BENHA MEDICAL JOURNAL bmj

Print ISSN 1110-208X

Online ISSN 2357-0016

Original article

The Possible Ameliorating Effect of Exosomes Derived Mesenchymal Stem Cells and L- Carnitine on Amiodarone Induced Thyroid Follicular Cells Toxicity in Adult Male Albino Rats: Histological and Immunohistochemical Study

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Received: 6 December 2022

Accepted: 26 January 2023

ABSTRACT

Background: Amiodarone is an antiarrhythmic drug. It causes thyroid dysfunction. **Objective:** To explore the possible therapeutic effect of L-carnitine and exosomes on amiodarone induced thyroid follicular cells toxicity. **Materials and Methods:** Sixty-two rats divided into 7 groups. Group I (control), group II (they were treated with amiodarone orally in a dose of 60 mg/kg for 6 weeks, group III (they were treated with L-carnitine in a dose of 500 mg/ kg for 4weeks),and group IV (they were treated with exosomes which were given in 3 doses of exosomes. Some rats from this group were sacrificed immediately after the last dose. While, The remaining rats stayed another 10 days formed group VII, group V (they were treated with L-carnitine and exosomes together), group VI (recovery group) and Group VII (exosomes withdrawal). **Results:** Groups II and VI showed loss of normal follicular architecture. The follicular epithelial cells showed more than one layer with small dark nuclei. The interfollicular tissue showed congested vessels and infiltrates. Ultrathin sections of these groups showed follicular epithelium with irregular nuclei. The cytoplasm showed dilated rough endoplasmic reticulum and degenerated mitochondria. Furthermore, there was intense positive collagen and Ki-67 in these groups ($p< 0.01$). Groups III, IV, V and VII showed improvement. **Biochemical Results:** a significant increase in TSH in groups II and VI in comparison to control. While, there was a significant decrease in the levels of FT3 and FT4. Serum TSH levels were decreased in groups III, IV, VII and V. while, free T3 and T4 levels were increased. **Conclusion:** L-carnitine and exosomes can improve thyroid dysfunction.

Key Words: Amiodarone; Thyroid dysfunction; L-carnitine; Exosomes.

Introduction

Amiodarone is an antiarrhythmic drug. It releases a 20 to 40 times higher iodine level**.** Amiodarone causes multiple hazardous effects as thyroid dysfunction[1].
Amiodarone has a cytotoxic effect on thyroid cells that is mainly due to either a direct effect of the drug on thyroid cells or due to the toxic action of excess iodine [2]**.**
L-carnitine is a natural tri-methylated amino acid that plays an important role in cell metabolism by mediating the transport of long-chain fatty acids across the inner mitochondrial membrane where, they are oxidized to produce ATP. L-carnitine has antioxidant and anti-inflammatory effects on various pathophysiological conditions [3].

Exosomes are small sized (30–120 nm) extracellular vesicles. They play an important role in cell-to-cell communication by its contents, including lipids, RNAs, DNAs and proteins. Exosomes when reach recipient cells deliver their cargoes, which can affect the physiological or pathological status of recipient cells [4].
The aim of this study was to explore the possible therapeutic effect of L-carnitine and bone marrow mesenchymal stem cells derived exosomes on amiodarone induced thyroid follicular cells toxicity in adult male albino rats.

Material and methods

Drugs and Chemicals

Amiodarone tablets:

They were obtained from (El Ezaby Pharmacies, Cairo, Egypt). Each tablet contains 200 mg of amiodarone hydrochloride [5].

L-carnitine ampoules:

L-carnitine was obtained in the form of ampoules, from (El Ezaby Pharmacies, Cairo, Egypt).It was taken at a dose of 500 mg/kg/day via intraperitoneal injection for four weeks [6]**.**

Exosomes:

Exosomes were prepared in the molecular biology unit, Cairo faculty of medicine. They were isolated from conditioned media of rat bone marrow derived MSC [7]**.**

Animals and Diet:

Sixty-two adult male albino rats of a weight range 180-250 grams were utilized in this work. Animal care was provided by animal house in pharmacology department, Benha faculty of medicine. The animals were housed in cages under strict care and hygiene and received balanced diet and water. All Benha faculty of medicine ethical protocols for animal treatment were followed. All animal experiments received approval from institutional animal care committee of Benha university, Egypt (study no. MD.3.10.2020).

Experimental Design:

An experimental study was done from January 2021 to April 2021. After one week of housing, 62 adult male albino rats were randomly divided into 7 groups as follow:

- **Group I (control group; n=12):** The rats were furtherly divided equally into 4 subgroups:

  Subgroup Ia: The rats were left without intervention.

  Subgroup Ib: The rats received orally 4ml of distilled water/kg (vehicle of amiodarone group).

  Subgroup Ic: The rats were injected intraperitonially with 2.5 ml of distilled water/kg (vehicle of L-carnitine group).

  Subgroup Id: The rats were injected intravenously with 3 doses of 0.5 ml PBS (vehicle of exosome group).

- **Group II (amiodarone treated group; n=10):** The amiodarone tablet was dissolved in 10 ml of distilled water. It was administered orally in a daily dose of 60 mg/kg for 6 consecutive weeks.
- **Group III (L-carnitine treated group; n=10):** The rats received amiodarone for 6 weeks, then the rats were given L- carnitine by intraperitoneal injection in a dose of 500 mg/ kg daily for four weeks.
- **Group IV (exosomes treated group; n=10):** The rats received amiodarone for 6 weeks, then they were given the first dose of exosomes at a concentration of 100μg diluted in 0.5 ml PBS, injected over 5 min via tail vein, then after 2 weeks they were given the second dose, then after 2 other weeks they were given the last dose (exosomes were given for 4 weeks divided into 3 doses). Six rats from the ten were sacrificed after the last dose formed group IV. The other four formed a new group (group VII)
- **Group V (L-carnitine and exosomes treated group; n=10):** The rats received the same dose of amiodarone for 6 weeks, then they were given the three doses of exosomes concomitant with L-carnitine until they were sacrificed.
- **Group VI (recovery group; n=10):** The rats were received amiodarone, then they were sacrificed at the end of the study without taking any treatment.
- **Group VII (Exosomes withdrawal group):** The four rats remaining from group IV stayed another 10 days from the last dose of exosomes to determine whether the improvement which occurred by exosomes would change after 10 days or not.

Sampling:

Adult male albino rats were sacrificed after 6 weeks from the first day of administration of amiodarone in group II. Rats of groups I, III, IV, V, VI were sacrificed after 4 weeks after induction of thyroid follicular cells toxicity by amiodarone. While, the rats of group VII were sacrificed after 10 days from the last dose of exosomes. Specimens of the thyroid gland were fixed in 10% buffered formalin. They were embedded in paraffin. Serial sections of 5–7 μm thickness were cut for ordinary, special staining and immunostaining.

Induction of thyroid injury or disorder:

It was induced by using amiodarone tablets as mentioned in group II.

Preparation of MSCs-derived exosomes:

Exosomes were isolated from the supernatant of MSCs. First, rat bone marrow-derived MSCs were prepared in the molecular biology unit, Cairo University. MSCs were cultured in Dulbecco's Modified Eagle with 0.5% human serum albumin. Cells were plated at 4000 cells/cm^2 for 7 days then trypsinized, counted, and replated in expansion medium at a density of 2,000 cells/cm^2 for another 7 days, then centrifuged at 2,000 g for 20 min, and then additional centrifuged at 100,000 g for 1 hour at 4 ◦C ,then washed in serum free medium 199 [8] .

Electron microscopy for the exosomes:

Exosomes were cultured in Modified Eagle Medium (alpha-MEM). They were fixed with 2.5% glutaraldohyde in human serum albumin (HSA) for 2h. After washing, exosomes were ultracentrifuged and suspended in 100 mL HSA. An absolute of 20 mL of exosomes was loaded

onto a formvar/carben-coated grid, negatively stained with 3% aqueous phospher-tungstic acid for 1 min, and observed by TEM in the molecular biology unit, Cairo University.

Histological and Immunohistochemical Studies:

Paraffin sections of thickness (5-7 µm), mounted on glass slides for H&E and Masson trichrome staining for demonstration of collagen fibers deposition. Other sections were mounted on positive charged slides for immunohistochemical staining for Ki-67. Ki-67 antigen is a HMW nuclear protein. It is generally accepted as the most reliable cell proliferative marker[9]. Ki-67 (positive) cells showed brown nuclei.

Biochemical Investigations:

Blood samples were drawn for assaying serum TSH, FT3 and FT4 levels. Then, they were taken into serum tubes and centrifuged at 3000 rpm for 10 min. The FT3 and FT4 levels were determined using a radioimmunoassay kit. While, TSH levels by enzyme-linked immunosorbent assay, performed at Clinical Pathology Lab, Al-Kasr Al-Ainy Medical School[10.]

Morphometrical study:

The image analysis system (Leica Q 500 MC program) at the Faculty of Science, Tanta University, was used to determine the mean area % of the stained collagen fibers and ki67 immuno-histochemical expression in the thyroid of different experimental groups.

Statistical analysis:

All the data collected from the experiment was recorded and analyzed at the Faculty of Science, Tanta University, using IBM SPSS Statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc LSD test was used to compare differences among the groups. In each test, the data was expressed as the mean value, standard deviation and differences were significant (p<0.01).

MSCs- Exosomes Characterization:

A transmission electron microscopic examination of purified exosomes demonstrated their characteristic spheroid double-membrane bound morphology with a diameter of 90-100 nm **(Fig., 1).**

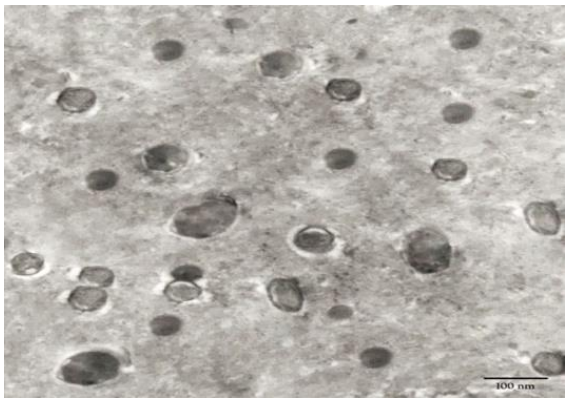


Fig. (1): A transmission electron micrograph of exosomes demonstrated their characteristic spheroid double-membrane bound morphology with a diameter of 90-100 nm **(TEM, X2500).**

H & E Stain results:

Examination of all subgroups of the group I (control group) showed similar histological architecture. Group I (control group): Sections of the thyroid gland of control rats revealed that it was formed of multiple variable sized follicles. Each follicle was lined by a

single layer of cuboidal epithelial cells with centrally placed round nuclei. Follicular Lumina were filled with homogenous eosinophilic colloid. There was a thin connective tissue containing capillary bed between follicles **(Fig., 2a).**

Group II (amiodarone treated group) (affected group): Sections of thyroid gland of rats of group II showed loss of normal follicular architecture. The follicles were disorganized and variable sized. Some follicles were empty, while, others had scanty colloid. The follicular epithelial cells showed more than one layer with small dark nuclei in some areas. Cells were ballooned and severely vacuolated. The epithelium showed focal disruption at other points and some follicles showed desquamated epithelium forming clusters in the lumen. The interfollicular tissue showed markedly congested blood vessels and mononuclear cellular infiltrates **(Fig., 2b).**

Group III (L-carnitine treated group): Sections of the thyroid gland of rats of group III showed mild structural improvement with disorganized, variable sized thyroid follicles lined with more than one layer of follicular epithelial cells in some areas. Cells were vacuolated with abnormal dark nuclei. Clusters of nuclei inside the lumen of follicles indicating desquamated epithelium. The interfollicular tissue showed congested blood vessels and mononuclear cellular infiltrates **(Fig., 2c).**

Group IV (exosomes treated group): Sections of the thyroid gland of rats of group IV showed moderate structural improvement with variable sized thyroid follicles lined with vacuolated follicular epithelium, some of them showed apparently normal nuclei. While others showed abnormal dark nuclei. Interfollicular tissue showed mononuclear cellular infiltrates **(Fig., 2d).**

Group V (L-carnitine and exosomes treated group): Sections of the thyroid gland of rats of group V showed marked structural improvement. Thyroid follicles appeared more or less as control. But there was widening in inter follicular connective tissue and blood vessels showed mild congestion **(Fig., 2e).**

Group VI (recovery group): Sections of the thyroid gland of rats of recovery group showed disorganized thyroid follicles lined with abnormal epithelium containing abnormal dark nuclei. Some follicles showed desquamated epithelium forming clusters in the lumen. Blood vessels showed marked congestion. Interfollicular tissue showed mononuclear cellular infiltrates **(Fig., 2f).**

Group VII (exosomes withdrawal group): Sections of the thyroid gland of rats of group VII showed moderate structural improvement. Variable sized thyroid follicles lined with vacuolated epithelium with apparently normal nuclei in some areas and abnormal dark nuclei in other areas. Interfollicular tissue showed mononuclear cellular infiltrates **(Fig., 2g).**

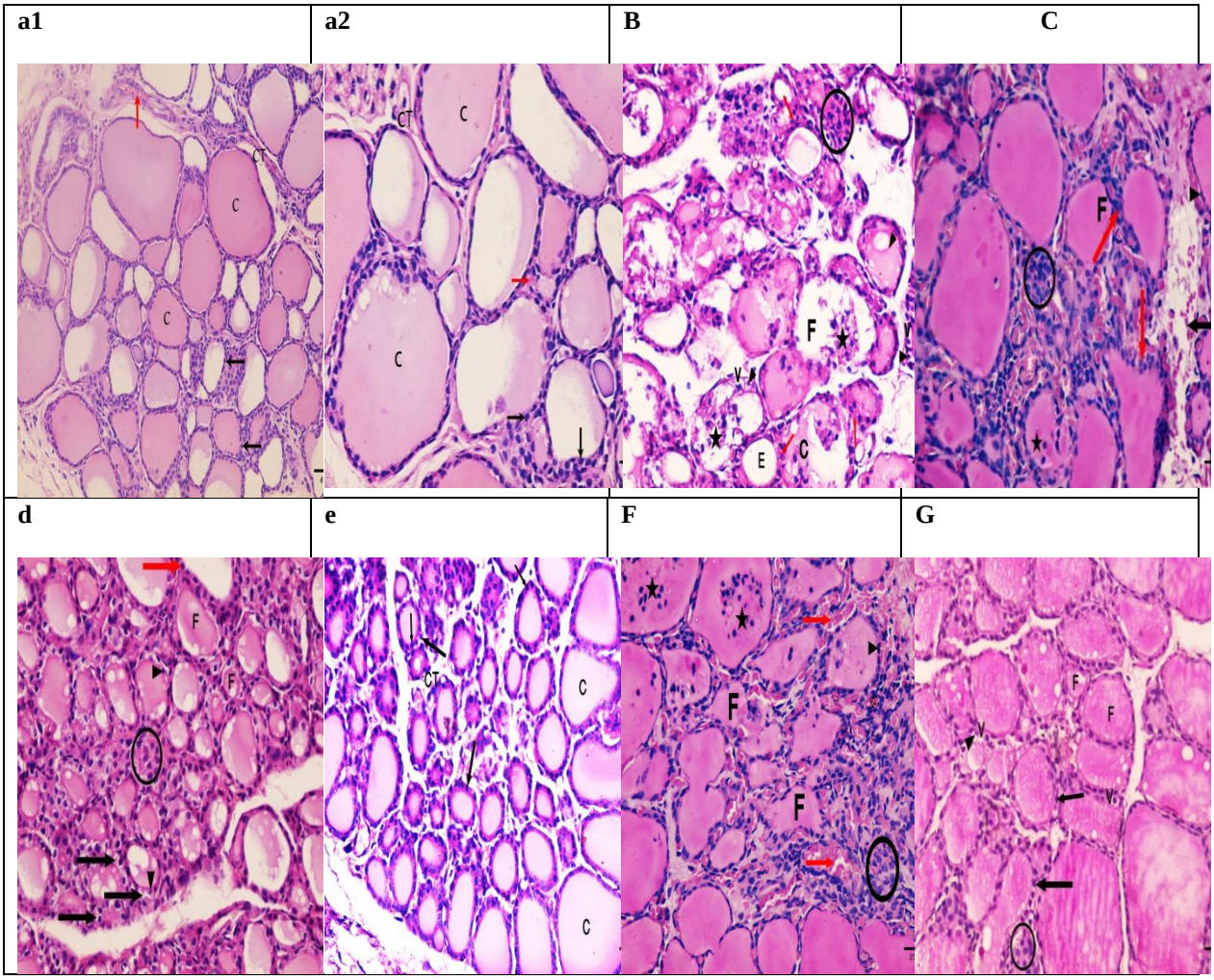


Fig. (2): (a1) A photomicrograph of a section in the thyroid gland of a rat of the control group (group I) showing thyroid follicles lined with cuboidal epithelium with round central nuclei (black arrows).The lumen is filled with homogenous eosinophilic colloid (C). Follicles are separated by thin connective tissue (CT) containing capillary beds (red arrow) **(H&E X200), (a2)** A higher magnification of a photomicrograph (a1) **(H&E X400), (b)** affected group (amiodarone treated group) (group II) showing disorganized, ruptured and fused thyroid follicles (F) lined with vacuolated epithelium (V) showing more than one layer of cells in some areas (red arrows). Cells contained small dark nuclei in some areas (arrow heads). Some follicles are empty (E) or show different densities of colloid (C). Clusters of nuclei inside the lumen of follicles indicating desquamated epithelium are also present (stars). Interfollicular tissue shows mononuclear cellular infiltrates (circle), **(c)** L-carnitine treated group (group III) showing disorganized and variable sized thyroid follicles (F) lined with more than one layer of follicular epithelial cells (red arrows) with small dark nuclei in some areas (arrow head).Clusters of nuclei inside the lumen of follicles (stars). Blood vessels show some congestion (black arrow). Widening of Interfollicular tissue showing mononuclear cellular infiltrates (circle), **(d)** exosomes treated group (group IV) showing variable sized thyroid follicles (F) lined with vacuolated epithelium (black arrows), some of them showing apparently normal nuclei (red arrow).While, others showing small dark nuclei (arrow heads). Interfollicular tissue shows mononuclear cellular infiltrates (circle), **(e)** L-carnitine and exosomes treated group (group V) showing Thyroid follicles appear more or less as control (black arrows). But there is widening in inter follicular connective tissue (CT) and Blood vessels shows mild congestion (red arrow), **(f)** recovery group (group VI): showing disorganized thyroid follicles (F) lined with abnormal epithelium containing abnormal dark nuclei in some areas (arrow heads). Some follicles show desquamated epithelium forming clusters in the lumen (stars). Blood vessels show marked congestion (red arrows). Interfollicular tissue shows mononuclear cellular infiltrates (circle), **(g)** exosomes withdrawal group (group VII) showing variable sized thyroid follicles (F) lined with vacuolated epithelium (V) with apparently normal nuclei in some areas (black arrows) and abnormal dark nuclei in other areas (black arrow head). Interfollicular tissue shows mononuclear cellular infiltrates (circle) **(H&E,X400).**

Masson trichrome staining results:

- **Group I** (control group): showed fine collagen fibers between thyroid follicles **(Fig., 3a).**
- **Group II** (amiodarone treated group) (affected group): showed marked inter-follicular deposition of collagen fibers **(Fig., 3b).**
- **Group III** (L-carnitine treated group): showed moderate inter-follicular deposition of collagen fibers **(Fig., 3c).**
- **Group IV** (exosome treated group): showed mild inter-follicular deposition of collagen fibers **(Fig., 3d).**
- **Group V** (L-carnitine and exosomes treated group): showed minimal inter-follicular deposition of collagen fibers **(Fig., 3e).**
- **Group VI** (recovery group): showed marked inter-follicular deposition collagen fibers (**Fig., 3f).**
- **Group VII** (exosome withdrawal group): showed mild inter-follicular deposition of collagen fibers. (**Fig., 3g).**

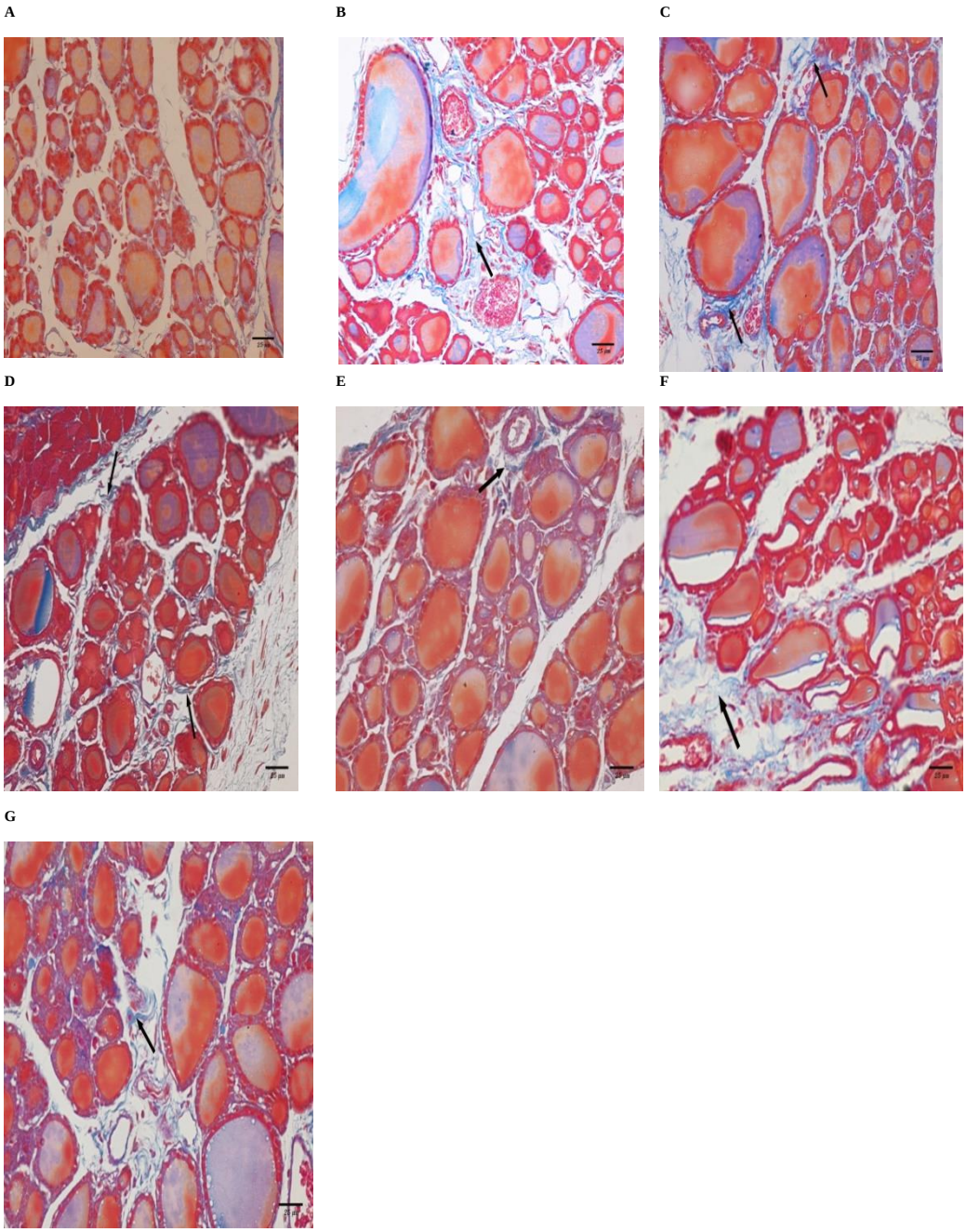


Fig.(3):(a) A Photomicrograph of a section in the thyroid gland of a control rat (group I) showing fine collagen fibers between thyroid follicles, **(b)** group II (amiodarone treated group) showing marked deposition of collagen fibers (black arrow), **(c)** group III (L-carnitine treated group) showing moderate inter-follicular deposition of collagen fibers (black arrows), **(d)** group IV (exosomes treated group) showing mild inter-follicular deposition of collagen fibers (black arrows), **(e)** group V (L-carnitine and exosome treated group) showing minimal inter-follicular deposition of collagen fibers (black arrow), **(f)** group VI (recovery group) showing marked inter-follicular deposition of collagen fibers (black arrow), **(g)** group VII (exosomes withdrawal group) showing mild inter-follicular deposition of collagen fibers (black arrow) **(Masson trichrome staining X400).**

Ki-67 immunostaining results:

- **Group I** (control group): showed thyroid follicles with negative nuclear immunostaining reaction for Ki 67 (**Fig., 4a).**
- **Group II** (amiodarone treated group) (affected group): showed thyroid follicles with strong positive nuclear immunostaining reaction for Ki67 in the form of dark brown granules indicating hyperplasic epithelium **(Fig., 4b).**
- **Group III** (L-carnitine treated group): showed thyroid follicles with moderate positive nuclear immunostaining reaction for Ki67 in the form of brown granules **(Fig., 4c).**
- **Group IV** (exosomes treated group): showed thyroid follicles with mild positive nuclear immunostaining reaction for Ki67 in the form of brown granules **(Fig., 4d).**
- **Group V** (L-carnitine and exosomes and treated group): showed thyroid follicles with minimal positive nuclear immunostaining reaction for Ki-67 in the form of lightly stained brown granules (**Fig., 4e).**
- **Group VI** (recovery group): showed thyroid follicles with strong positive nuclear immunostaining reaction for Ki 67 in the form of dark brown granules **(Fig., 4f).**
- **Group VII** (exosomes withdrawal group): showed thyroid follicles with mild positive nuclear immunostaining reaction for Ki67 in the form of brown granules **(Fig., 4g).**

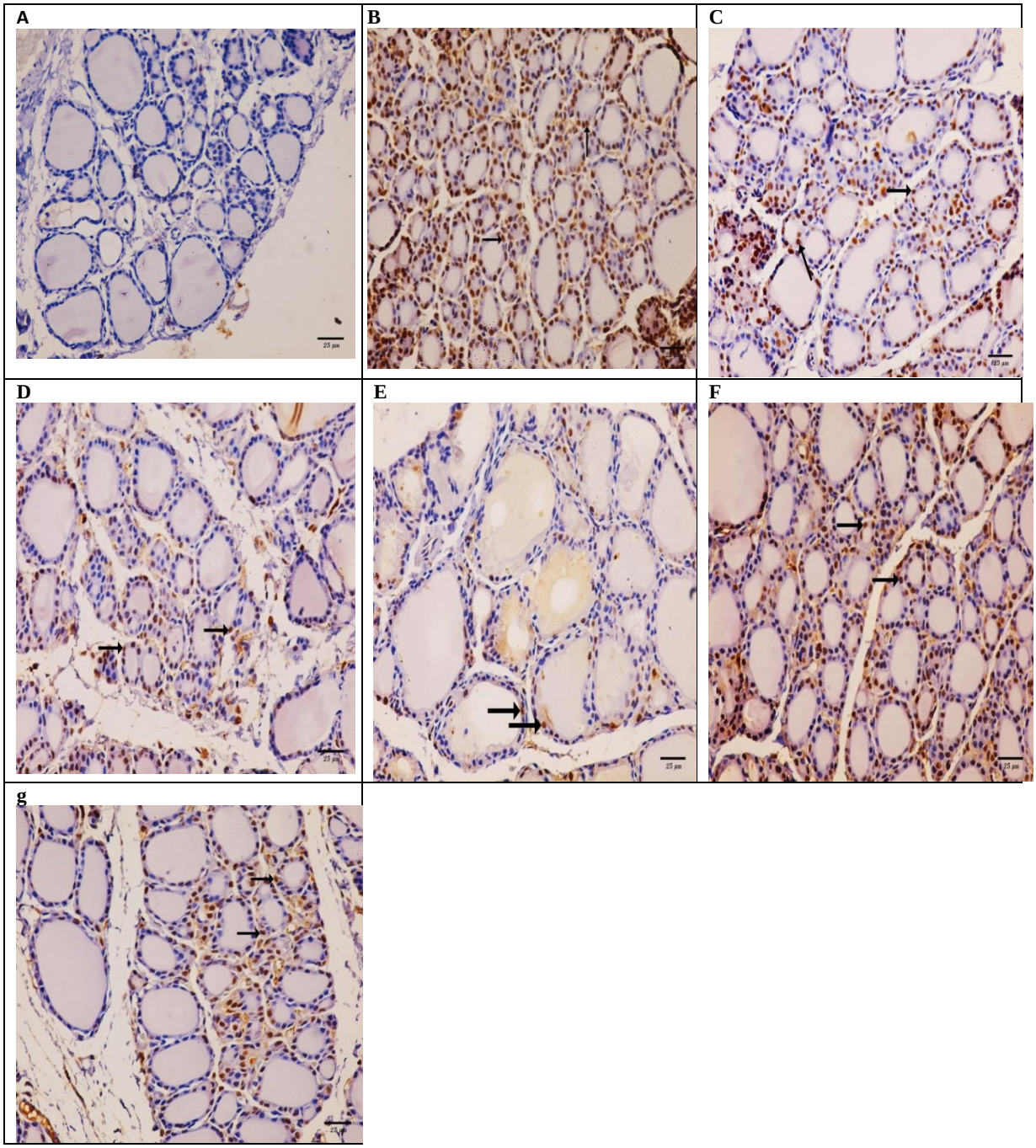


Fig. (4): (a) A Photomicrograph of a section in the thyroid gland of a control rat (group I) showing follicles with negative immunostaining reaction Ki67, **(b)** group II (amiodarone treated group) showing most thyroid follicles with strong positive nuclear immunostaining reaction for Ki-67 (black arrows), **(c)** group III (L-carnitine treated group) showing thyroid follicles with moderate positive nuclear immunostaining reaction for Ki-67 (black arrows), **(d)** group IV (exosomes treated group) showing thyroid follicles with mild positive nuclear immunostaining reaction for Ki-67 (black arrows), **(e)** group V (L-carnitine and exosomes treated group) showing thyroid follicles with minimal positive nuclear immunostaining reaction for Ki-67 (black arrows), **(f)** group VI (recovery group) showing thyroid follicles with strong positive nuclear immunostaining reaction Ki-67 (black arrows), **(g)** group VII (exosomes withdrawal group) showing thyroid follicles with mild positive nuclear immunostaining reaction Ki-67(black arrows) **(Ki-67, X400).**

Transmission electron microscope results:

- **Group I** (Control group)**:** Examination of ultrathin sections of the thyroid gland of control rats showed thyroid follicle lined with cubical follicular epithelium situated on thin basal lamina. The nuclei were with euchromatic and peripheral chromatin. They are surrounded by rough endoplasmic reticulum. The cytoplasm showed apparently normal mitochondria, many lysosomes, colloid vesicles and many secretory vesicles. The apical free surface showed microvilli protruding in follicular lumen containing colloid. Junctional complexes are also present between adjacent cells **(Fig., 5a).**
- **Group II** (amiodarone treated group) (affected group)**:** Examination of ultrathin sections of the thyroid gland of rats of group II (Amiodarone treated) showed follicular epithelium with irregular nuclei to markedly shrunken ones containing abnormal dispersed chromatin. The cytoplasm showed marked vacuolization with markedly dilated rough endoplasmic reticulum. The mitochondria were degenerated with lost cristae. Many lysosomes were also distributed within the cytoplasm. Some parts of cytoplasm were rarified. The free surface showed apical broken microvilli protruding in the follicular lumen containing colloid. The lumen showed clusters of desquamated follicular cells (**Fig., 5b).**
- **Group III** (L-carnitine treated group)**:** Examination of ultrathin section of thyroid gland of rats of group III showed follicular epithelium with irregular nuclei. Some nuclei were indented with abnormal chromatin. The cytoplasm showed dilated rough endoplasmic reticulum, degenerated mitochondria and many lysosomes. The free surface showed an apical broken microvilli protruding into follicular lumen containing colloid **(Fig., 5c).**
- **Group IV** (exosomes treated group)**:** Examination of ultrathin section of thyroid gland of rats of group IV showed follicular epithelium with irregular nucleus. The cytoplasm showed mildly dilated rough endoplasmic reticulum, degenerated mitochondria and many lysosomes. The free surface showed partially broken microvilli **(Fig., 5d).**
- **Group V** (L-carnitine and exosomes treated group)**:** Examination of ultrathin section of thyroid gland of rats of group V showed follicular epithelium with regular nuclei with euchromatic peripherally located chromatin. The cytoplasm showed apparently normal rough endoplasmic reticulum, mitochondria and many lysosomes. Free surface showing many apical microvilli protruding in the follicular lumen containing colloid (**Fig., 5e).**

- **Group VI** (recovery group)**:** Examination of ultrathin sections of the thyroid gland of rats of group VI showed follicular epithelium with irregular nuclei. The cytoplasm showed marked vacuolization with markedly dilated rough endoplasmic reticulum, degenerated mitochondria and many lysosomes. The free surface showed apical broken microvilli. The lumen showed clusters of desquamated follicular cells **(Fig., 5f).**

- **Group VII** (exosome withdrawal group)**:** Examination of ultrathin section of thyroid gland of rats of group VII showed follicular epithelium with irregular nuclei. Some nuclei were indented. The cytoplasm showed dilated rough endoplasmic reticulum, degenerated mitochondria and many lysosomes. The free surface showed partially broken microvilli protruding in the follicular lumen **(Fig., 5g).**

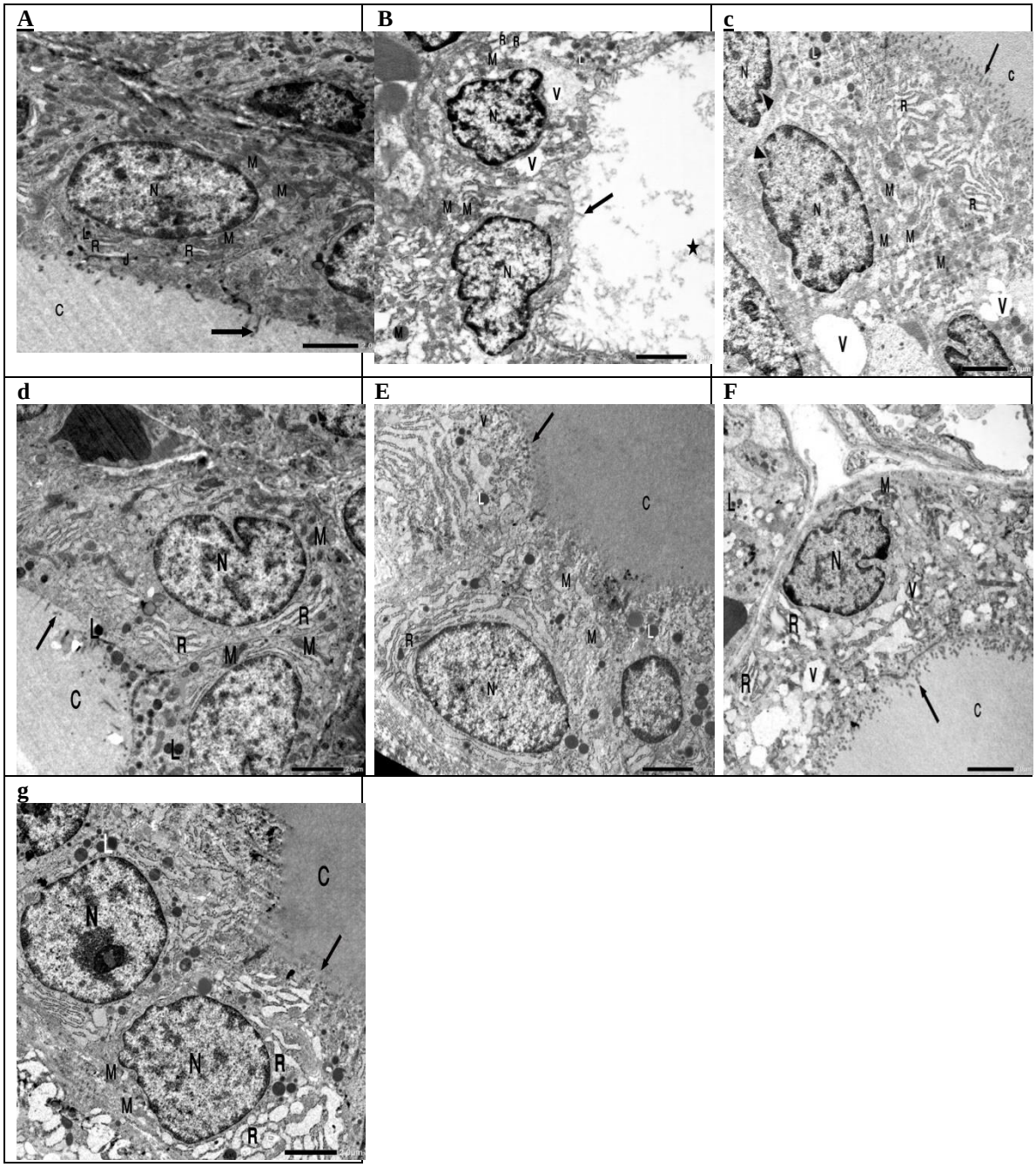


Fig. (5): (a) A transmission electron micrograph of ultrathin section in the thyroid gland of an adult male albino rat of control group (group I) showing thyroid follicle lined with cubical follicular epithelium situated on thin basal lamina. Nuclei (N) are euchromatic with peripheral chromatin. They are surrounded by rough endoplasmic reticulum (R). The cytoplasm shows apparently normal mitochondria (M) and lysosomes (L). The apical free surface shows long apical microvilli (black arrow) protruding in follicular lumen containing colloid (C). Junctional complexes between cells are also present (J), **(b)** group II (affected group) (amiodarone treated group) showing nuclei had many shapes ranging from irregular nuclei to markedly shrunken ones with abnormal dispersed chromatin (N). The cytoplasm shows marked vacuolization (V) with markedly dilated rough endoplasmic reticulum (R). The mitochondria (M) were degenerated with lost cristae. Lysosomes (L) were also distributed within the cytoplasm. The free surface shows destructed or broken apical microvilli (black arrow). The lumen shows clusters of desquamated follicular cells (star), **(c)** group III (L-carnitine treated group) showing follicular epithelium with abnormal nuclei (N) with irregular outlines and indentations (arrow heads) . The cytoplasm shows vacuolization (V), dilated rough endoplasmic reticulum (R), degenerated mitochondria (M) and lysosomes (L). The apical free surface shows broken microvilli (arrow) protruding into colloid (C), **(d)** group IV (exosomes treated group) showing follicular epithelium with slightly irregular nuclei (N). The cytoplasm shows mildly dilated rough endoplasmic

reticulum (R), apparently normal mitochondria (M) and many lysosomes (L). The apical free surface shows apical broken microvilli (black arrow) protruding into the colloid (C), **(e)** group V (L-carnitine and exosomes treated group) showing follicular epithelium with regular nuclei (N) with euchromatic peripherally located chromatin. The cytoplasm shows apparently normal rough endoplasmic reticulum (R), mitochondria (M) and lysosomes (L). Free surface shows many apical microvilli (black arrow) protruding into the follicular colloid (C), **(f)** group VI (recovery group) showing distorted and shrunken follicular epithelium with irregular nuclear outlines with abnormal dispersed chromatin (N). The cytoplasm shows vacuolization (V) with markedly dilated rough endoplasmic reticulum (R), degenerated mitochondria (M) and lysosomes (L). The free surface shows apical broken microvilli (black arrow) protruding in the follicular lumen containing colloid (C), **(g)** group VII (exosomes withdrawal group) showing follicular epithelium with irregular nuclear outlines (N). The cytoplasm shows dilated rough endoplasmic reticulum (R), mitochondria (M) and many lysosomes (L). The apical free surface shows partially broken microvilli (black arrow) protruding into the follicular lumen containing colloid (C) **(TEM, X 2500)**.

Morphometric and Statistical Results:

The mean area percentage ± SD of collagen fibers deposition and Ki-67 immunostaining are represented in t**ables 1 and 2 as well as histograms 1&2.** The mean area percentage of collagen fibers deposition and Ki-67were significantly increased ($p < 0.01$) in groups II and VI compared with group I. The mean area percentage of collagen fibers deposition and Ki-67 immunostaining were significantly decreased ($p < 0.01$) in groups III, IV, V and VII compared with group II, as mentioned in **tables 1 & 2 and in histograms 1 & 2.**

Biochemical Results:

There was a significant increase ($p<0.05$) in serum TSH in groups II and VI. While, there was a significant decrease in FT3 and FT4 in the same groups in comparison to control group. Serum TSH was gradually decreased in groups III, IV, VII and V. While, free T3 and T4 levels were increased in the same groups**, (Table 3 and histogram 3).**

Table (1): Showing the mean area % of collagen fibers ± SD in all experimental groups by **ANOVA with Post Hoc LSD** test.

Groups	**Group I**	**Group II**	**Group III L.**	**Group IV exo**	**Group V Syn**	**Group VI W**	**Group VII W. exo**
mean area %	5.15	36.57	22.83	17.25	9.79	30.32	19.61
SD	1.82	0.35	1.85	0.44	1.66	2.71	0.93
significant ($p<0.01$)	**2,3,4,6,7**	**1,3,4,5,7**	**1,2,6**	**1,2,6**	**2,3,4,6,7**	**1,3,4,5,7**	**1,2,6**

Data expressed as mean and SD = Standard deviation; 1= sig. with group I; 2= sig. with group II; 3= sig. with group III; 4= sig. with group IV; 5= sig. with group V; 6= sig. with group VI; 7= sig. with group VII

Table (2): Showing the mean area % of (Ki67) immuno-expression ± SD in all experimental groups by **ANOVA with Post Hoc LSD** test.

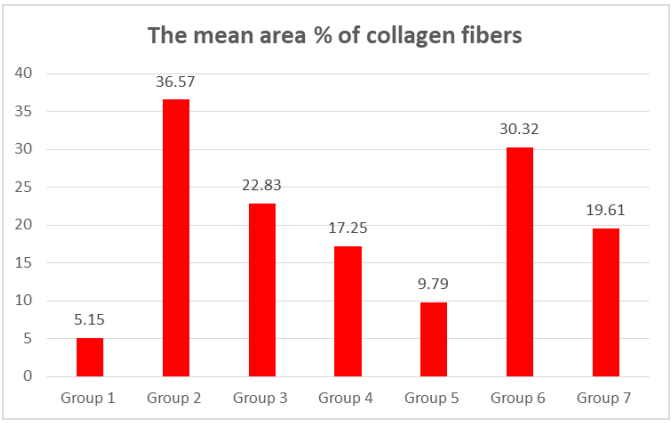
Groups	**Group I**	**Group II**	**Group III L.**	**Group IV exo**	**Group V Syn**	**Group VI**	**Group VII W. exo**
mean area %	3.59	44.83	29.94	18.27	7.81	38.92	23.33
SD	0.37	0.28	0.14	0.59	0.25	0.61	0.75
significant (P<0.01	**2,3,4,6,7**	**1,3,4,5,7**	**1,2,4,5,6,7**	**1,2,3,5,6,7**	**2,3,4,6,7**	**1,3,4,5,7**	**1,3,4,5,6**

Data expressed as mean and SD = Standard deviation; 1= sig. with group I; 2= sig. with group II; 3= sig. with group III; 4= sig. with group IV; 5= sig. with group V; 6= sig. with group VI; 7= sig. with group VII

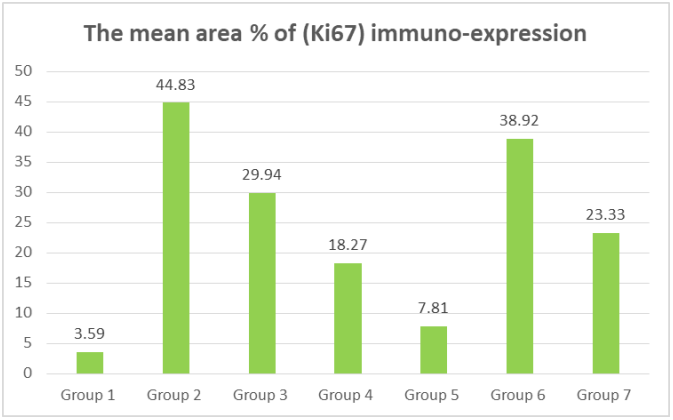
Table (3): Showing the mean of FT3, FT4 and TSH hormones in all experimental groups by **ANOVA** test.

Groups	Mean of FT3 ± SD	Mean of FT4 ± SD	Mean of TSH ± SD
Group I	3.27±0.2	2.31±0.14	1.85±0.12
Group II	1.95*±0.13	0.8*±0.19	29.8*±1.91
Group III	2.32±0.16	1.48±0.2	17.23±2.01
Group IV	2.92±0.11	1.89±0.19	9.42±0.98
Group V	3.05±0.17	2.15±0.23	3.17±0.17
Group VI	2.05*±0.18	0.83*±0.17	27.29*±2
Group VII	2.88±0.16	1.63±0.23	11.31±1.12

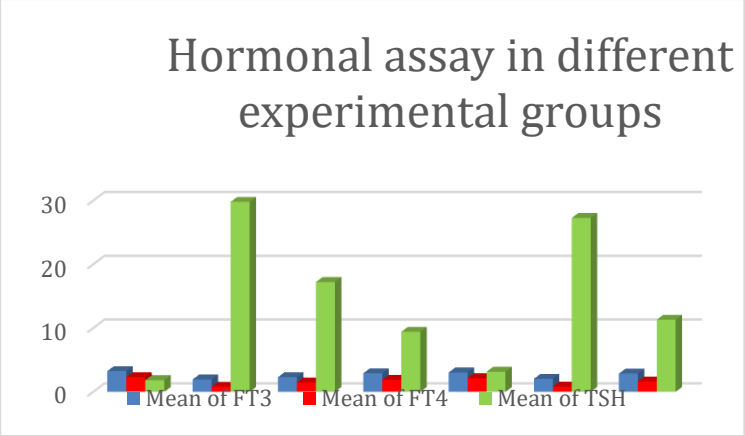
Data are expressed as mean ± standard division; *p* value *< 0.05 is significant versus control.



Histogram (1): Showing the mean area % of collagen fibers deposition of all groups.



Histogram (2): Showing the mean area % of Ki-67 immunostaining in all groups.



Histogram (3): Showing the mean of FT3, FT4 and TSH hormones in all experimental groups.

Discussion

Although amiodarone has been proved to be highly effective in controlling cardiac arrhythmias, its clinical use is mainly limited due to its hazardous effect on the thyroid gland [11].

In the present study, group II (affected group) showed disorganized and variable sized follicles. Follicles showed focal disruption of some follicular cells. The follicular epithelium showed more than layer of cells in some areas. While, others showed marked vacuolation. Many follicular cells revealed small dark nuclei. The interfollicular tissue showed markedly congested blood vessels, infiltrates and a significant increase (p<0.01) of collagen fibers compared to control group. It also showed a significant increase (p<0.01) in Ki-67 immunostaining.

The mechanism of thyroid dysfunction caused by amiodarone was related to iodine overload or direct toxic effect of amiodarone. It induced apoptosis through a p53 independent mechanism involving oxidative stress and associated with production of reactive oxygen species [12].

These results agreed with some investigators [13,14,15] who stated that amiodarone induced degenerative follicular lesions with focal fibrosis .This was due to inflammatory reaction secondary to direct toxic effects of amiodarone and its metabolites. Lymphocytic infiltration occurred secondary to autoimmune thyroiditis.

Examination of ultrathin sections of this group showed that the nuclei were irregular. There was also marked dilatation of rough endoplasmic reticulum. This dilatation occurred due to the accumulation of iodinated thyroglobulin which resists proteolytic destruction. This was in agreement with [12,13] who observed similar changes in the thyrocytes of amiodarone treated rats. Similar changes were also observed by [16,17] who reported nuclear changes in rat hepatocytes exposed to amiodarone . The mitochondria were degenerated. This was in agreement with [18,19] who stated that amiodarone was a well-known mitochondrial toxin. Amiodarone increases the permeability of the mitochondrial membrane.

As regards the biochemical results in this group, there were a significant increase (p<0.05) in serum TSH and a significant

decrease in free T3 and T4 levels compared to the control group.

This was agreed Batcher et al., 2007 and Basaria and Cooper, 2005 [20, 21] who found that the amiodarone-induced hypothyroidism is due to iodine induced cellular damage.

Group III (L-carnitine treated) showed disorganized, variable sized thyroid follicles lined with vacuolated follicular epithelium with abnormal dark nuclei in some areas. The Interfollicular tissue showed cellular infiltrates. There was also a significant decrease (p<0.01) in both collagen fibers deposition and Ki-67 staining expression compared to group II.

Examination of ultrathin sections of this group showed follicular epithelium with irregular nuclei. The cytoplasm showed dilated rough endoplasmic reticulum and degenerated mitochondria. The free surface showed apical broken microvilli.

As regards the biochemical results emerged from group III, there were a decrease in the levels of serum TSH and an increase in free T3 and T4 levels compared to group II.

The mild improvement in this group was due to several important mechanisms of antioxidant action of carnitine. Firstly, carnitine is shown to scavenge free radicals. Secondly, carnitine can chelate metals as iron, preventing their participation in reactive oxygen species formation. Thirdly, L-carnitine was found to inhibit specific enzymes responsible for free radical production [22.] .These results concur with other studies [23,24,25] who, reported that carnitine facilitates the translocation of long-chain fatty acids acyl groups from cytosol into mitochondria for production of ATP.

Group IV (exosomes treated) showed variable sized thyroid follicles lined with vacuolated follicular epithelium containing apparently normal nuclei but some areas still showing abnormal dark nuclei. Interfollicular tissue showed infiltrates. There was also a significant decrease (p<0.01) in both collagen fibers deposition and Ki-67staining expression compered to groups II and III.

Examination of ultrathin sections of this group showed follicular epithelium with irregular nucleus. The cytoplasm showed mildly dilated rough endoplasmic reticulum, apparently normal mitochondria. The free surface showed partially broken microvilli.

As regards the biochemical results emerged from group IV, there were a decrease in serum TSH and an increase in free T3 and T4 levels in group IV compared to groups II and III.

This was agreed with Zhang et al. and Gnecchi et al. [26,27] who reported that exosomes have genetic and protein material that when transferred to recipient cells can initiate various repair mechanisms. This was also agreed with Shetgaonkar et al. [28] who reported that exosomes can reach recipient cells and deliver their cargoes which affect the physiological or pathological status of the recipient cells.

Group V (exosomes and L-carnitine treated) showed follicles which appeared more or less as control. But, there was widening in interfollicular connective tissue and blood vessels showed mild congestion. This group also showed significant decrease (p<0.01) in both collagen fibers deposition and Ki-67 staining expression compered to groups II, III and IV.

Examination of ultrathin sections in this group showed follicular epithelium with regular nuclei. The cytoplasm showed

apparently normal rough endoplasmic reticulum and mitochondria. Free surface showed many apical microvilli.

As regards the biochemical results emerged from this group, there were a decrease in serum TSH and an increase in free T3 and T4 levels in group V compared to groups II, III and IV.

This effect may be due to synergism between the effect of both exosomes and L- carnitine. The conjugation between exosomes and some drugs would enhance the efficacy of these drugs.

Moreover, Wang et al. and Shi et al. [29,30] suggested that the exosomes when loaded with drugs could facilitate skin wound healing in rats by promoting the re-epithelialization and by enhancing angiogenesis.

Osterma et al. [31] also reported that exosomal curcumin was absorbed 3-4 times more rapidly compared to free curcumin.

In the present study, group VI (recovery group) showed disorganized thyroid follicles lined with abnormal epithelium containing abnormal dark nuclei. Some follicles showed desquamated epithelium in the lumen. Blood vessels showed marked congestion. Interfollicular tissue showed mononuclear cellular infiltrates. This group also showed a significant increase ($p<0.01$) in the collagen fibers and a strong positive nuclear immunostaining reaction for Ki67 compared to the control group.

Examination of ultrathin sections of this group showed vacuolization with markedly dilated rough endoplasmic reticulum and degenerated mitochondria. The free surface showed partially broken microvilli.

As regards the biochemical results emerged from this group, there were a significant increase ($p<0.05$) in serum TSH and a significant decrease in free T3 and T4 levels compared to the control group.

These results agreed with Van Erven and Schalij and Naryana et al. [32,33] who said that the patients will need more than 3-4 months from stopping amiodarone to become euthyroid again.

In the present study, group VII (exosomes withdrawal) showed variable sized thyroid follicles lined with vacuolated epithelium with apparently normal nuclei in some areas and abnormal dark nuclei in other areas. Interfollicular tissue showed infiltrates. There was a significant decrease in both collagen fibers deposition and Ki-67staining expression compered to groups II and III.

Examination of ultrathin sections of this group showed follicular epithelium with irregular nuclei. The cytoplasm showed mildly dilated rough endoplasmic reticulum and apparently normal mitochondria.

As regards the biochemical results emerging from this group, there were a decrease in serum TSH and an increase in free T3 and T4 levels compared to groups II and III.

In this study, there was no improvement or deterioration occurred compared to group IV.

Conclusion

The L-carnitine and exosomes together revealed more increase in efficiency in the repair of amiodarone induced thyroid follicular cells toxicity.

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To cite this article: Esraa H. Khairat, Aisha E. Mansy, Ola M. Mohamed, Mohamed Abd El Hamid, Amira E. El-Alfy. The Possible Ameliorating Effect of Exosomes Derived Mesenchymal Stem Cells and L- Carnitine on Amiodarone Induced Thyroid Follicular Cells Toxicity in Adult Male Albino Rats: Histological and Immunohistochemical Study. BMFJ 2023;40 (academic issue): 62-81.