to 0,01mol/l citrate buffer (pH 6.0) in order to reveal the antigen. Endogenous peroxidase activity was eliminated in the sections by incubating them in 0.3 percent H_2O_2 for 30 minutes. For reduction the nonspecific immunoreactions, the sections were blocked with 5% horse serum for two hours at room temperature. Then, slides were first treated for 18–20 hours at 4ºC with the primary antibodies (1:100 polyclonal rabbit inducible nitric oxide synthase iNOS (Catalog#MA5-17139 Thermo Fisher Scientific, CA, USA) and tumor necrosis factor alpha TNFα (Catalog #PA1-40281, Thermo Fisher Scientific, CA, USA), followed by a wash and an additional incubation with biotinylated secondary antibodies and 0.05 percent diaminobenzidine. Mayer's hematoxylin was utilized as a counterstain then the slides were dehydrated, cleared, and mounted[17]. The same procedure was used for the negative controls, but no primary antibody was used. The stomach was employed as positive controls for TNFα immunohistochemical markers, whereas the lung was used for iNOS.

Morphometric and Statistical studies

In the different studied groups, Image-J software v1.51a 2016 (NIH, Bethesda, MD, USA) was employed to quantify the thickness of myofibers in the heart and gastrocnemius muscle as well as area percent of the collagen fibres, positive reaction of iNOS and TNFα utilizing ten non-overlapping fields per specimen with an objective lens that magnifies x40.

The final data was shown as mean ± standard deviation (SD). The one-way ANOVA test was applied to assess the groups' statistical significance. A *p-value* of less than 0.05 showed that the data were significant.

RESULTS

When the negative control group and the positive control group underwent light and electron microscopic examination, similar morphological results were discovered.

The effect of Rotenone and Folic acid on the histological structure of the heart

Light microscopic results

Using H & E stain

Light microscopic evaluation of the group I demonstrated typical architecture of the branching and anastomosing myofibers with typical transverse striations and intercalated discs. The thin layers of connective tissue separated the myofibers and contained fibroblasts with flat nuclei. Vesicular nuclei of the cardiomyocytes were detected (Figure 1a).

On the contrast, the group III showed wavy arrangement of thin myofibers with loss of their striations and intercalated discs. Vacuolated nuclei of the cardiomyocytes, eosinophilic hyaline materials and dilated blood vessels were noticed (Figure s 1 b,c).

Interestingly, the group IV showed restoring the typical architecture of the branching and anastomosing myofibers with typical transverse striations. The thin layer of connective tissue separated the myofibers and contained fibroblasts with flat nuclei. Vesicular nuclei of the cardiomyocytes, dilated blood vessels and extravasation of the red blood cells were detected (Figure 1d).

Using picrosirius red stain

Assessment of picrosirius red stain in the group I showed few collagen fibres between cardiac myofibers (Figure 2a).

However, the group III demonstrated many collagen fibres between cardiac myofibers (Figure 2b).

While, in the group IV few collagen fibres between cardiac myofibers like the control group were observed (Figure 2c).

A significant variation was found in the area percent of the collagen fibers between the different groups (Table 1).

Electron microscopic results

Ultrastructural examination of the cardiac tissues in the group I demonstrated parallel lines of regularly spaced cardiac myofibers displayed rows of many mitochondria placed in between the myofibers, alternating dark bands and light bands, and regular Z lines in-between light bands. Also, the presence of normal appearing intercalated discs, large oval vesicular nuclei of the cardiomyocytes were observed (Figures 3 a,b).

On the other hand, the group III showed thinning and damage of the cardiac myofibers with wide spaces between them. Damaged mitochondria, and shrunken nuclei of the cardiomyocytes were observed (Figures 3 c,d).

Nevertheless, the group IV showed normal appeared cardiac myofibers displayed rows of many mitochondria placed in between the myofibers, alternating dark bands and light bands, and regular Z lines in-between light bands. The presence of normal appeared intercalated discs and large oval vesicular nuclei of the cardiomyocytes were noticed. Atrophy and disruption of few myofibers were observed (Figures 3 e,f).

The effect of Rotenone and Folic acid on iNOS and TNFα immunohistochemistry in the heart

Assessment of immunohistochemical staining of the heart in the group I showed negative iNOS reaction (Figure 4a).

However, the group III showed sever positive cytoplasmic iNOS reaction (Figure 4b).

Surprising, in the group IV moderate positive cytoplasmic iNOS reaction was detected (Figure 4c).

A significant difference was found in the area percent of iNOS reaction between the different groups (Table 1).

Additionally, the group I showed mild positive cytoplasmic TNFα reaction in the cardiac tissues (Figure 5a).

On the contrary, the cardiac tissues of the group III revealed sever positive cytoplasmic TNFα reaction (Figure 5b).

While the group IV showed moderate positive cytoplasmic TNFα reaction (Figure 5c).

A significant variation was found in the area percent of TNFα reaction between the different groups (Table 1).

The effect of Rotenone and Folic acid on the thickness of cardiac myofibers

Morphometrical analysis of the thickness of the cardiac myofibers in the group III showed a significant decline with comparison to the group I, while the group IV revealed a significant elevation compared to the group III (Table 1).

The effect of Rotenone and Folic acid on the histological structure of the gastrocnemius muscle

Light microscopic results

1- Using H& E stain

Light microscopic evaluation of the gastrocnemius muscle in the group I revealed cylindrical parallel non-branched muscle fibers with many flat nuclei underneath the sarcolemma, acidophilic sarcoplasm and regular striations. The muscle bundles were divided by connective tissue called perimysium (Figure 6a).

Conversely, the group III showed splitting of the muscle fibers with loss of striations and nuclei. Pale stained areas and degenerated areas in many muscle fibres were also observed (Figure 6b). In addition, wavy arrangement of the muscle fibers, haemorrhage inside some muscle fibers and sarcolemma disruption were also seen (Figure 6c).

Whereas sections from the group IV revealed parallel non-branched muscle fibers with many flat nuclei underneath the sarcolemma, acidophilic sarcoplasm and regular striations like the control, but wavy arrangement of some muscle fibers was noticed (Figure 6d).

2- Using picrosirius red stain

Assessment of picrosirius red stain in the group I showed few collagen fibres between the muscle fibers of the gastrocnemius muscle (Figure 7a).

On contrast, the group III showed many collagen fibres between the muscle fibers and around the blood vessels (Figures 7 b,c).

The group IV demonstrated few collagen fibres between the muscle fibers like the control group (Figure 7d).

A significant variation was found in the area percent of the collagen fibers between the different groups (Table 2).

Electron microscopic results

Examination of the ultra-thin sections of the gastrocnemius muscle in the group I demonstrated parallel lines of regularly spaced myofibers displayed rows of many mitochondria placed in between the myofibers, alternating dark bands and light bands as well as regular Z lines in-between light bands. Aso, the presence of elongated peripheral heterochromatic nuclei was seen (Figure 8a).

However, the group III showed disarrangement of the myofibers with degenerated areas, accumulations of damaged vacuolated mitochondria of different sizes and shape as well as vacuolated nucleoplasm of peripheral nuclei (Figures 8 b,c).

The group IV revealed restoration of parallel lines of regularly spaced myofibers which displayed alternating dark bands and light bands as well as regular Z lines in-between light bands, but mitochondria appeared damaged (Figure 8d).

The effect of Rotenone and Folic acid on iNOS and TNFα immunohistochemistry in the gastrocnemius muscle

Assessment of immunohistochemical staining of the gastrocnemius muscle in the group I showed mild positive cytoplasmic iNOS reaction (Figure 9a) and TNFα reaction (Figure 10a).

However, the group III showed sever positive cytoplasmic iNOS reaction (Figure 9b) and TNFα reaction (Figure 10b).

Interestingly, the group IV showing moderate positive cytoplasmic iNOS reaction (Figure 9c) and TNFα reaction (Figure 10c).

significant variation was found in the area percents of iNOS reaction and TNFα reaction between the different groups (Table 2).

The effect of Rotenone and Folic acid on the thickness of the myofibers in the gastrocnemius muscle

Morphometrical analysis of the thickness of the myofibers of the gastrocnemius muscle in the group III showed a significant decline with comparison to the control group, while the group IV demonstrated a significant elevation compared to the group III (Table 2).

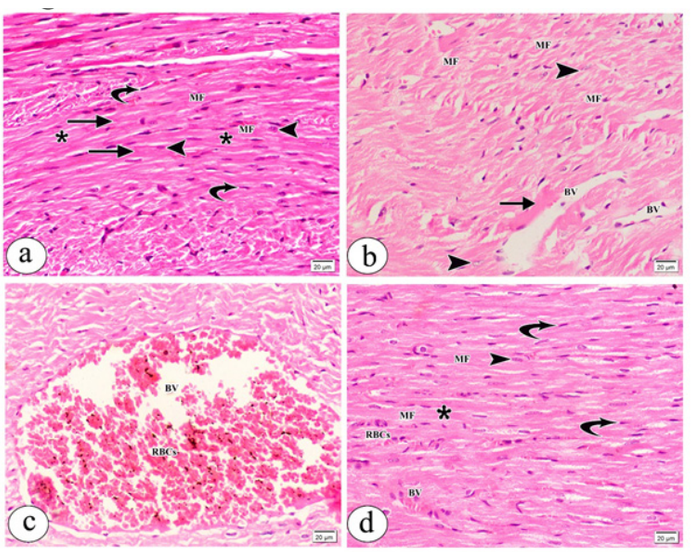

Fig. 1: The photomicrograph of the longitudinal sections in the heart of (a) The control group showing typical architecture of the branching and anastomosing myofibers (MF) with typical transverse striations (asterisk) and intercalated discs (arrow). The thin layer of connective tissue separates the myofibers and contains fibroblasts with flat nuclei (curved arrow). Vesicular nuclei (arrowhead) of the cardiomyocytes are detected. (b) The Rotenone treated group showing wavy arrangement of thin myofibers (MF) with loss of their striations and intercalated discs. Vacuolated nuclei (arrowhead) of the cardiomyocytes are detected. An eosinophilic hyaline material (arrow) and dilated blood vessels (BV) are noticed. (c) The Rotenone treated group showing dilated blood vessels (BV) congested with red blood cells (RBCs). (d) The Rotenone+ folic acid treated group showing restoring the typical architecture of the branching and anastomosing myofibers (MF) with typical transverse striations (asterisk). The thin layer of connective tissue separates the myofibers and contains fibroblasts with flat nuclei (curved arrow). Vesicular nuclei (arrowhead) of the cardiomyocytes are detected. Notice dilated blood vessels (BV) and extravasation of the red blood cells (RBCs). (H& E, X400, scale bar=20 µm)

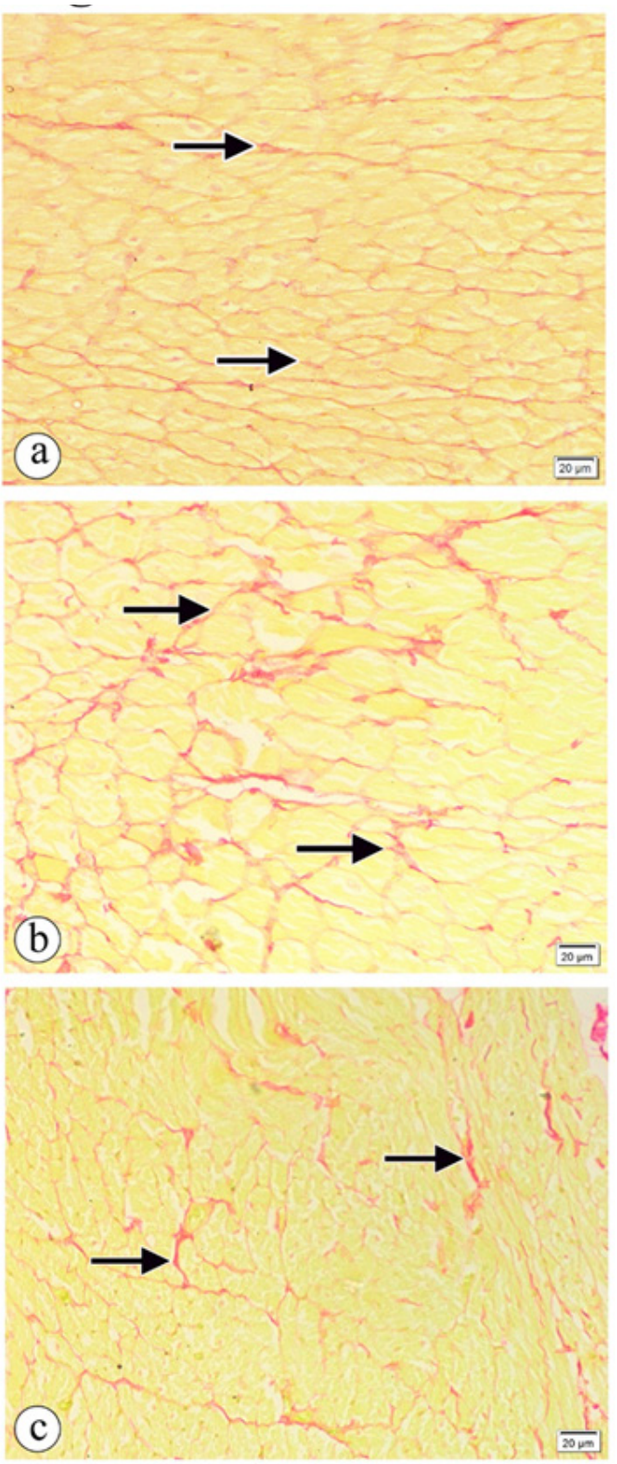

Fig.2: The photomicrographs of the sections of the heart in (a) the control group showing few collagen fibres (arrow) between cardiac myofibers. (b) The Rotenone treated group showing many collagen fibres (arrow) between cardiac myofibers. (c) The Rotenone+ folic acid treated group showing few collagen fibres (arrow) between cardiac myofibers like the control group. (Picrosirius red × 400, Scale bar = 20 µm)

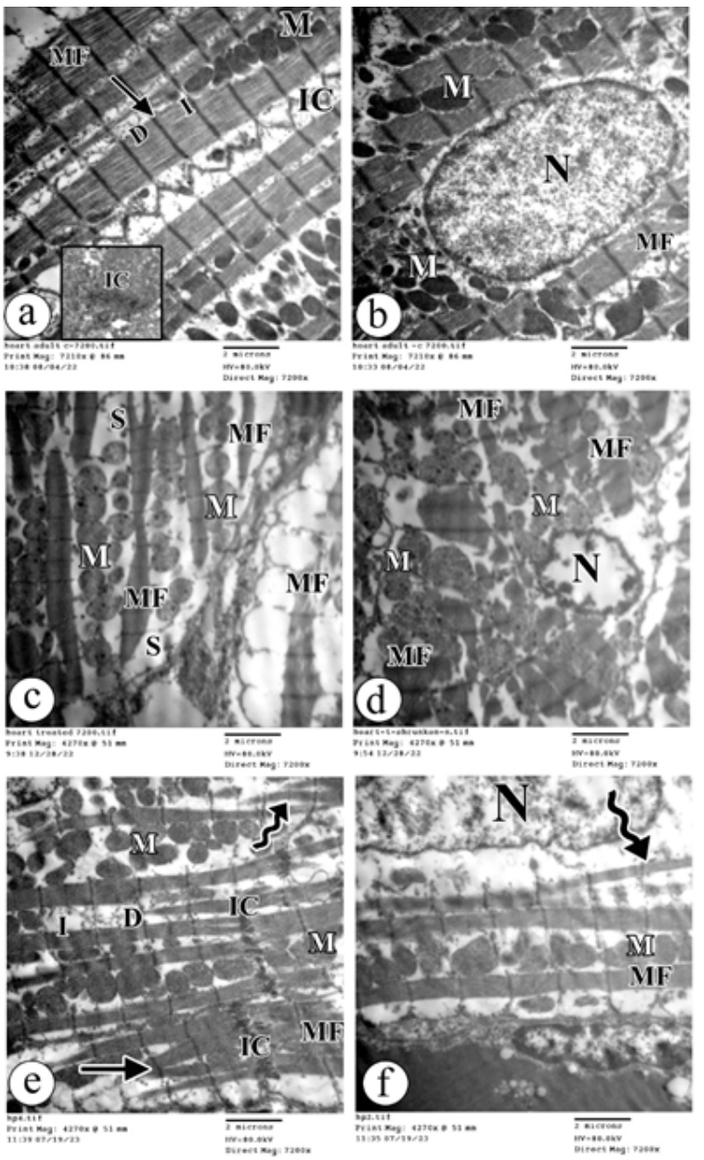

Fig. 3: The electron photomicrographs of the ultra-thin sections of the heart in (a) the control group showing parallel lines of regularly spaced cardiac myofibers (MF) display a row of many mitochondria (M) placed in between the myofibers, alternating dark band (D) and light band (I), and regular Z lines (arrow) in-between light band. Note the presence of the intercalated disc (ID). Inset: showing normal appeared intercalated disc (ID). (b) the control group showing large oval vesicular nucleus (N) of the cardiomyocytes, normal arranged myofibers (MF) and many normal mitochondria (M). (c) The Rotenone treated group showing thinning and damage of the cardiac myofibers (MF) with wide spaces (S) between them. Damaged mitochondria (M) are observed. (d) The Rotenone treated group showing shrunken nucleus (N) of the cardiomyocytes, disrupted cardiac myofibers (MF) and damaged mitochondria (M). (e) The Rotenone+ folic acid treated group showing normal appeared cardiac myofibers (MF) display a row of many mitochondria (M) placed in between the myofibers, alternating dark band (D) and light band (I), and regular Z lines (arrow) in-between light band. Note the presence of the intercalated disc (ID). Atrophy and disruption of few myofibers (wavy arrow) are observed. (f) The Rotenone+ folic acid treated group showing large oval vesicular nucleus (N) of the cardiomyocytes, and many normal mitochondria (M). Some normal arranged myofibers (MF) are seen. Atrophy of other myofibers (wavy arrow) is observed. (TEM, ×7200, scale bar =2 µm)

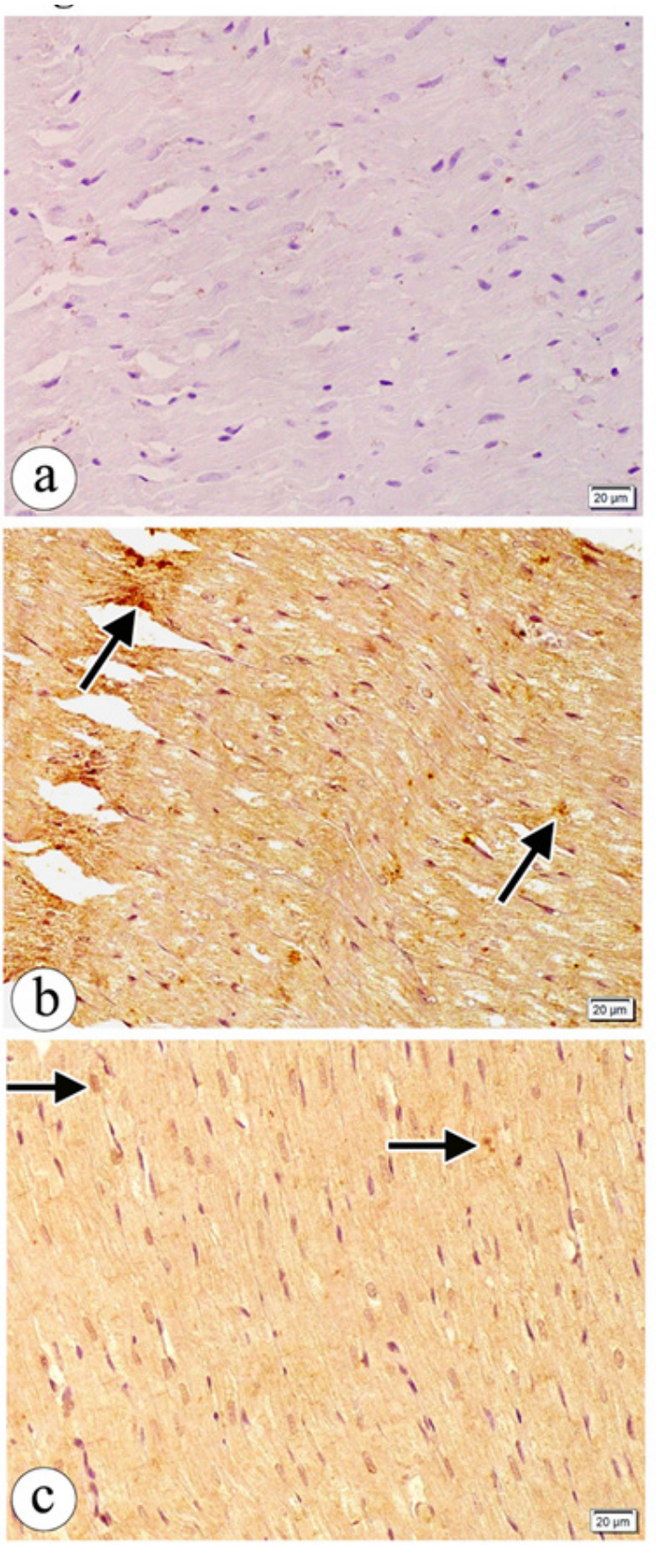

Fig. 4: The photomicrographs of the heart in (a) the control group showing negative iNOS reaction. (b) The Rotenone treated group showing sever positive cytoplasmic iNOS reaction (arrow). (c) The Rotenone+ folic acid treated group showing moderate positive cytoplasmic iNOS reaction (arrow). (iNOS counterstained with haematoxylin, ×400, scale bar=20 µm)

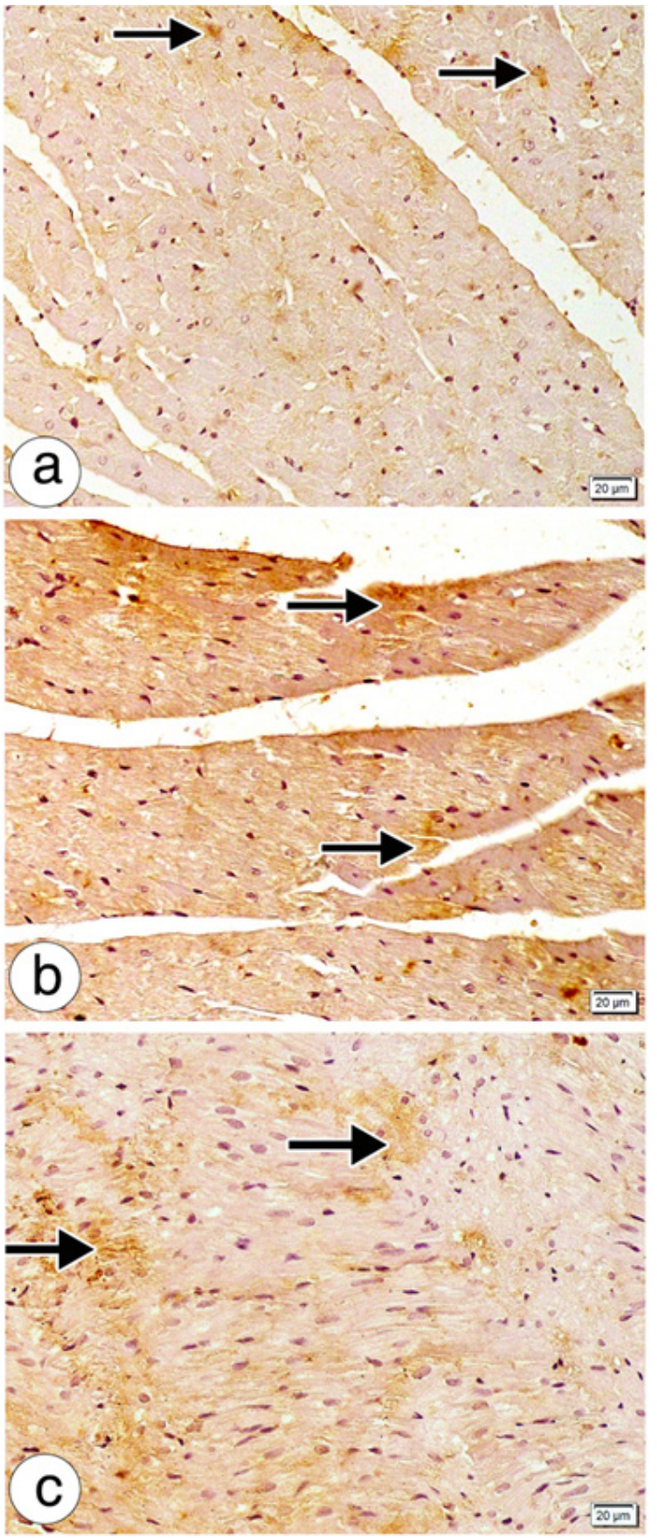

Fig. 5: The photomicrographs of the heart in (a) the control group showing mild positive cytoplasmic TNFα reaction (arrow). (b) The Rotenone treated group showing sever positive cytoplasmic TNFα reaction (arrow). (c) The Rotenone+ folic acid treated group showing moderate positive cytoplasmic TNFα reaction (arrow). (TNFα counterstained with haematoxylin, ×400, scale bar=20 µm)

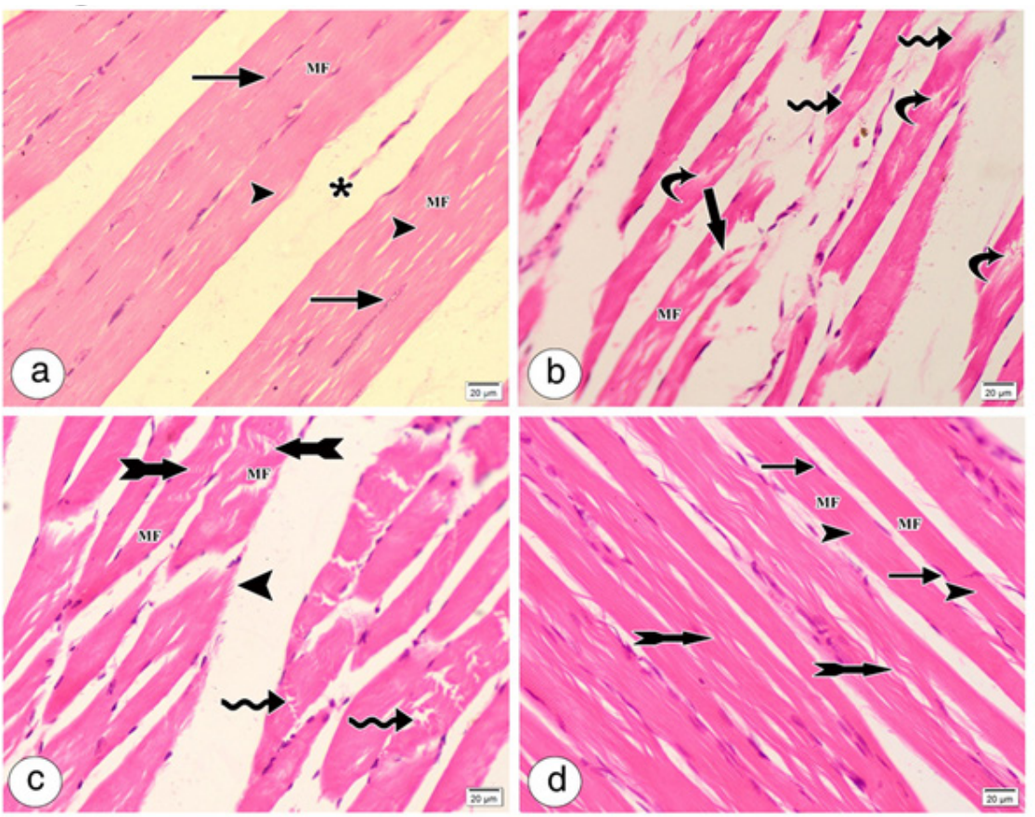

Fig. 6: The photomicrograph of the longitudinal sections in the gastrocnemius muscle of (a) The control group showing cylindrical parallel non-branched muscle fibers (MF) with many flat nuclei (arrow) underneath the sarcolemma, acidophilic sarcoplasm and regular striations (arrowhead). The muscle bundles are divided by connective tissue called perimysium (asterisks). (b) The Rotenone treated group showing splitting (thick arrow) of the muscle fibers (MF) with loss of striations and nuclei. Pale stained areas (wavy arrow) and degenerated areas (curved arrow) in many muscle fibres are observed. (c) The Rotenone treated group showing wavy arrangement (tailed arrow) of the muscle fibers (MF). Note the presence of haemorrhage (wavy arrow) inside some muscle fibers and sarcolemma disruption (arrow head). (d) The Rotenone+ folic acid treated group showing parallel non-branched muscle fibers (MF) with multiple flat nuclei (arrow) underneath the sarcolemma, acidophilic sarcoplasm and regular striations (arrowhead) like the control. Wavy arrangement (tailed arrow) of some muscle fibers is noticed. (H& E, X400, scale bar=20 µm)

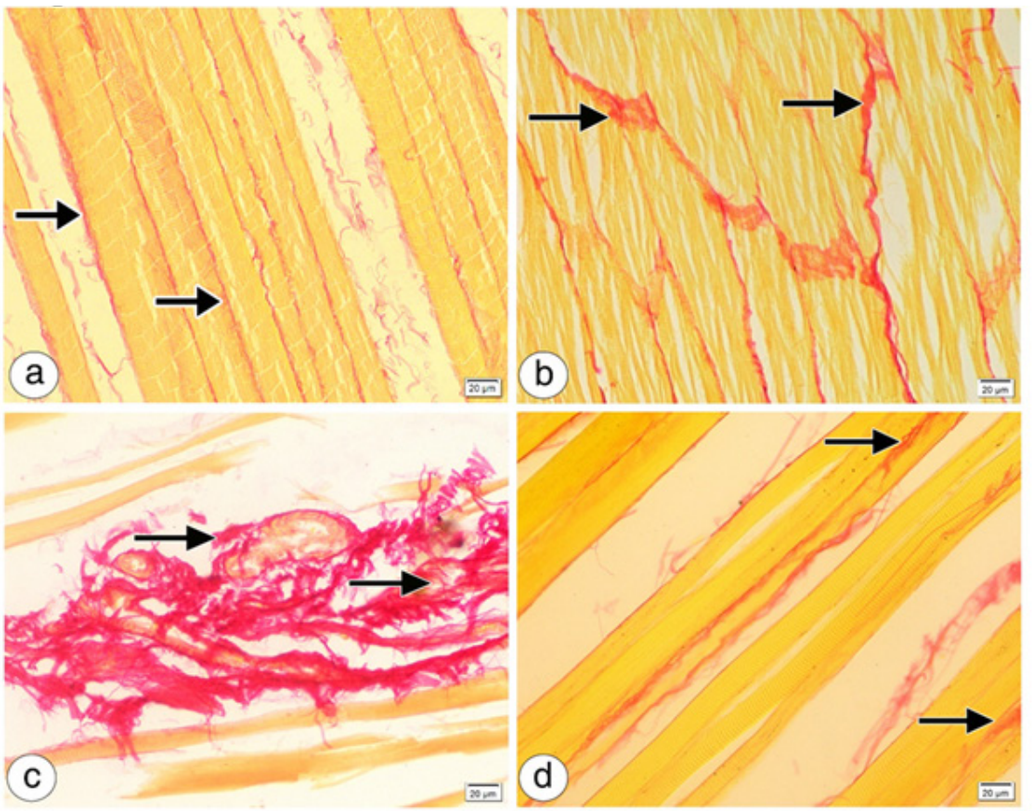

Fig. 7: The photomicrographs of the sections of the gastrocnemius muscle in (a) the control group showing few collagen fibres (arrow) between the muscle fibers. (b) The Rotenone treated group showing many collagen fibres (arrow) between the muscle fibers. (c) The Rotenone treated group showing many collagen fibres (arrow) around the blood vessels. (d) The Rotenone+ folic acid treated group showing few collagen fibres (arrow) between the muscle fibers like the control group. (Picrosirius red × 400, Scale bar = 20 µm)

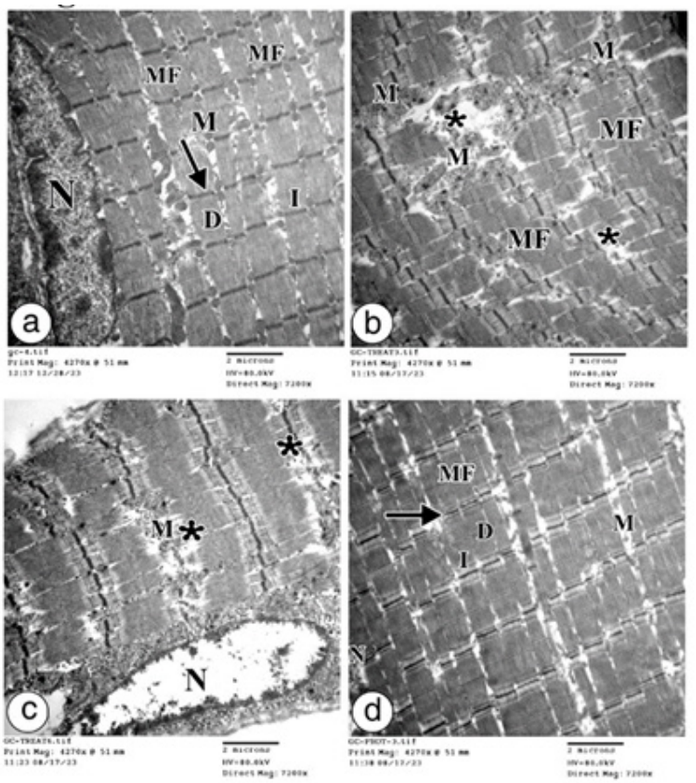

Fig. 8: The electron photomicrographs of the ultra-thin longitudinal sections of the gastrocnemius muscle in (a) the control group showing parallel lines of regularly spaced myofibers (MF) display a row of many mitochondria (M) placed in between the myofibers, alternating dark band (D) and light band (I), and regular Z lines (arrow) in-between light band. Note the presence of elongated peripheral heterochromatic nucleus (N). (b) The Rotenone treated group showing disarrangement of the myofibers (MF) with degenerated areas (asterisk) and accumulations of damaged vacuolated mitochondria (M) of different sizes and shape. (c) The Rotenone treated group showing degenerated areas (asterisk) of the myofibers, damaged mitochondria (M) and vacuolated nucleoplasm of peripheral nucleus (N). (d) The Rotenone+ folic acid treated group showing restoration of parallel lines of regularly spaced myofibers (MF) display alternating dark band (D) and light band (I), and regular Z lines (arrow) in-between light band. Mitochondria (M) appear damaged. Note the presence of part of peripheral nucleus (N). (TEM, ×7200, scale bar =2 μm)

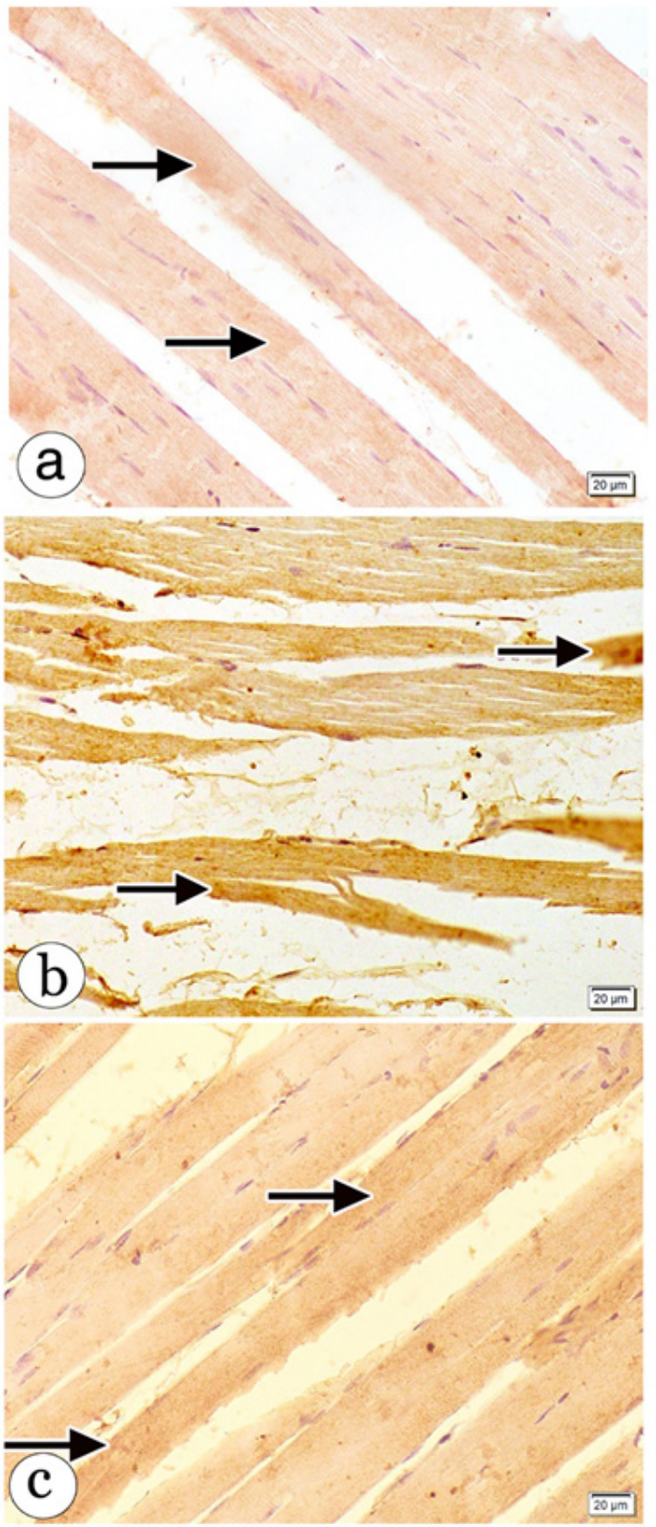

Fig. 9: The photomicrographs of the gastrocnemius muscle in (a) the control group showing mild positive cytoplasmic iNOS reaction (arrow). (b) The Rotenone treated group showing sever positive cytoplasmic iNOS reaction (arrow). (c) The Rotenone+ folic acid treated group showing moderate positive cytoplasmic iNOS reaction (arrow). (iNOS counterstained with haematoxylin, ×400, scale bar=20 µm)

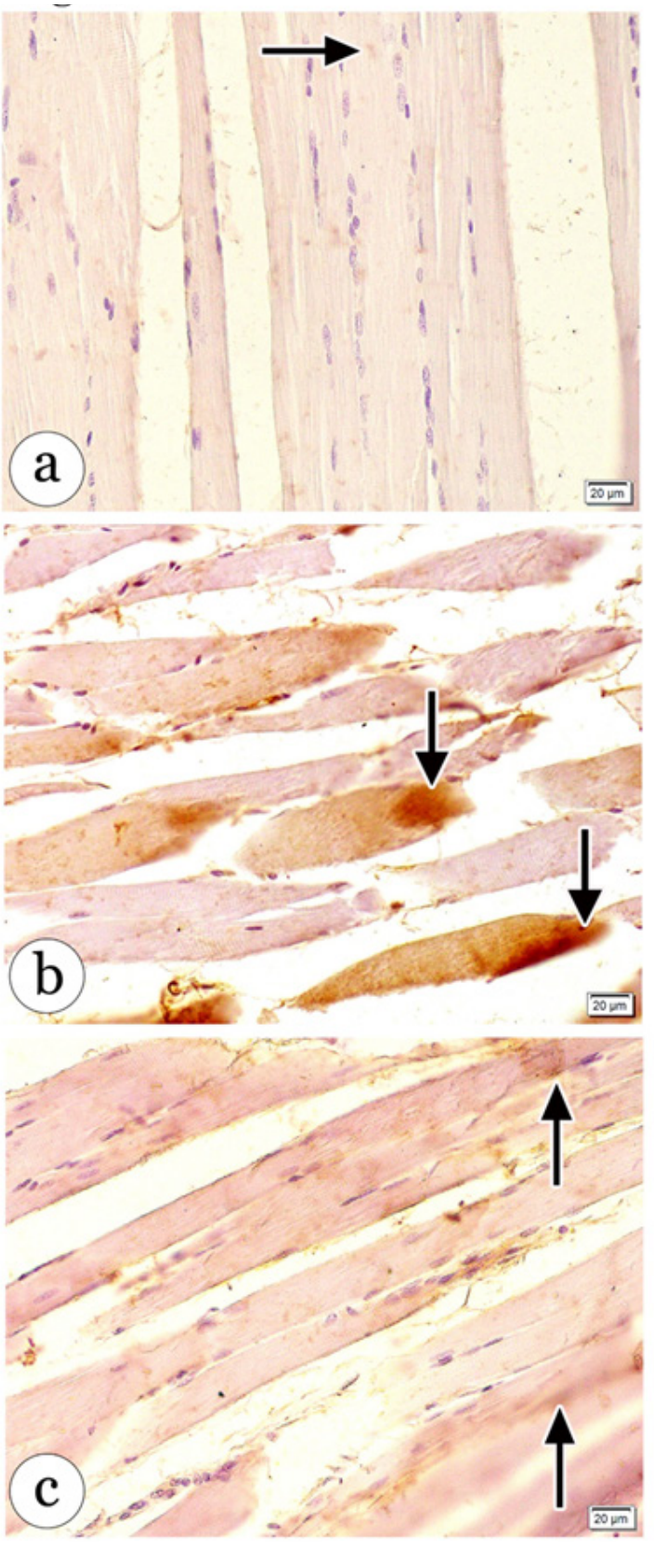

Fig. 10: The photomicrographs of the gastrocnemius muscle in (a) the control group showing mild positive cytoplasmic TNFα reaction (arrow). (b) The Rotenone treated group showing sever positive cytoplasmic TNFα reaction (arrow). (c) The Rotenone+ folic acid treated group showing moderate positive cytoplasmic TNFα reaction (arrow). (TNFα counterstained with haematoxylin, ×400, scale bar=20 µm)

Table 1: The comparison of the thickness of cardiac myofibers (µm), the area percent (area %) of collagen deposition, the area percent (area %) of iNOS reaction and area percent (area %) of TNFα reaction in the heart

Groups	Thickness of cardiac myofibers (µm)	Area % of collagen deposition	Area % of iNOS reaction	Area % of TNFα reaction
Group I	2.008±0.50	8.873± 0.83	2.323± 0.66	3.508± 0.77
Group II	1.903±0.60	9.329± 1.82	2.200± 0.67	3.261± 0.90
Group III	0.881±0.30 [a,b]	19.28± 1.16[a,b,d]	18.40± 0.89 [a,b,d]	17.56±0.91 [a,b,d]
Group IV	1.342±0.37 [a,b]	10.85± 1.06 [a,b,c]	8.494±0.96 [a,b,c]	11.41± 0.77 [a,b,c]
P-value	<0.0001*	<0.0001*	<0.0001*	<0.0001*

Data are represented as Mean ± SD.* means statistically significant difference
a statistically significant as compared with the group I, $P< 0.05$
b statistically significant as compared with the group II, $P< 0.05$
c statistically significant as compared with the group III, $P< 0.05$
d statistically significant as compared with the group IV, $P< 0.05$

Table 2: The comparison of the thickness of myofibers (µm) , the area percent (area %) of collagen deposition, the area percent (area %) of iNOS reaction and area percent (area %) of TNFα reaction in the gastrocnemius muscle

Groups	Thickness of myofibers (µm)	Area % of collagen deposition	Area % of iNOS reaction	Area % of TNFα reaction
Group I	1.348±0.36	7.189± 0.92	11.67± 0.81	12.90± 0.75
Group II	1.242±0.42	8.850± 1.04	12.27± 0.85	11.20± 1.13
Group III	0.478±0.12 [a,b,d]	16.00± 0.96 [a,b,d]	25.91± 1.02 [a,b,d]	29.20±1.03 [a,b,d]
Group IV	0.844±0.09 [a,b,c]	9.419± 0.99 [a,b,c]	13.66 ±0.96 [a,b,c]	18.66± 1.07 [a,b,c]
P-value	<0.0001*	<0.0001*	<0.0001*	<0.0001*

Data are represented as Mean ± SD.* means statistically significant difference
a statistically significant as compared with the group I, $P< 0.05$
b statistically significant as compared with the group II, $P< 0.05$
c statistically significant as compared with the group III, $P< 0.05$
d statistically significant as compared with the group IV, $P< 0.05$

DISCUSSION

Rotenone is a natural insecticide derived from Leguminosa plants and widely used to induce Parkinson's disease models in *vivo*. Also, it is mostly used in agriculture to paralyze insects and other pests by interfering with their neurological systems[5].

Several studies demonstrated the negative effects of rotenone on the liver[18], the prefrontal cortex[5], the midbrain[19], hippocampus[20] and intestine[21].

People's concern over pesticide residues has grown in response to the severity of the food safety issue. Rotenone is a traditional insecticide; studies of its residue have been published. It is unclear, though, if exposure to rotenone affects the heart and skeletal muscles and if folic acid can protect them against any damage. Therefore, this study was designed to assess effect of rotenone exposure on the heart and skeletal muscle as well as to evaluate folic acid's potential cardio-protective and myo-protective effects. The present findings were of particular interest as they offered for the first-time convincing proof of the preventive and protective abilities of folic acid against the cardiotoxic and myotoxic effects of rotenone exposure in adult rats.

The present work found that rotenone induced structural changes in the heart like wavy arrangement of thin myofibers with loss of their striations and intercalated discs. In addition, many collagen fibres between cardiac myofibers, thinning and damage of the cardiac myofibers with wide spaces between them, damaged mitochondria, shrunken nuclei of the cardiomyocytes, as well as strong positive cytoplasmic iNOS and TNFα reactions in the heart.

Regarding effect of rotenone on the gastrocnemius muscle, we found that splitting of the muscle fibers, degenerated areas in many muscle fibres, wavy arrangement of many muscle fibers, haemorrhage inside some muscle fibers, sarcolemmal disruption, accumulations of damaged vacuolated mitochondria, and vacuolated nucleoplasm of peripheral nuclei. Additionally, many collagen fibres between muscle fibers as well as strong positive cytoplasmic iNOS and TNFα reactions were observed.

Interestingly, folic acid co-administration led to preserve the typical architecture of the myofibers with typical transverse striations in the heart and the gastrocnemius muscle. Moreover, few collagen fibres between cardiac and muscle fibers like the control group, decrease in the positive reaction of iNOS and TNFα.

In line with our data, using Masson's trichrome, H&E, and PAS staining and transmission electron microscopy prior authors found that rotenone led to myocardial fibrosis, apoptosis, structural and electrical cardiac alterations in rats[2].

In the same context, early authors found that rotenone reduced the amount of ATP produced by cardiomyocytes, produced mitochondrial reactive oxygen species, increased

lactate dehydrogenase release, and significantly cell death in a dose-dependent manner[8].

Our data are consistent with a previous study of Gonchar *et al*. (2021) who demonstrated that administration of rotenone sets off a chain of events that eventually result in hyperfunction of the heart's mitochondria, including increases in the generation of superoxide anion and thiobarbituric acid - reactive substances (TBARS) that are the subsequent products of lipid peroxidation. Protein oxidative modification, reduced glutathione (GSH) concentration, glutathione/oxidized glutathione ratio (GSH/GSSG), and glutathione peroxidase (GPx) activity were also present during these events with rotenone exposure. These changes may be linked to DJ-1 gene and DJ-1 protein deficit[22].

In addition, using immunoblotting and immunofluorescence studies, cardiomyocytes treated with rotenone demonstrated an increase in the autophagy marker LC3-II[23]. Moreover, a previous work found that rotenone exposure in rats caused cardiovascular impairments, decreased sympathetic activity, mean blood pressure and plasma catecholamine levels[24].

Observed present cardiac fibrosis is a developing condition process which result in long-term heart function deterioration. Fibrotic tissue patches impair heart function, obstruct coordinated electrical conduction, and raise the risk of arrhythmias. The main cell responsible for cardiac fibrosis is the cardiac fibroblast[25]. According to cumulative evidence, oxidative stress was responsible for the development of cardiac fibrosis since levels of the protein carbonyls, lipid peroxidation product, and superoxide anion were increased significantly[26].

According to the interpretation of other literatures, fibroblasts were activated by tumor growth factor-beta (TGF-β1), which in turn led to cardiac fibrosis. This resulted in structural deposition of the extracellular matrix proteins[27]. Also, the same mechanism may be the cause of observed fibrosis in the skeletal muscle[28].

Deformed intercalated discs were seen in the current study when the myocardium from the rotenone treated group was evaluated. In most of the affected locations, deformed intercalated discs result in a loss of effective contraction force[29]. Desmosomes, adherens junctions, gap junctions, and ion channels make up the structure of the intercalated disc. Desmosomes and adherens junctions are two distinct junctional structures that mechanically unite cells by connecting with their cytoskeleton[30].

One possible explanation for the current observed vacuoles formation in the cardiac tissue is an enlargement of the cell membranes brought on by an imbalance in electrolytes and water. Nonetheless, the rough sarcoplasmic reticulum's dilatation may be the cause of this[31].

It was suggested that the main mechanism of cardiac toxicity associated with rotenone treatment is the suppression of respiratory complex I of mitochondria. The respiratory complex of the mitochondria is what ultimately decides the quality of the mitochondria and the fate of the cardiomyocytes. Complex I of mitochondria deficit is present at the beginning and during the progression of ischemia/reperfusion damage, heart hypertrophy, and problems related to diabetes[8].

After reviewing our light and electron microscopic data, it was determined that rotenone had harmful effects on the structure of the gastrocnemius muscle and led to muscular injury. One possible explanation for splitting of muscle fibers could be inadequate exchange of metabolites and oxygen to the larger and hypertrophied fibers as mentioned by[32].

The present data regarding the effect of rotenone on the gastrocnemius muscle support the previous findings of the authors who found that rotenone reduced mitochondrial complex I and II activity in the skeletal muscle tissues, minor alterations in the activity of antioxidant enzymes and the lipidome of the skeletal muscle as well as motor disabilities[33].

In addition, previous researchers reported that rotenone treatment caused motor impairment and muscle incoordination examined by open field and rotarod tests[34]. Another study had revealed that continuous rotenone administration for 35 days caused oxidative stress in the brain, muscle rigidity, catalepsy, as well as a decrease in body weight, rearing behaviour, and locomotor activity[35]. Motor impairments caused by rotenone that resemble Parkinson's disease symptoms[36].

It was documented that rotenone reduced the ability of mitochondria to synthesise ATP, and decreased the activity of mitochondrial ATP enzyme[37]. Thus, rotenone caused damage to the mitochondria by generation the oxidative stress[18].

In addition, rotenone worsens the state of oxidative stress, which triggers the activation of the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4) by blocking mitochondrial complex I. Tumor necrosis factor-alpha (TNF-α), interleukin (IL) IL-6, and IL-1β are among the proinflammatory cytokines released when TLR4 and RAGE are stimulated. This enhances the neuroinflammatory cascade by activating nuclear factor kappa B (NF-κB p65)[34].

Our immunohistochemical study demonstrated that rotenone exposure caused a rise in iNOS expression indicating the ability of rotenone to induce apoptosis in the myocardium and gastrocnemius muscle. These data were in agreement with early authors who mentioned that in comparison to the control group, rotenone resulted in an 83.9 percent rise in brain nitric oxide levels[38]. Also, prior authors found that rotenone had the ability to both up- and down-regulate the expression of proteins that promote apoptosis[18]. In addition, early research indicated that long-term rotenone administration raised NO levels in the rat brain's cortical and striatal areas, which significantly harm the nigrostriatal pathway[37].

Noteworthy, stimulation of iNOS increases NO production, which has the potential to influence the inflammatory response at an early stage then generate apoptosis. By means of the enzyme nitric oxide synthase (NOS), nitric oxide is produced from L-arginine[39]. Inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS) are the three isoforms of NOS. Both iNOS and eNOS participate in the catalytic production of NO in the myocardium[40].

Furthermore, growing evidence proves that the term " reactive nitrogen species " (RNS) describes a class of chemicals with strong oxidative activity that are produced when NO interacts with substances that include reactive oxygen species, like peroxynitrite anion. NO depletion is most likely the result of the interaction between NO and uperoxide, which compromises the endothelium-dependent vasodilatory function. Moreover, maintaining healthy cardiovascular and cerebral tissue function depends on a normal NO concentration and its bioavailability[41]. Furthermore, reactive oxygen species (ROS) easily convert NO to peroxynitrite, which damages cardiomyocytes in a secondary manner[39].

The process by which rotenone poisoning occurs in the present study is that oxidative stress weakens or destroys the organelles like the mitochondria and endoplasmic reticulum, which in turn causes the apoptosis[18].

It was postulated the equilibrium between oxidants and antioxidants dictates the cellular redox state. An imbalance between these two, which can lead to necrosis or apoptosis, characterizes the oxidative status of the cell. Despite being a generally nonreactive material, oxygen may be broken down by the body to produce a variety of extremely reactive free radicals, including superoxide anions and hydroxyl radicals[42].

We found that rotenone exposure increased the expression of TNF-α in the heart and skeletal muscles. Therefore, there was a link between rotenone exposure; muscular damage (cardiac and skeletal) and inflammation. This is similar to an early study which reported that rotenone exposure is related to oxidative stress and inflammation in *vivo*[3].

It was documented that a general defensive reaction that aids in the restoration of injured tissues is inflammation. Inflammation begins with activated inflammatory cells releasing inflammatory mediators into the surrounding tissue[43]. One important transcriptional regulator in charge of the inflammatory process is NF-κB. Different proinflammatory cytokines, including TNF-α, can be triggered by NF-κB. TNF binds to both TNFR1 and TNFR2, however only TNFR1 has been shown to have most of its biological action in humans and mice. This is because only TNFR1 has the ability to cause cell death[44].

It is interesting to note that folic acid is a synthetic version of folate. Water-soluble vitamin B9, or folate, is necessary to maintain human health[45]. Folic acid is a vital nutrient that is needed for one-carbon biosynthetic metabolism to promote cell growth and division. Its molecular formula is C19H19N7O6, and it is widely known for its critical function in neural tube formation[46].

Foods like leafy green vegetables naturally contain lower forms of folate. For transportation, in the intestinal lumen folate must hydrolyze into monoglutamate form. The intestinal brush border enzyme glutamate carboxypeptidase II is responsible for converting polyglutamate derivatives of tetrahydrofolate into monoglutamates. The majority of naturally occurring folate that is absorbed is converted in the gut and/or liver to 5-methyltetrahydrofolate (5-MTHF). The conversion of folic acid and natural folate into 5-MTHF is their fate[45]. 5-MTHF is intimately associated with the viability of the embryo and the capacity to sustain DNA methylation patterns in replicating cells. 5-MTHF donates its methyl group to assist DNA methylation via DNA methyltransferase enzymes[47].

Subsequent investigation revealed that folic acid could hasten the regeneration of the skeletal muscles and stimulate the migration of C2C12 cells by means of interaction of Folate receptor 1 with transforming protein RhoA then altered the location of microfilaments to enhance C2C12 cell motility by stimulating the RhoA/ROCK/LIMK/cofilin signaling pathway[12].

Due to its anti-inflammatory and antioxidant qualities, folic acid is garnering attention as a possible treatment for muscular diseases. As a result, we investigated the impact of co-administering folic acid in the heart and skeletal muscle of the rat treated with rotenone. We found that folic acid mitigated the increase in the level of iNOS, TNFα, the apoptosis and inflammation. It also improved the histological structure of the heart and gastrocnemius muscle.

Our findings are in harmony with a former study showed that treatment with folic acid improved heart structure, reduced the intercellular spaces, enhance the survival of cardiac cells and lowered the level of apoptotic proteins in the heart of mice with triple-transgenic late-stage Alzheimer's disease[48].

Besides, folic acid supplementation has ability to decrease reactive oxygen species production, reduce Caspase-3 activation, as well as improve mitochondrial dynamics by increasing ATP synthesis and mitochondrial membrane potential in the mice[10].

Moreover, folic acid led to notable improvements in blood pressure, heart rate, cardiac enzymes, histopathological alterations, collagen fiber accumulation, oxidative stress, inflammation, and programmed cell death in diabetic cardiomyopathy rat model[49].

In a study on the isolated rat heart, it was discovered that perfusion of the heart with folic acid greatly enhanced the flow through the coronary arteries and decreased the production of superoxide anion, but also increased the index of lipid peroxidation[50].

Despite the fact that vitamin B6 and folic acid are employed in the primary prevention of coronary heart disease, it was observed a considerable thickening of the left ventricular wall diameter resulting from B6 and folic acid treatment. It may be attributed to direct effects of B6 and folic acid on the heart or as a result of cardiac remodelling occurring after coronary heart disease[51].

Moreover, other authors reported that the findings suggest that specific dietary choices like folic acid can control muscle level of Interleukin-6 and Tumor Necrosis Factor-alpha , hence decreasing the detrimental effects of chronic inflammation in the muscles that result in chronic disease[52].

In the same context, folic acid was discovered to lessen the number of injured muscle fibers per field by 20% when histological sections were analysed. In comparison to rats that were not given folic acid, the Rotarod test demonstrated that folic acid also improved muscle function when it is determined by how long the animals run along the rod[53].

On the contrary, it was found that high dose of folic acid treatment in the rats causes degeneration of cardiomyocytes, boosted mononuclear cellularity, apoptosis, clearly lowers coronary flow, depresses the contractility of the heart, as well as increased activity of cardiac myeloperoxidase, TNF-alpha and IL-1 secondary to renal ischemia[54].

Taken together, folic acid, in our opinion, is the promising agent that appears to preserve normal histological structure of the heart and skeletal muscle and decrease iNOS and TNFα level in rotenone treated rats. More data on the therapeutic effectiveness of folic acid against rotenone- induced cardiac and muscular toxicity need from appropriate animal models and clinical studies in the future.

CONCLUSION

Our findings suggest that folic acid has a protective influence on cardiac and muscular architecture in rats treated with rotenone most likely via mechanisms dependent on iNOS and TNFα. As a result, we concluded that folic acid can be taken by persons exposed to rotenone in order to potentially mitigate the histological changes caused by the drug. To support this, additional investigation is necessary. Also, increasing rotenone's efficiency and lowering its risk in the future is something that deserves our consideration.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

الدور الوقائي المحتمل لحمض الفوليك على عضلة القلب وعضلات الساق في حالات الضرر الناتج عن الروتينون في ذكور الجرذان البيضاء البالغة: دراسة نسيجية وهستوكيميائية مناعية

امنية ابراهيم اسماعيل، نهي احمد راشد، هالة محمد حسانين

قسم التشريح الآدمي وعلم الأجنة، كلية الطب، جامعة أسيوط

الخلفية: يُعدّ الروتينون أحد الملوثات البيئية، ويُستخدم كمبيد حشري على نطاق واسع. لأكثر من ١٥٠ عامًا، استُخدم الروتينون تجاريًا في الزراعة وإدارة مصائد الأسماك.

هدف العمل: الكشف عن الآثار الوقائية المحتملة لحمض الفوليك في الوقاية من سمية الروتينون الخلوية في القلب والعضلات الهيكلية.

المواد والطرق: اختيرت أربع مجموعات من أربعين فأرًا أبيض بالغًا ذكرًا عشوائيًا، واستُخدمت فئران المجموعة الأولى كمجموعة ضابطة طبيعية. تلقت المجموعة الثانية حمض الفوليك عن طريق الفم بجرعة ٢٠ ملغم/كغم من وزن الجسم لمدة ٣٥ يومًا، وتلقت المجموعة الثالثة حقنة تحت الجلد مرة واحدة يوميًا لمدة ٣٥ يومًا بجرعة ١,٣ ملغم/كغم من وزن الجسم من الروتينون. تلقت فئران المجموعة الرابعة كلاً من الروتينون (كالمجموعة الثالثة) وحمض الفوليك (كالمجموعة الثانية). لإجراء دراسة مجهرية ضوئية باستخدام صبغة الهيماتوكسيلين والإيوزين والبيكروسيريوس الحمراء، ودراسة التركيب الدقيق، والفحص المناعي النسيجي باستخدام أجسام مضادة لـ iNOS وTNFα، تم تحضير عينات من عضلة القلب وعضلة الساق.

النتائج: تسبب العلاج بالروتينون في تنكس وانقسام ألياف القلب والعضل مع فقدان الخطوط، وترتيب متموج للعديد من ألياف العضلات، وتلف الميتوكوندريا، وتقلص نوى خلايا عضلة القلب، ووجود العديد من ألياف الكولاجين بين ألياف القلب والعضل، وانخفاض ملحوظ في سمك الألياف العضلية، بالإضافة إلى تفاعلات سيتوبلازمية إيجابية قوية لـ iNOS وTNFα في عضلة القلب وعضلة الساق. حافظ الإعطاء المتزامن لحمض الفوليك على البنية النسيجية الطبيعية للقلب وعضلة الساق.

الاستنتاج: يمكن لحمض الفوليك أن يحسن التنكس وله تأثير وقائي على بنية القلب والعضل في الفئران المعالجة بالروتينون على الأرجح من خلال آليات تعتمد على iNOS وTNFα.