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THYMOQUINONE NANOTHERAPY ABROGATES HEPATOTOXICITY-INDUCED BY DOXORUBICIN IN MALE ALBINO RATS

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Received: 16 April 2023; **Accepted:** 7 May 2023

ABSTRACT

Doxorubicin (DOX) is an antitumor drug that causes hepatotoxicity by release of free radicals and injury of the liver tissues. Nano-thymoquinone (nano-TQ) is considered a potent antioxidant that abrogates hepatotoxicity. The aim of the current investigation was to assess the liver toxicity of DOX and the protective effects of nano-TQ on chronic hepatotoxicity in male rats. Sixty rats were divided into four even groups: DOX treated group (group 1) received 3.750 mg/kg b.wt. intraperitoneally at days 10,17,24 and 31 from the beginning of the experiment to make a cumulative dose of 15 mg/kg b.wt., Group (2) received DOX as group 1 beside nano-thymoquinone daily from day one in a dose of 10 mg/kg b.wt. until the end of the experiment, Group (3) received nano-TQ only, group (4) is considered a negative control group. Serum samples were used for estimating oxidative stress markers and liver function enzymes. Liver specimens were used for histopathological and Transmission electron microscopic (TEM) examination. Doxorubicin administration induced an increase in liver enzymes and lipid peroxidation products. Nano-TQ treatment improved those altered parameters. Microscopic examination of the DOX-treated liver sections revealed vascular and parenchymatous alterations as congestion, thrombosis of the blood vessels, vacuolar degeneration, hepatocellular necrosis and fibrosis of the liver. While nano-TQ improved such pathological alteration of the hepatic parenchyma. Transmission electron microscope of the liver revealed infiltration of lymphocyte in the necrotic areas and presence of fat globules in the cytoplasm of the hepatocytes in DOX treated group while TEM revealed a unique finding in DOX and nano-TQ treated group expressed by hypertrophy of Kupffer cells. It could be concluded that nano-TQ has a potent antioxidant effect that protected the liver damage via its free radicals scavenging protection.

Keywords: Doxorubicin, nano-TQ, hepatotoxicity, histopathology, transmission electron microscope, male albino rats

INTRODUCTION

Anthracyclines, such as doxorubicin (DOX) stayed the main cancer therapy for a long time despite broad-spectrum antitumor activity (Globcan, 2012). Liposome injection of doxorubicin hydrochloride was the first clinically approved liposome-encapsulated anticancer agent and is effective against a variety of malignancies, including solid tumors, transplantable leukemias, and lymphomas (Slingerland *et al.,* 2012). Doxorubicin is most frequently used to treat Hodgkin's lymphoma, multiple myeloma, soft tissue sarcoma, bladder, breast, stomach, lung, ovary, and thyroid cancers. Adriamycin

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may be present in commonly used doxorubicin-based treatments. (Rivankar, 2014).

Doxorubicin exerts its antitumor activity primarily through DNA intercalation and inhibition of topoisomerase-2 (TOP 2), as cancer cells need this enzyme to divide and grow. Fast proliferation leads to cancer cell death (Zhao and Zhang, 2017). The liver is where doxorubicin undergoes the majority of its main metabolite formation as cytotoxic aglycone metabolites and doxorubicinol (Ballet *et al.*, 1987).

Doxorubicin induces inflammatory changes in the liver in the form of granulomatous lesions and periportal fibrosis (Saad *et al.*, 2001). Furthermore, Murat *et al.* (2007) stated that DOX induced histological alterations such as hepatocyte degeneration, necrosis, congestion and hemorrhage.

Nagi and Mansour, (2000) studied the protective effect of thymoquinone against DOX. They found that TQ is a potent superoxide radical scavenging power and has an inhibitory effect on lipid peroxidation. In addition, TQ has anticancer activity by targeting oncogenic signaling molecules and immunomodulatory activities (Hassanein and El-Amir, 2018). Recently, TQ nanotherapy is currently being utilized to treat cancers, pulmonary disease, and other chronic illnesses. However, because of its physicochemical characteristics and injection route, formulation challenges with TQ are what is preventing it from being transferred to clinical studies (Al-Garbi *et al.*, 2021). This study aimed to investigate the histopathology, biochemical parameters and ultrastructure of DOX-induced hepatotoxicity in rats. Also, to explore the protective effect of nano-thymoquinone on DOX-induced toxicity.

MATERIALS AND METHODS

Ethical approval:

This study was approved by the National Ethics Committee, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.

Materials:

Chemical used:

Doxorubicin hydrochloride was purchased from RMPL PHARMA LLP, India. Chitosan-loaded thymoquinone nanoparticles were purchased from Nano-gate Company Egypt (Yadav *et al.*, 2016). Malondialdehyde (MDA) kit and total antioxidant capacity (TAC) kit were obtained from Biodiagnostic Company, Egypt. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total protein (TP), albumin (AL) and globulin (GL) were obtained from Egyptian Company for Biotechnology (SAE).

Nano-thymoquinone:

Thymoquinone chitosan nanoparticles were prepared and evaluated by the providing company. Particle size and its calibration curves are shown in (Fig. 1).

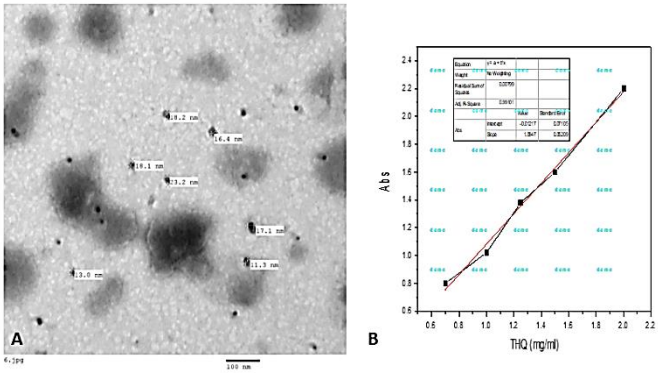

Fig. 1: A) TEM micrograph showing the particle size of the prepared TQ-loaded chitosan nanoparticles. B) Shows the curve of TQ, initial conc. =1.2mg/ml, free conc. abs =0.2177 =0.21mg/ml, EE =1.2-0.21/1.2*100 =82.5%, E capacity =8.25%w/w.

Experimental animals and design:
At the Faculty of Veterinary Medicine at Assiut University, sixty mature male albino rats were purchased. The rats weighed between 180 and 200 g and were in good health. Animals were kept in 25°C cages with humidity and temperature controls. All animals received unlimited access to tap water and laboratory food. Prior to the experiment, they were kept in the lab for at least a week to get used to it. The four groups of rats were randomly assigned in accordance with the following experimental design.

Doxorubicin treated group (Group 1):
This group consisted of 15 rats. The rats were given DOX in a dose of (3.750 mg/kg b.wt.) (Wape *et al.,* 2015). DOX was dissolved in saline and given intraperitoneally at days 10,17,24 and 31 from the beginning of the experiment to make a cumulative dose of 15 mg/kg b.wt.

Doxorubicin + nano-TQ treated group (Group 2):
This group consisted of 15 rats. The rats were given DOX as group (1) on days 10,17,24 and 31 then rats were given nano-TQ. Rat in this group was administrated daily by a gastric tube of Nano-TQ from day one (10 mg/kg b.wt) until the end of the experiment.

Nano-TQ treated group (Group 3):
This group consisted of 15 rats. The rats were administrated nano-TQ Only as (2).

Control group (Group 4):
This group consisted of 15 rats that were administrated normal saline I/P injections on days 10, 17, 24 and 31.

Rats in all groups were sacrificed at the end of the experiment on day 38 from the beginning of the experiment.

Methods:

Histopathological examination
Liver samples were fixed in 10% neutral-buffered formalin after sacrificing different groups of rats according to the assigned schedule. All tissue samples were routinely processed for histological analysis following fixation. After being rinsed in tap water, tissue samples were submerged in ethyl alcohol that ranged in concentration from 70% to 100% to dehydrate them. Xylene was used to clean the tissue samples before they were embedded in paraffin wax and blocked with recently melted paraffin. For histological analysis, five-micron sections were cut and stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and Masson's trichrome stains. (Bancroft *et al.,* 1996). The slides were then examined microscopically with an Olympus CX31 microscope and photographed with an Olympus SC30 camera adapted to the microscope.

Biochemical estimation:
Serum samples were separated from all different groups with a micropipette in Eppendorf tubes and stored frozen at -20 °C until biochemical parameter estimation.

The following biochemical parameters were evaluated using a JENWAY 6705 UV, Vis Spectrophotometer: Malondialdehyde (MDA): according to Ohkawa *et al.* (1979), MDA was measured using a colorimetric assay kit. According to Koracevic *et al.* (2001), total antioxidant capacity (TAC) was assessed using a colorimetric assay kit. According to Young DS (1990), alanine aminotransferase (ALT/GPT) was measured using a colorimetric assay kit. According to Young DS (1990), aspartate aminotransferase (AST/GOT) was measured using a colorimetric test kit. A modified Bromocresol Green Colorimetric Assay Kit was used to measure albumin (Doumas *et al.,* 1971).

Transmission electron microscope:
Liver samples were fixed in 5% cold glutaraldehyde for 24-48 hours immediately after animal necropsy. Samples were then

washed 3-4 times with cacodylate buffer (pH 7.2) for 20 minutes each, post-fixed with 1% O_4S_4 for 2 hours, and then washed 4 times with the same buffer. Dehydration with respective ascending alcohol content (30, 50, 70, 90, and 100% for 2 hours) was done in accordance with the Electron Microscope unit Assiut University protocol (Bozzol and Russell, 1991). and embedded in the epon mixture. Semi-thin sections 0.5-1 microns thick were cut from embedded blocks using an LKB ultramicrotome, A Leica AG ultramicrotome was used to slice the tissue into ultra-thin sections that were 500–700 Å thick and contrasted as usual with uranyl acetate and lead citrate. The tissue was orientated and shot using an SC30 Olympus camera. CCD digital camera Model XR-41 and JEM 100 CXII electron microscope at 80 KV were used to inspect and take pictures. Using the program Photo Filter 6.3.2, we digitally colored the TEM pictures in order to identify various cell and structural kinds.

Statistical analysis:

Data were analyzed with Program SPSS (version 16) software. Comparisons between several experimental groups were made using a one-way analysis of variance (ANOVA), and Duncan's test was performed as a post hoc analysis. The acceptable level of statistical significance was P <. 0.05. All data were expressed as mean ± standard error (SE).

RESULTS

Biochemical results:

Oxidative stress index **(MDA, TAC):**

Measurement of serum MDA levels showed that his MDA levels were significantly increased in the DOX-treated group compared to the control and nano groups. A significant decrease was observed in the serum level of MDA in the control, DOX + nano-TQ and nano-TQ treated group as compared with DOX treated group. Estimation of serum level of TAC exhibited that there was a significant decrease in DOX +Nano, DOX and Nano as compared with the control group (Table 1).

Table 1: Values of indicators of oxidative stress (MDA and TAC) in all experimental groups.

Groups	MDA(µmol/g)	TAC (mM/L)
Control	7.8 ± 0.4^{b}	8.27 ± 0.20^{a}
DOX	8.7 ± 0.8^{a}	6.9 ± 0.1^{b}
DOX + nano-TQ	7.9 ± 0.2^{b}	7.5 ± 0.12^{b}
nano-TQ	7.7 ± 0.3^{b}	7.8 ± 0.23^{b}

Means within the same column with different superscript letters were significantly different at P< 0.05. Data were expressed as the mean ± S.E and n of each group = 5.

Liver Function parameters (AST, ALT, TP, AL, GL):

Doxorubicin and DOX+ nano-TQ treated group revealed a significant increase in serum level of ALT in comparison to other groups. Doxorubicin and DOX+ nano-TQ treated group revealed no significant increase in serum level of AST in comparison to other groups. The total protein results showed a significant increase in DOX treated group in comparison to other groups. Doxorubicin + nano-TQ treated group reported a significant decrease in TP in comparison to DOX treated group and reported a significant increase in comparison to the nano group only. Concerning the serum level of albumin and globulin, there was no significant change between all groups (Table 2).

Table 2: Values of Liver function parameters (AST, ALT, TP, AL, GL) in all experimental groups.

Groups	AST(U/ml)	ALT(U/ml)	TP(g/dl)	AL(g/dl)	GL(g/dl)
Control	8.3±1.83 [b]	6.65±1.07 [b]	4.15±0.15 [b]	3.32 ± 0.17 [a]	0.87±0.07 [a]
DOX	23.4 ±5.6 [ab]	32.8±5.9[a]	5.9±0.32 [a]	4.28 ± 0.26[a]	1.66±0.22[a]
DOX + Nano-TQ	14.8±0.6 [ab]	25.20±1.3[a]	4.72±0.30 [b]	3.70 ± 0.31[a]	1.02±0.07[a]
Nano-TQ	6.5±2.6[b]	8.0±0.8[b]	4.46±0.78[b]	3.66±0.2[a]	0.8±0.10[a]

Means within the same column with different superscript letters were significantly different at $P< 0.05$. Data were expressed as the mean ± SE and n of each group = 5.

Histopathological findings:

Histopathological examination of the liver in DOX-treated group showed vascular and parenchymal changes. The vascular changes were in the form of congestion and thrombosis of the blood vessels. Congestion of the central vein and injury of its endothelium. Some cases were associated with perivascular fibrosis which was confirmed by Masson trichrome stain as the fibrous tissue appeared blue. Thrombosis of portal blood vessels associated with perivascular mononuclear inflammatory cell infiltration. The hepatic changes ranged from degeneration and necrosis of the hepatocytes. Most cases revealed vacuolar degeneration of the hepatocyte with pericellular fibrosis. Focal areas of necrosis of coagulative type infiltrated with mononuclear cells and proliferation of Kupffer cells were also noticed. Mononuclear cell infiltration was also seen in the portal area with portal fibrosis. Some cases showed focal areas of lytic necrosis infiltrated with mononuclear cells (Fig. 3A, B). Examination of the liver of DOX + nano-TQ treated group showed minimal changes in the form of congestion of the central vein, vacuolar degeneration and coagulative necrosis (Fig.3. C, D). The liver of the nano-TQ treated group and control group showed a normal appearance of the hepatocytes (Fig. 3E, F). Table 3 summarized the lesion score of the histopathological results based on the incidence of liver lesions in 30 examined sections of the 15 rats in each experimental group.

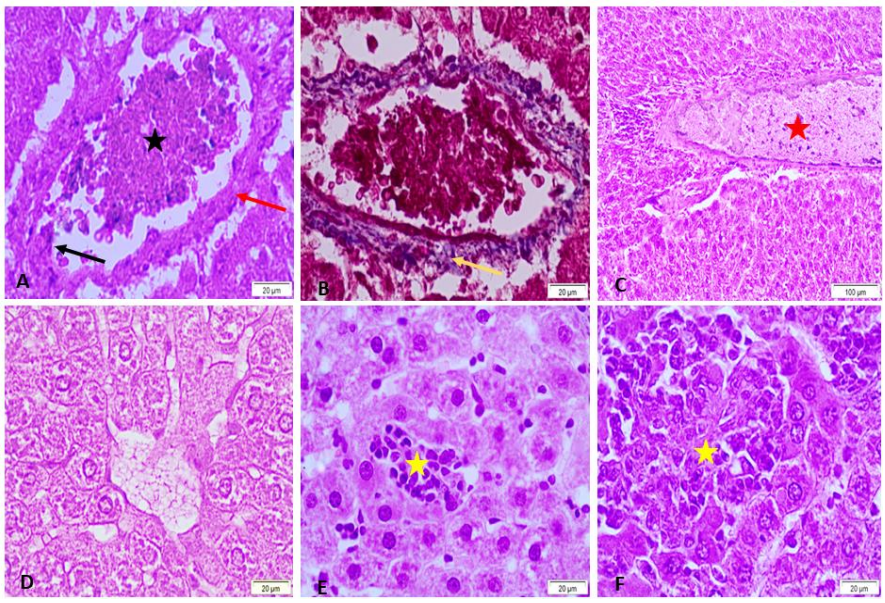

Fig. 2: Photomicrograph of the liver in DOX treated group showing A) congestion of the central vein (black star), injury of the endothelium (black arrow) and perivascular fibrosis (red arrow). B) Perivascular fibrosis stained by Masson trichrome (yellow arrow). C) Thrombosis at blood vessels in the portal area (red star) and perivascular mononuclear inflammatory cells. D) Vacuolar degeneration of the hepatocyte and pericellular fibrosis. E, F) Focal area of necrosis infiltrated with mononuclear cells (yellow star). H&E.

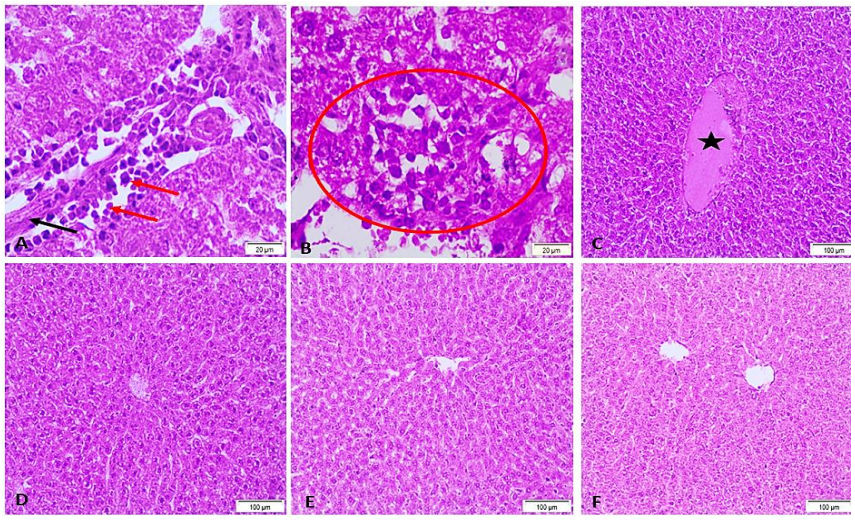

Fig. 3: Photomicrograph of the liver stained by H&E. A, B) Doxorubicin treated group showing mononuclear cell infiltration of the portal area (red arrows), portal fibrosis (black arrow) and focal area of lytic necrosis infiltrated with mononuclear cell (Circle). C, D) Liver of doxorubicin + nano-TQ treated group showing congestion and dilatation of the central vein (star), the liver cells slightly normal appearance. E) Liver of nano-TQ treated group showing normal appearance of the hepatocyte. F) Control group showing normal appearance of the hepatocytes.

Table 3: Effect of nano-TQ on the histopathological lesions in liver of rats treated with DOX.

Lesions	Control -ve	DOX	DOX+Nano-TQ	Nano-TQ
Liver lesions:				
Congestion of bl.vs	-	+++	++	-
Injury of endothelial lining bl.vs	-	+++	+	-
Perivascular fibrosis	-	++	-	-
Thrombosis	-	++	-	-
Vacuolar degeneration	-	+++	+	-
Coagulative necrosis	-	++	+	-
Lytic necrosis	-	++	-	-
Kupffer cell proliferation	-	+++	+	-
Inflammatory cell infiltration	-	+++	+	-
Portal fibrosis	-	++	-	-

(- No lesions, + lesions present in 2-5 sections, ++ lesions present in 6-15 sections, +++ lesions present in 16-30 sections per 15 rats).

Transmission Electron Microscopic findings:

Examination of the semithin sections of the liver stained by toluidine blue in the DOX-treated group showed focal areas of necrosis infiltrated with mononuclear cells. Also, single cells appeared shrunk and deeply stained with nuclear pyknosis (Fig. 4A, B). DOX + nano-TQ treated group revealed prominent Kupffer cell proliferation (Fig. 4C). Digitally colored TEM in DOX treated group revealed the presence of lymphocytes in the area of necrosis with large nucleus and rim of cytoplasm. The hepatocytes showed fat globules in the cytoplasm. Some hepatocytes showed necrosed nuclei with fragmentation of chromatin and fat globules in the cytoplasm (Fig. 5A, B). Doxorubicin + nano-TQ treated group revealed the presence of hypertrophied Kupffer cells (Fig. 5C).

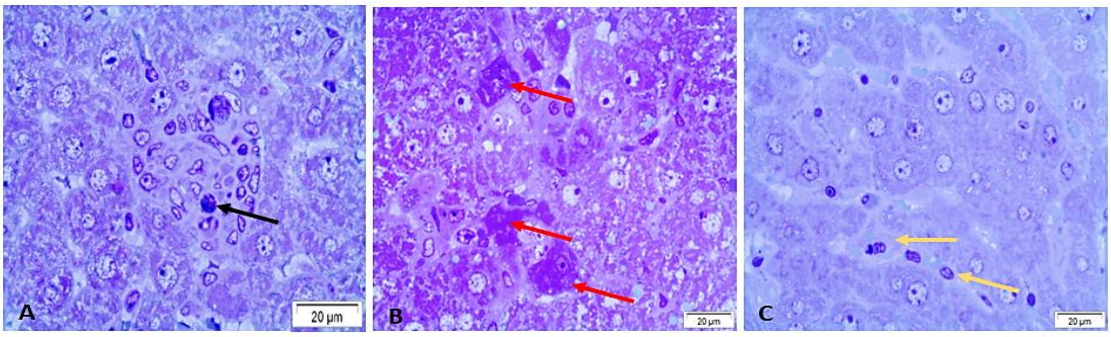

Fig. 4: Semithin section of the liver stained by toluidine blue. A, B) Doxorubicin treated group showing focal areas of necrosis infiltrated with mononuclear cells (black arrow), single cells with deeply stained, shrined and pyknotic nuclei (red arrows). Doxorubicin + nano-TQ treated group showing Kupffer cell proliferation (yellow arrows).

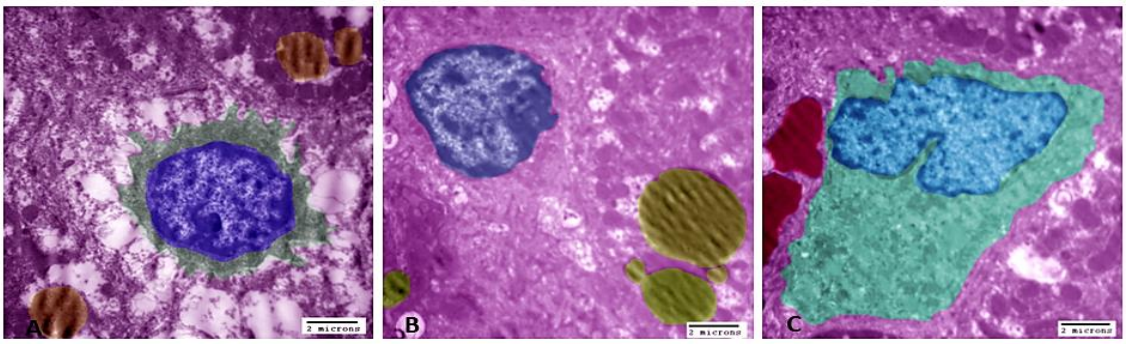

Fig. 5: Digitally colored TEM showing A) Doxorubicin treated group showing an area of necrosis infiltrated with lymphocyte (blue nucleus and rim of cytoplasm with green color). and also, the presence of fat globules (yellow). B) Doxorubicin treated group showing necrosis of hepatocyte, a pyknotic nucleus with fragmented chromatin (blue) and fat globules (yellowish color). C) Doxorubicin + nano-TQ treated group showing hypertrophied Kupffer cell (nucleus with blue color and cytoplasm with green color).

DISCUSSION

In our work, we evaluated the protective effect of nano-TQ on DOX-induced liver toxicity in male rats. DOX remains the anti-tumor drug of choice (Outomuro *et al.*, 2007). DOX caused tissue damage in the liver, and damage was substantiated by the biochemical studies in this study.

The current study reported that DOX induced hepatotoxicity and increased the biochemical parameters increase in liver enzymes and lipid peroxidation products in comparison to other groups. The TAC was significantly decreased in the DOX-treated group in comparison to other groups. Similar results were obtained by many authors (Abdel-Daim *et al.*, 2017; Alam *et al.*, 2018; Khedre *et al.*, 2022). Liver damage was confirmed pathologically by the presence of vascular and hepatocellular changes such as congestion, and thrombosis of blood vessels which is associated with perivascular mononuclear inflammatory cell infiltration. There was vacuolar degeneration of hepatocytes, necrosis and Kupffer cell proliferation. Many authors demonstrated hepatic dysfunction, an inflammatory cascade and histopathology of the liver after DOX treatment in rats agreed with our findings (Murat *et al.*, 2007; El-Sayed *et al.*, 2009; Chaudhary *et al.*, 2016). Le Couteur *et al.* (2005) confirmed that this drug must first pass the hepatic sinusoidal endothelium to enter the space of Disse and access the hepatocytes. Also, Pedrycz *et al.* (2005) said that the perisinusoidal space was often dilated and swollen with cytoplasmic contents of injured hepatocytes, and connective tissue proliferation was noticed. By TEM, DOX treated group revealed the presence of lymphocytes in the area of necrosis, the hepatocytes showed fat globules in the cytoplasm and necrosis with fragmentation of chromatin. A similar result was obtained by Kalender *et al.* (2005) who reported ultrastructure changes in the liver treated with DOX as mitochondrial vacuolization, mitochondrial swelling, necrosis with pyknotic nucleus and dilation of intercellular space.

Treatment with nano-TQ lead to a significant decrease in liver enzymes, LPO and a significant elevation of TAC compared to the DOX-treated group. Similarly, Hassanein and El-Amir (2017) reported that TQ treatment reversed titanium dioxide nanoparticles-induced hepatotoxicity's elevated levels of LPO, AST, and ALT, probably as a result of its potent antioxidant action. This is explained by its anti-inflammatory properties and antioxidant effects in cell culture systems and animal models (Mansour *et al.*, 2002). Thymoquinone can be bio-transformed to dihydrothymoquinone (DHTQ) by hepatic

quinone reductase. Both TQ and DHTQ can inhibit free radical-induced lipid peroxidation (Nagi et al., 1999). In the present study, DOX + nano-TQ treated group showed minimal changes as congestion of the central veins and vacuolar degeneration of the hepatocytes. Similar results were described by (El-sayed *et al.*, 2009 and Al Aboud *et al.*, 2021).

The present study showed that rats treated with DOX demonstrated pathological changes in the liver, associated with oxidative stress and altered liver enzymes. nano-TQ improved the toxic effect associated with DOX administration. The protective effect of nano-TQ could be due to its strong antioxidant and anti-inflammatory properties.

ACKNOWLEDGEMENT

We express our gratitude to the technical staff of the Department of Pathology & Clinical Pathology and the technical staff of the Electron Microscope Unit, Assiut University, Assiut, Egypt.

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العلاج بالنانوثيموكينون يبطل السمية الكبدية المستحثة بالدوكسوربوسين في ذكور الجرذان البيضاء

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الدوكسوربوسين هو دواء الأنثراسيكلين المضاد للأورام الذى يسبب السمية الكبدية باطلاق الشوارد الحرة وإصابة أنسجة الكبد ويعتبر النانوثيموكينون أحد مضادات الأكسده القوية التى تخفف من السمية الكبدية. تم تصميم الدراسة الحالية للكشف عن الأثار السامة للدوكسوربوسين فى الكبد و تقييم التأثير الوقائى للنانوثيموكينون ضد السمية الكبدية فى ذكور الجرذان وتم تقسيم ٦٠ جرذ الى ٤ مجموعات وتلقت المجموعه الأولى المعالجة بالدوكسوربوسين ٣,٧٥٠ مجم/ كجم من وزن الجسم بالحقن داخل تجويف البطن فى اليوم ١٠و١٧و٢٤و٣١ من بدايه التجربة لعمل جرعة تراكمية ١٥ مجم/ كجم وتلقت المجموعة الثانية الدوكسوربوسين و النانوثيموكينون من اليوم الاول بجرعه ١٠ مجم/ كجم من وزن الجسم حتى نهاية التجربة و تلقت المجموعة الثالثة النانوثيموكينون فقط والمجموعة الرابعة كانت مجموعة التحكم. وتم استخدام عينات المصل كعلامات للإجهاد التأكسدى و اختبارات وظائف الكبد واستخدامها للفحص الهستوباثولوجى والمجهر الالكترونى النافذ. و يتسبب اعطاء الدوكسوربوسين فى زيادة إنزيمات الكبد و نواتج أكسدة الدهون وأدى اعطاء النانوثيموكينون إلى التحسن فى تلك المستويات وأظهرت الفحوصات النسيجية المرضية للمجموعة المعالجة بالدوكسوربوسين إلى بعض التغييرات فى الأوعية الدموية مثل احتقان و تجلط الأوعية الدموية وتنكس و نخر وتليف الكبد. بينما يحسن النانو ثيموكينون مثل هذا التغيير المرضي للخلايا الكبدية. وكشف المجهر الالكترونى النافذ عن وجود الخلايا الليمفاوية فى المناطق الميتة ووجود كريات دهنية فى سيتوبلازم الخلايا الكبديه فى المجموعة المعالجة بالدوكسوربوسين بينما أظهر المجهر الإلكترونى النافذ عن اكتشاف فريد للمجموعة المعالجة بالدوكسوربوسين و النانوثيموكينون فى صورة تضخم فى خلايا كوفر. يمكن أن نستنتج أن نانوثيموكينون له تأثير مضاد للأكسدة قوي يحمي تلف الكبد من خلال الوقاية من الشوارد الحرة.