

Hepato-protective Effect of Green Tea Extract on Cyclosporine a Treated Rabbits: Histological and Ultrastructural Study

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Abstract: Cyclosporine A (CsA) has a biologic activities, including anti-parasitic, fungicidal and anti-inflammatory effects by inhibiting T-cell activation. CsA and its metabolites are taken and excreted by the hepatocytes into the bile and a small portion into the blood. Green tea contains several antioxidants and has a role as an antioxidant. **Objective:** The present work aimed to study the structural hepato-protective effect of green tea extract on rabbits treated with cyclosporine A (CsA). **Materials and methods:** Three groups of adult rabbits were used for this study; the 1st group was used as a control, the 2nd group was treated with cyclosporine A (CsA) as oral solution in a dose of 15 mg/kg for 4 weeks and the 3rd group was treated with cyclosporine A in the same dose simultaneously with green tea extract for the same period. The animals were anaesthetized and liver specimens were obtained and semi-thin sections of 1 μ m thick were obtained, stained with 1% toluidine blue and examined by light microscopy and ultrathin sections for electron microscopic examination. **Results:** The cyclosporine A had adverse effects on the liver cells of cyclosporine A-treated animals in comparison with that of the control group where, the hepatocytes showed extensive vacuolization and variable areas of fatty degeneration. Also, the cytoplasm showed reduced organelles, as few mitochondria with loss of their transverse cristae, few glycogen particles and loss of endoplasmic reticulum. In some cells the cytoplasm showed loss of the organelles and depletion of glycogen content. The nucleus appeared irregular with deformed nuclear membrane. A concomitant administration of green tea extract to cyclosporine A treated rabbits markedly prevented the adverse effects of cyclosporine A where the hepatocytes preserved their normal appearance. The cytoplasm showed numerous mitochondria with well developed transverse cristae, smooth and rough endoplasmic reticulum, Golgi apparatus and abundant amount of glycogen particles. The nucleus appeared round and light with well formed nuclear membrane. Also, bile canaliculi and the microvilli projecting from the cells were preserved. **Conclusion:** The green tea extract had beneficial effects against cyclosporine A induced hepatic toxicity and should be used in combination with cyclosporine A transplant treatment to improve the cyclosporine A induced oxidative stress parameters and other adverse effects.

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1. Introduction

Cyclosporine A (CsA), a neutral lipophilic cyclic undecapeptide isolated from the fungus *Tolypocladium inflatum gams*, was first identified in 1976 during screening for novel antibiotic agents. It exerted a wide spectrum of biologic activities, including anti-parasitic, fungicidal and anti-inflammatory effects. It was subsequently discovered to be a powerful immunosuppressive agents⁽¹⁾. The drug exerts its major therapeutic effects by inhibiting T-cell activation⁽²⁾. The use of cyclosporine A has improved quality of life and survival of transplant patients^(3,4). CsA has largely contributed to the decrease in morbidity, rejection episodes and hospitalization days in these patients. The immunosuppressive properties of CsA allowed its utilization in several diseases as uveitis, rheumatoid arthritis, nephrotic syndrome and others^(5,6). It is preferentially bound in blood to cells and lipoproteins. Openings in the liver capillaries allow

blood cells and lipoproteins to enter Disse's space and to get into close contact to the hepatocytes membrane and then diffuses into the hepatocytes⁽⁷⁾. In the liver, CsA and its metabolites are taken up into the hepatocytes. The metabolites are mainly excreted from the hepatocytes into bile, a small portion into blood⁽⁸⁾. Hydrogen peroxide generation and enhancement of the lipid peroxidation have been proposed as causative factors associated with the potential of CsA in inducing structural and functional damage⁽⁹⁾. More attentions have been paid to the protective effects of natural antioxidant against drug-induced toxicities. Tea has been one of the most commonly consumed beverages since ancient times, and it is still the beverage most available to general populations in many countries all over the world⁽¹⁰⁾. As a naturally safe beverage, it has many advantages over chemical preventive agents. It contains several antioxidants, including polyphenols of the catechin (green tea) and theaflavin (black tea) groups. Green

tea has recently received a lot of attention owing to its role as an antioxidant and free radicals scavenger^(11,12).

The present study aimed to study the histological and ultrastructural hepato-protective effects of green tea extract on CsA treated rabbits.

2. Materials and Methods

Animals:

Twenty adult rabbits weighing 1.5-2 kg were used in this study. The animals were maintained in cages with food and water *ad libitum* under controlled conditions of light, humidity and temperature. They were obtained from Animal House Colony of Faculty of Medicine, Zagazig University. All experimental procedures were conducted in accordance with the guide for the care and use of laboratory animals and in accordance with the local Animal Care and Use Committee.

Green tea extract:

The green tea was made according to Maity et al.⁽¹³⁾. Briefly, 10 gram of instant green tea powder (lyophilized water extract) were soaked in 1 L of boiling water for 5 minutes and filtered to make 1% instant green tea solution. The filtrate, designated as green tea extract (GTE), was provided to rabbits as their sole source of drinking water.

Chemicals:

Cyclosporine A used in the present study was in the form of oral solution (Sandimmun Neoral 100 g/ml) and given to the animals in a distilled water by gavage using sterile equipments. CsA was administered in a dose of 15 mg/kg of body weight for 4 weeks according to Rezzani⁽⁸⁾ who stated that daily administration of CsA ranging from 15 to 25 mg/kg was well tolerated and showed immunosuppressive effects like those observed in transplant patients. Also, Thomson et al.⁽¹⁴⁾ and Blair et al.⁽¹⁵⁾ found that, impairment of liver architecture has been reported in rats given CsA for 3-15 weeks.

Experimental design:

The rabbits were divided into 3 groups (6-7 rabbits for each) and treated as follow: the 1st group served as control group and received distilled water, the 2nd group (CsA-treated rabbits) were daily administered 15 mg/kg of body weight for 4 weeks while those of the 3rd group (CsA treated rabbits co-administered with GTE) were treated with CsA in the same dose (15 mg/kg) simultaneously with green tea extract for the same period (4 weeks). The animals were anaesthetized by intra-abdominal injection of thiopental, then rapid dissection of the abdomen was done and the liver specimens were obtained and cut into small blocks less than 1 mm. The specimens were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2

hours at 4°C and then washed with the phosphate buffer, postfixed in 1% osmium tetroxide in the same buffer for one hour at 4°C. After washing in phosphate buffer, specimens were dehydrated with ascending grades of ethanol and then were put in propylene oxide for 30 minutes at room temperature, impregnated in a mixture of propylene oxide and resin (1:1) for 24 hours and in a pure resin for another 24 hours. Then, the specimens were embedded in embed 812 resin in BEEM capsules at 60°C for 24 hours^(16,17). Semi-thin sections of about one micron thick were obtained by glass knives and stained with 1% toluidine blue and examined by light microscopy. Ultrathin section of about 50-70 nm thick were cut using diamond knives and mounted on a copper grids, stained with uranyl acetate and lead citrate^(16,17) and examined using a JEOL JEM 1010 transmission electron microscope in Electron Microscope Research Laboratory (EMRL) of Histology Department, Faculty of Medicine, Zagazig University.

3. Results

In control animals, the liver lobule examined by light microscopy appeared surrounding the portal triad; branch of the portal vein, branch of hepatic artery and bile canaliculi. The lobules were formed of plates of cells separated from each other by blood sinusoids (Fig. 1). The wall of the sinusoids are formed of hepatocytes and Kupffer cells. The cytoplasm of the hepatocytes appeared finely granulated and the nuclei appeared lightly stained and vesicular with prominent nucleoli, the Kupffer cells are small with pale cytoplasm and small nuclei (Fig. 2). By electron microscopic examination, the liver cell appeared polyhedral in shape. Their cytoplasm showed copious smooth endoplasmic reticulum in the entire perinuclear space and also membrane of rough endoplasmic reticulum. Numerous mitochondria were observed in the cytoplasm in addition to few glycogen particles. The nuclei of the cells appeared light, round with chromatin bodies inside and the nuclear membrane appeared regular and well formed (Figs. 3, 4 & 6). Numerous microvilli appeared protruding into the space of Disse and the Kupffer cells were observed to surround the sinusoids (Figs. 5 & 6).

In animals treated with CsA, the liver sinusoids were widened and surrounded by the cells of the liver cords when examined by light microscopy. The hepatocytes showed extensive vacuolization of different sizes in most of the cells with loss of the fine granulation (Figs. 7 & 8). By electron microscopic examination, the cytoplasm of the hepatocyte showed multiple vacuolization with variable areas of fatty degeneration. Few organelles

were observed as few mitochondria with loss of their transverse cristae, few glycogen particles and loss of endoplasmic reticulum (Figs. 9 & 11). In some cells the cytoplasm showed loss of organelles and depletion of glycogen content. The nucleus appeared irregular in shape with irregular and deformed nuclear membrane and thick heterochromatin material inside (Figs. 9 & 10).

In CsA treated animals co-administered with green tea extract, the hepatocytes examined by light microscopy were found to preserve most of their normal arrangement. The liver cords were separated by normal sized sinusoids. The cytoplasm appeared finely granulated and the nucleus appeared round, lightly stained with prominent nucleolus (Figs. 12 & 13).

Electron microscopic examination of the CsA treated animals co-administered with green tea extract revealed that the hepatocytes regained their normal appearance. Bile canaliculi appeared inbetween the hepatocytes and showed apparent junctional complexes with each other (zonula occludens and macula adherens) (Figs. 14 & 16). The cytoplasm showed numerous mitochondria with well developed transverse cristae, smooth and rough endoplasmic reticulum, Golgi apparatus and abundant amount of glycogen particles. The nucleus appeared round and light with chromatin bodies inside. The nuclear membrane appeared regular and well formed (Figs. 14 & 15). Numerous microvilli were observed projecting from the hepatocyte in the space of Disse towards the sinusoid (Fig. 15).

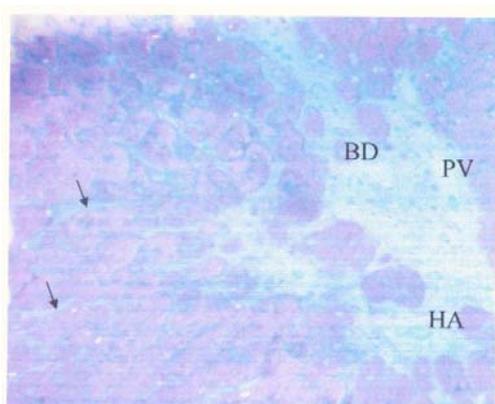


Fig. (1): A photomicrograph of a semi-thin section in the liver of rabbit (control) showing parts of liver lobules surrounding the portal triad in an area of connective tissue. The portal triad contains a branch of hepatic artery (HA), a branch of portal vein (PV) and bile ductule (BD). The lobules are formed of plates of cells separated by sinusoids (arrows). (Toluidine blue X 400)

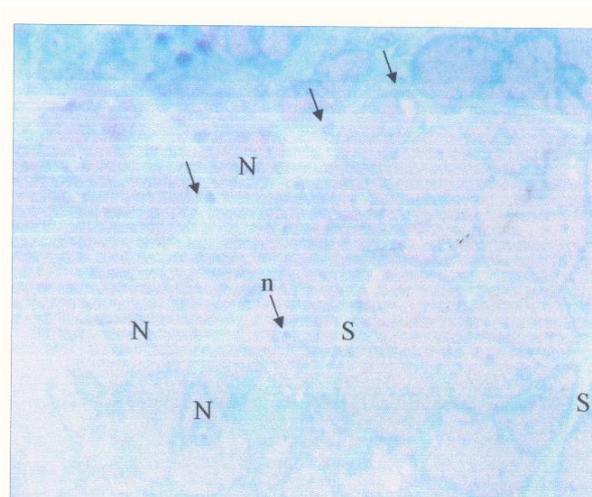


Fig. (2): A photomicrograph of a semi-thin section of a higher magnification in the liver of rabbit (Control) showing the cells of liver cords separated by sinusoids (S). Kupffer cells (arrows) appear as part of sinusoidal wall. The cytoplasm of the cells appear finely granulated and the nuclei (N) are lightly stained with prominent nucleoli (n). (Toluidine blue X 1000)

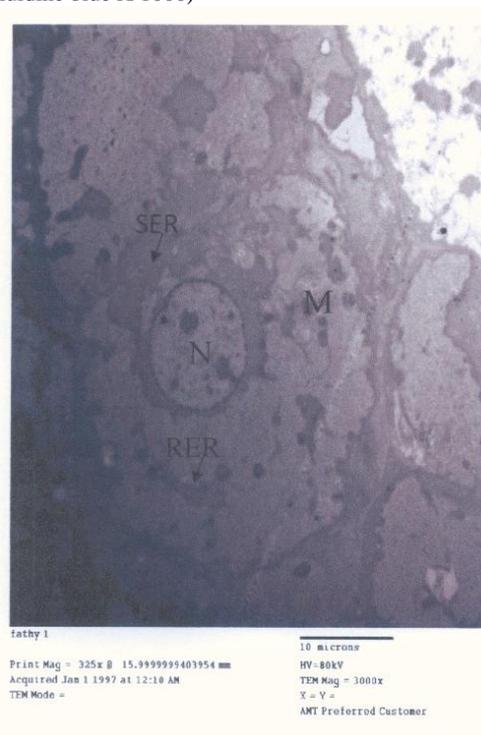


Fig. (3): An electron photomicrograph of ultrathin section in the liver of rabbit (control) showing the liver cell as polyhedral. The cytoplasm shows copious smooth endoplasmic reticulum (SER) in the entire perinuclear space and also there are membranes of rough endoplasmic reticulum (RER). The cytoplasm contains numerous mitochondria (M). The nucleus (N) appears light, round with chromatin bodies inside.

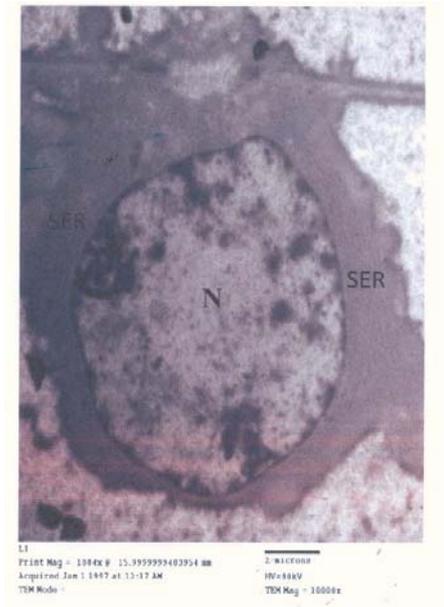


Fig. (4): An electron photomicrograph of a higher magnification of ultrathin section in the liver of rabbit (control) showing copious smooth endoplasmic reticulum (SER) in the entire perinuclear space. The nucleus (N) appears round, lightly stained and chromatin bodies inside. The nuclear membrane appears regular and well formed.

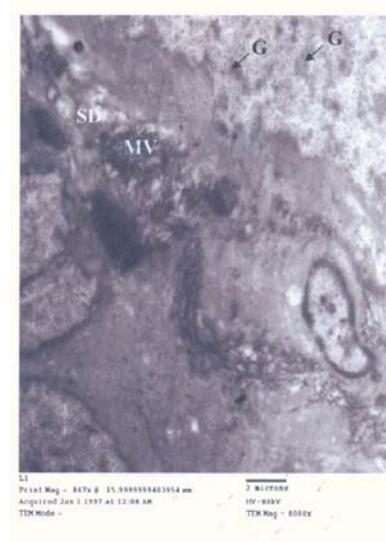


Fig. (6): An electron photomicrograph of ultrathin section in the liver of rabbit (control) showing glycogen particles (G) in the cytoplasm and numerous microvilli (MV) projecting into the space of Disse (SD).

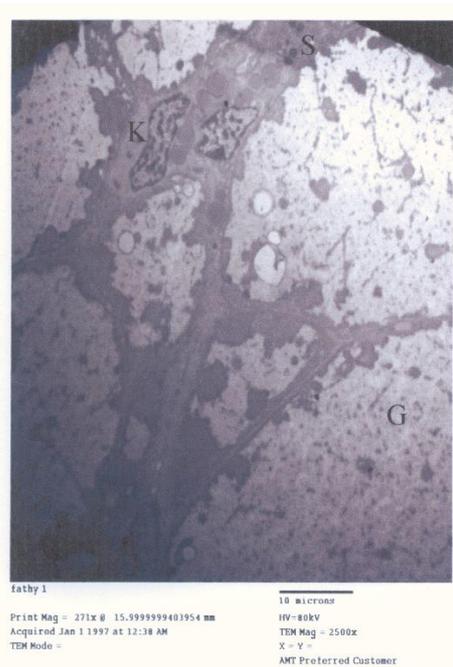


Fig. (5): An electron photomicrograph of ultrathin section in the liver of rabbit (control) showing hepatocytes with sinusoid (S) inbetween. Kupffer cells (K) appear surrounding the sinusoid. Glycogen (G) deposits appear in the cytoplasm.

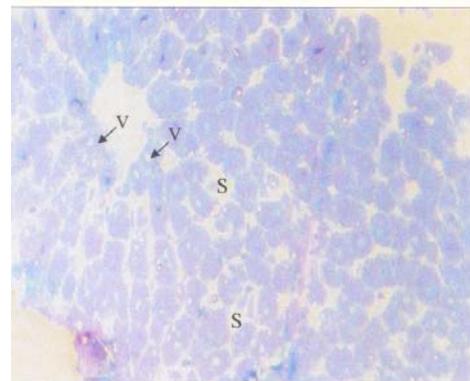


Fig. (7): A photomicrograph of a semi-thin section in the liver of rabbit treated with CsA showing the cells of the liver cords and separated by wide sinusoids (S). The hepatocytes show extensive vacuolization (V) in most of the cells. (Toluidine blue X 400)

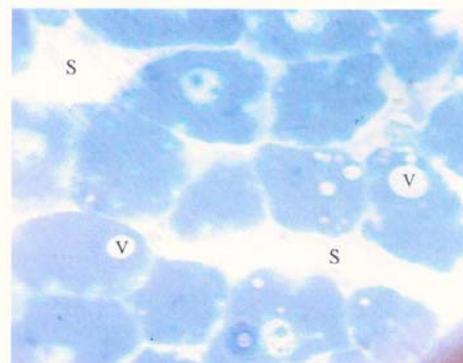


Fig. (8): A photomicrograph of a semi-thin section in the liver of rabbit treated with CsA showing the cells of the liver cord at a higher magnification. The cells show numerous vacuoles (V) at different sizes with loss of the fine granulation. The sinusoids (S) appear wide. (Toluidine blue X 1000)

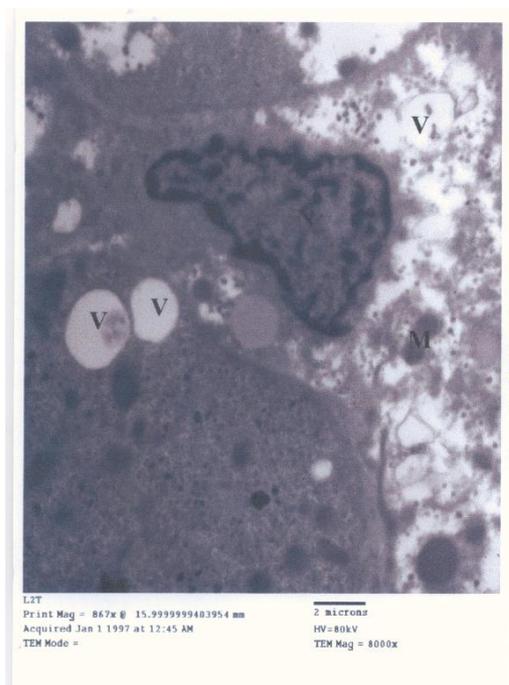


Fig. (9):An electron photomicrograph of ultrathin section in the liver of rabbit treated with CsA showing liver cells. The cytoplasm shows multiple vacuolization (V) and few mitochondria (M) with loss of transverse cristae. The nucleus (N) is irregular in shape.

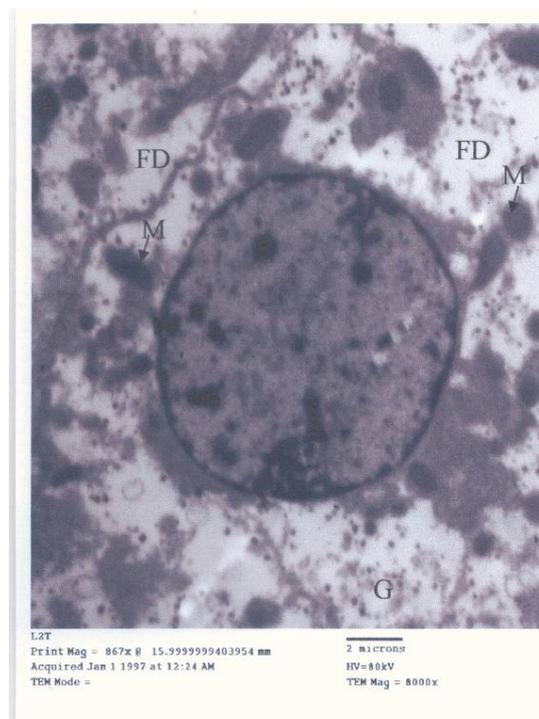


Fig. (11):An electron photomicrograph of ultrathin section in the liver of rabbit treated with CsA showing the liver cell. The cytoplasm shows wide areas of fatty degeneration (FD). Some areas show few mitochondria (M) with loss of transverse cristae and few glycogen particles (G) with loss of endoplasmic reticulum.

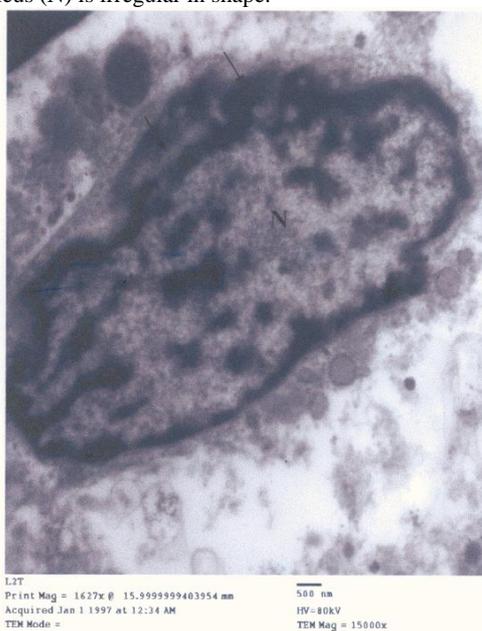


Fig. (10): An electron photomicrograph of ultrathin section at a higher magnification in the liver of rabbit treated with CsA showing the liver cell. The nucleus (N) is irregular in shape with deformed nuclear membrane (arrows) and irregularly placed thick heterochromatin.

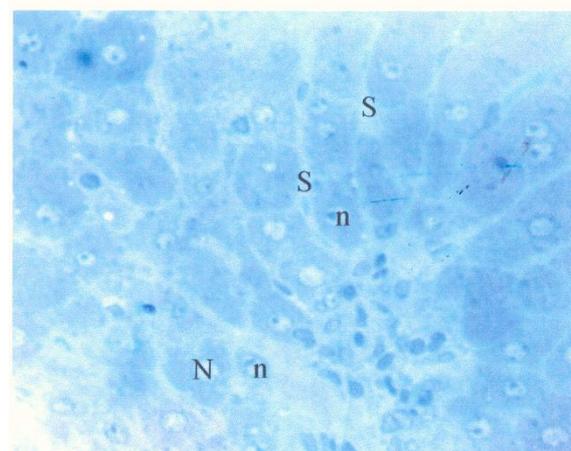


Fig. (12): A photomicrograph of a semi-thin section in the liver of rabbit of CsA treated rabbit co-administered with green tea extract showing that, the hepatocytes preserved their normal arrangement. The liver cords are separated by normal sized sinusoids (S). The hepatocytes show vesicular nuclei (N) with prominent nucleoli (n). (Toluidine blue X 400)

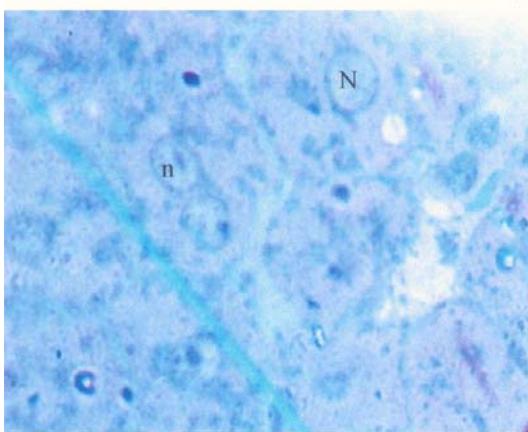


Fig. (13): A photomicrograph of a semi-thin section of a higher magnification in the liver of CSA treated rabbit co-administered with green tea extract showing hepatocytes. The cytoplasm appears finely granulated and the nucleus(N) appears rounded, lightly stained with prominent nucleoli (n). (Toluidine blue X 1000)

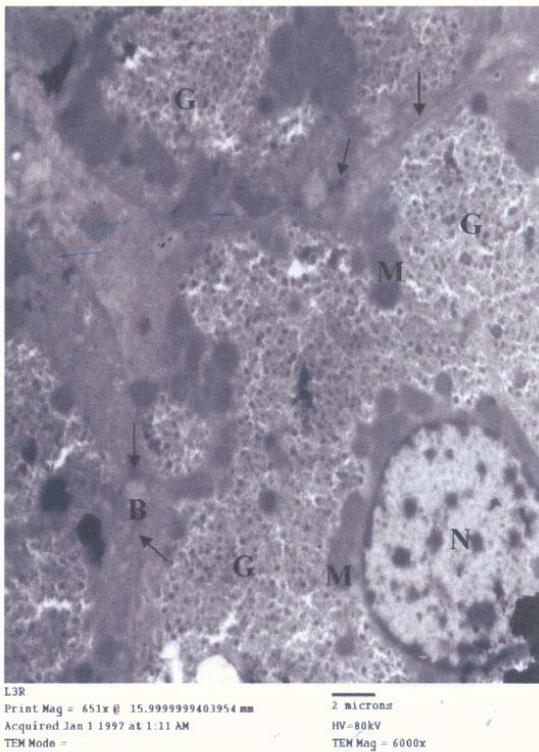


Fig. (14): An electron photo-micrograph of ultrathin section in the liver of CsA treated rabbit co-administered with green tea extract showing 3 neighbouring hepatocytes with bile canaliculi (B) inbetween. The intercellular junctions in close proximity to bile canaliculi consists of junctional complexes (arrows), as zonula occludens and macula adherens. The cytoplasm shows numerous mitochondria (M) and a much of glycogen particles (G). The nucleus (N) appears rounded and light with chromatin bodies inside.

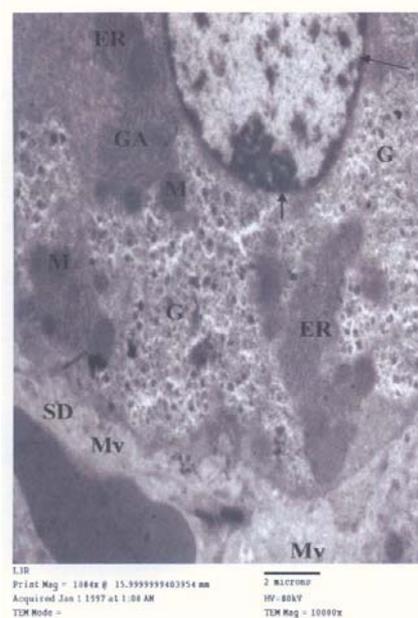


Fig. (15): An electron photomicrograph of ultrathin section of a higher magnification in the liver of CsA treated rabbit co-administered with green tea extract showing the hepatocyte. Numerous microvilli (Mv) project from the cell in the space of Disse (SD) towards the sinusoid. The cytoplasm shows smooth and rough endoplasmic reticulum (ER), Golgi apparatus (GA), numerous mitochondria (M) and a lot of glycogen particles (G). The mitochondria appear with well formed transverse cristae. The nuclear membrane appears regular and well formed (arrows).



Fig. (16): An electron photomicrograph of ultrathin section in the liver of CsA treated rabbit co-administered with green tea extract showing hepatocytes. The cytoplasm shows abundant amount of glycogen particles (G), numerous mitochondria (M). The mitochondria appear with well developed transverse cristae. Bile canaliculi (B) with junctional complexes appear inbetween the hepatocytes (arrows).

4. Discussion

In the present study, the CsA had adverse effects on the liver cells where the cytoplasm showed multiple vacuolization with variable areas of fatty degeneration and few organelles were observed. In some cells, the cytoplasm showed loss of organelles and depletion of glycogen content. This is in agreement with many authors; Thomson et al.⁽¹⁴⁾, Blair et al.⁽¹⁵⁾, Ryffel⁽¹⁸⁾ and Sweny and Tidman⁽¹⁹⁾ who reported the impairment of liver architecture in rats given CsA for 3-15 weeks. Ryffel⁽¹⁸⁾ added that fatty changes and dilatation of endoplasmic reticulum together with loss of ribosomes in several hepatocytes and individual hepatic necrosis were found in CsA treated rats. Also Abdurrauf⁽²⁰⁾ found that, CsA caused marked damage in the histopathological status in kidney, liver and heart tissues.

In this study, CsA treated rabbits showed that the mitochondria appeared few with loss of transverse cristae. This may be explained by de Arriba et al.⁽²¹⁾ who suggested that CsA induces several consequences in mitochondrial structure and function. Also CsA induces pores in mitochondrial membrane allowing the release of protein to cytosol and triggering the activation of caspases and in turn the process of programmed cell death. Fatty degeneration observed in the present study could be attributed to impaired protein synthesis as a result of rough endoplasmic reticulum damage and therefore inhibition of lipoprotein manufacture and its inhibition results in accumulation of fats in the cytoplasm⁽²²⁾.

In the present study, the nucleus appeared irregular in shape with irregular and deformed nuclear membrane and thick heterochromatin materials inside. Junqueira et al.⁽²³⁾ explained the nuclear degeneration of liver cells as a result of inhibition of the dihydrofolate reductase enzyme that interferes with the synthesis of DNA. Many studies revealed the functional impairment of the liver in transplant recipients receiving CsA. Lorber et al.⁽²⁴⁾ and Li et al.⁽²⁵⁾ observed impaired liver functions in a number of renal, cardiac, bone marrow transplant recipients. Pickrell et al.⁽²⁶⁾ reported the transient increase of bilirubin or hepatic aminotransferase levels which return to normal with reduction of CsA dosage. Rezzani⁽⁸⁾ observed hepatotoxicity in the first 90 post-transplant days.

Oxidative stress and an increase in different enzymatic activity (i.e. alkaline phosphatase, lactate dehydrogenase, NADPH-diaphorase) has been proposed as a causative factor in CsA induced toxicity in liver cells^(27,28,29). Hagar⁽³⁰⁾ and Rezzani⁽³¹⁾ stated that CsA generates reactive oxygen species (ROS) such as superoxide anion and hydroxyl radicals and stimulates renal, hepatic and cardiac

lipid peroxidation. Packer et al.⁽³²⁾ mentioned that lipid peroxidation leads to oxidative damage of cell component (e.g. proteins, lipids and nucleic acids). The normal balance between radical formations and antioxidants system protects the cells against oxidative stress⁽³³⁾. Turk et al.⁽³⁴⁾ found that, reduced activity of one or more antioxidant systems due to the direct toxic effect of CsA caused increase of lipid peroxidation and oxidative stress in transplanted and non-transplanted human being and animals.

In the present study, co-administration of GTE significantly prevented the injury in CsA treated animals where the hepatocytes regained their normal appearance. Bile canaliculi appeared inbetween the hepatocytes with junctional complexes. The cytoplasm showed numerous mitochondria with well developed transverse cristae, smooth and rough endoplasmic reticulum, Golgi apparatus and abundant amount of glycogen particles. The nucleus appeared round, light and the nuclear membrane appeared regular and well formed. Numerous microvilli were observed projecting from the cells in the space of Disse. This is concomitant with Fadhel and Amran⁽³⁵⁾ who reported that green and black tea have anti-oxidant effects and chemo-protective activity against CCL4-induced lipid peroxidation in liver, kidneys and testes. A similar study by Mohamadin et al.⁽³⁶⁾, observed the reno-protective potential of GTE in CsA-induced nephrotoxicity and added that, the anti-lipoperoxidative effect of GTE and its ability to restore the levels and activity of antioxidant machinery and lysosomal enzymes, all contribute to GTE's beneficial effects. Also some studies reported the effect of other antioxidants. Chen et al.⁽³⁷⁾ and Zal et al.⁽³⁸⁾ reported the role of vitamin E and Quercetin in decreasing the lipid peroxidation induced by CsA. Also, Olaleye and Rocha⁽³⁹⁾, Kanbur et al.⁽⁴⁰⁾ and Mohamed et al.⁽⁴¹⁾ reported the hepato-protective effects of medicinal plants and royal Jelly and that the mechanism by which they do this is by acting as antioxidants. Thus, from the above findings, it could be concluded that GTE had beneficial effects against cyclosporine A induced hepatic toxicity and may be used in combination with CsA transplant treatment to improve the CsA-induced oxidative stress parameters and other adverse effects.

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