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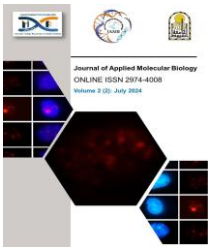

The Protective Effect of Omega-3 Fatty Acids against Genotoxicity and Hepatotoxicity of Doxorubicin Treatment in Male Albino Rats

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ABSTRACT

Doxorubicin (DOX) is a common antineoplastic drug, prescribed single or combined with different agents. Of its class, it remains having a wide range of activity. Nevertheless, DOX could adversely affect normal cells; therefore, its clinical use is limited. The present research aimed to investigate the genotoxic effects of DOX on liver tissues, the preventative role of omega-3 against DOX-induced hepatotoxicity in male albino rats. Rats (n=60) were split to 4 groups (15 individuals each). The control group was orally given corn oil at 0.5 ml. For a period of four weeks, group 2 received weekly intraperitoneal injections of DOX (4mg/kg b.w). For 30 days, group 3 received daily dose of omega-3 (400mg/kg b.w). Group 4 were administrated with DOX and omega-3 at the same doses as group 2 and 3, respectively. Many techniques were used to estimate the effect of omega-3 against a DOX. Comet assay showed that DOX treatment caused significant DNA damage in both the blood and liver. Biochemical assay showed elevation in serum alanine transaminase (ALT), asparate transaminase (AST) malondialdehyde (MDA), nitric oxide(NO) and reduction of antioxidant enzymes superoxide dismutase (SOD)and catalase (CAT). Histologically, the liver showed disarray, hyperemia, coagulative necrosis, hydropic degeneration and leukocytic infiltration. Treated animals with omega-3 along with DOX showed a decreased rate of DNA damage. Moreover, better biochemical changes, such as a reduction in the MDA and a rise in the antioxidant enzymes activity were observed. The current study has revealed a to protective effect of omega-3 against DOX-induced liver injury. Comet assay provided conclusive results for blood and liver genotoxicity.

1. INTRODUCTION

Cancers are caused by uncontrolled cell division which produce a huge number of immature cells. Carcinogenic agents may be present in the water, meals, air and frequently used chemical compounds [1] Chemotherapy is broadly used to face the spreading of tumors for most patients, which help to promot the treatment of cancer [2]. Exposure to different mutagens, chemicals, nanoparticles and radiation causes damage to cells. Doxorubicin is an anthracycline that was isolated from Streptomyces peucetius species. It has a variety of uses, including the treatment of solid tumors and haematological malignancies, as well as adult and pediatric cancers [3,4,5].

Unfortunately, despite being enormously powerful, DOX is non-selective and affects both cancer and normal cells, so its use is appreciably confined due to its toxicity. DOX toxicity was documented to impact different tissues including heart, brain, liver and kidneys, causing inflammatory alterations and oxidative damage in rats [6,7]. Free radical production, iron-dependent oxidative damage to biological macromolecules, membrane lipid peroxidation, and subsequent cellular membrane disintegration have all been implicated as possible mediators of DOX-induced toxicity [7,9,10]. Long-term implications may result from such toxicties [5,8].

Predicting the tumor cell response to radiation (radiotherapy) would give valuable information in the clinical oncology field. There are several techniques available, such as micro-array (are used in interpreting the data generated from experiments on DNA) and clonogenic (studying the effectiveness of specific agents on the survival and proliferation of cells) cell survival. However, these techniques have some limitations that can make the results complicated and time-consuming. Comet assays are used as a molecular biology tool to investigate DNA damage and repair kinetics. Tumor biopsy can be easily assessed using comet assay [11]. Different anti-cancer drugs, used for chemotherapy, can be screened to evaluate their levels of genotoxicity qualitatively, and quantitatively using comet assays [11]

Omega-3 polyunsaturated fatty acids (PUFAs) involve inolenic acid (ALA; 18:3-3), stearidonic acid (SDA; 18:4-3) and others. Numerous experiential and epidemiological research conducted over the past ten years have demonstrated that PUFAs. It has been suggested that omega-3 PUFAs reduce the chance of developing cancer and increase the effectiveness and tolerability to chemotherapy because it is a strong antioxidant [2,12].

The current study aimed to estimate the genotoxic potential of doxorubicin on liver tissues and blood cells, and the preventation effects of Omega-3 against doxorubicin-induced hepatotoxicity in male albino rats.

1. MATERIALS AND METHODS

2.1. Animals:

Adult male albino rats (body weight of 200±10 gm) were obtained from the Animal House, Faculty of Veterinary Medicine, Assuit University, Egypt. Animals were housed at room control (25-27 oC, with 12h light/dark cycle) for two weeks for adaptation before starting the experiment. They were fed a standard rat diet and tap water ad lipitum.

2.2. Chemicals:

DOX hydrochloride (Adriadox® vials) was purchased from RMPL PHARMA LLP (Mumbai, Maharashtra, India). Fish oil omega-3 (WINDMILL) was made in the U.S.A. from globally sourced ingredients.

2.3. Experimental design:

After adaptation male rats (n=60) were randomly split as 4 equal groups (15 rats each) as following:

Group 1: Control rats were administered 0.5 ml of corn oil via gastric tube.

Group 2: DOX-group, rats were treated with doxorubicin at 4 mg/kg b.wt. intraperitoneally, once weekly for four weeks.

Group 3: Omega 3 group, fish oil fatty acid of omega-3 (400 mg/kg) was given by gastric tube to the rats once every day for 30 days. [2]

Group 4: (omega-3+DOX) group, the rats were administered (400mg/kg) omega-3 daily for 30 days via gastric tube and simultaneously with doxorubicin (4 mg/kg b.wt.) intraperitoneally, once weekly for four weeks.

2.4. Blood and serum analyses:

The experimental animals were ketamine-anesthetized after four weeks. For the comet assay and the biochemical testing, blood samples were collected from the dorsal aorta in heparinized and non-heparinized tubes, respectively. Non-heparinized blood was centrifuged at 2500 rpm for 15 minutes to extract the blood serum, which was then separated and kept at -80. to determine the levels of liver enzymes in serum samples, Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial kits in accordance with industrialist's instructions.

2.5. Liver tissue collection and preparations:

Rats were slaughtered after blood was drawn, and the livers of the control and treatment animals were separated. For the comet assay, pieces of liver tissue were processed right away to check for DNA damage. For the quick assessment of antioxidants in the liver, additional liver tissue parts were processed. Another liver sample were fixed in 10% formalin for histological investigation.

2.6. Comet assay:

Comet assay were done according to (fouad et al;2015), 0.5 g of crushed liver samples were added to 1 ml ice cold Phosphate-buffered saline (PBS) . This suspension was stirred for 5 min and filtered . Cell suspension (100µl) was mixed with 600µl of low-melting (0.8% in PBS) . 100 µl of this mixture spread on pre-coated slides were immersed in lyses buffer (0.045 M TBE , pH 8.4 and contain 2.5 %SDS) for 15 min . The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS . The electrophoresis conditions were 2 V/cm for 2 min and 100 m A, staining with ethidium bromide 20 µg/ ml at 4oC . The observation were evaluated with fluorescence microscope (with excitation filter 420-490 nm (issue 510 nm) . The comets tails lengths were measured from the middle of the nucleus to the end of tail with 40 X increase for the count and measure the size of the comet . We use a comet 5 image analysis software developed by kinetic imaging Ltd . (liver pool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damaged in the cells by measuring the length of DNA migration and percentage of migrated .The results were presented as mean ± SE. [13]

2.7. Biochemical analysis of liver tissue:

In 4–8 ml of cold buffer (PBS buffer), minced livers were homogenized after being weighed. The clear supernatants from homogenates centrifuged at 10,000 g/min for 20 minutes at 4 oC The clear supernatants from homogenates centrifuged at 10,000 g/min for 20 minutes at 4 oC and used to calculate the oxidative stress and antioxidant

enzymes. The method of [14] was used to measure malondialdehyde (MDA), [15]; was used to measure superoxide dismutase (SOD), [16]; was used to measure nitric oxide (NO); and [17]; was used to measure catalase (CAT).

2.8. Histopathological examination:

Small cubes of tissue of livers were fixed in 10% formalin. They were washed after 24 hours in PBS, and dehydrated in ethyl alcohol series ascendinly. Then, they were cleared in xylene (2 changes, 60 minutes each). Samples were transferred to melted wax in an oven (60ºC) through 3 changes of paraffin wax for 3 h each. Finally, the liver cubes were embedded in wax. Then cut them to Sections (5-6µ) using a microtome (a Richert Leica RM 2125 Microtome, Germany). Slides were stained with eosin and counterstained with hematoxylin [18]. Selected photographs were taken by Olympus CX41 light microscope equipped with a digital Olympus camera and digitized images.

2.9. Statistical analysis:

The data were analyzed by (Prism 9, version 8.0.2(263)). Each value is expressed as the mean ± standard error (S.E.) of the mean. To compare differences in the mean values of the experiential groups, values were analyzed using one-way analysis of variance ANOVA. P-values ($P<0.05$) were regarded as significant.

2. RESULTS

Single-cell gel electrophoresis assay technique was executed to examine DOX-induced possible DNA damage in blood specimens as well as in liver samples (Tables 1,2 and Figures 1,2).

3.1. Blood

The results of blood comet assay of the control group displayed normal cells with typical nuclei (Figure 1a); while DOX treated group showed severe DNA damage observed as comets (Figure 1b). Omega-3 alone showed comparable results to the control (Figure 1c) and in the combined (Omega-3 + DOX) group, Omega-3 prevented the adverse effect of dox where the comet showed nearly normal nuclei of healthy cells (Figure 1d).

The combination of omega-3 with DOX revealed a significant decrease ($p<0.05$) in all DNA damage parameters in comet assay of blood compared to the DOX-treated group of rats. The decreased percentage of DNA damage upon supplementation with omega-3 indicates the protective action of omega-3 against the genotoxic injury of DNA induced by DOX treatment.

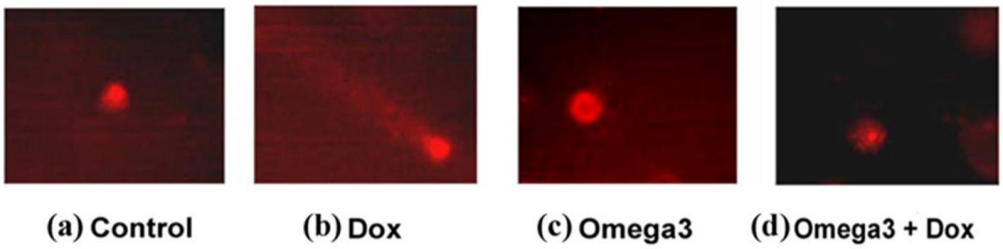

Figure 1. Representative photograph in blood of Albion rat showing (a) typical nuclei of healthy cells of the negative control group; (b) DNA damage appeared as comets in DOX (4 mg/kg) group; (c) Omega-3 group (400 mg/kg); and (d) combined omega-3+ DOX group which is characterized by a marked reduction of DNA damage.

3.2. liver tissue:

The results of liver tissue comet assay of the control group displayed typical nuclei of undamaged cells (Figure 2a); while DOX treated group showed severe DNA damage observed as comets (Figure 2b). Omega-3 alone showed comparable results to the control (Figure 2c) and in the combined (Omega-3 + DOX) group, omega-3 prevented the adverse effect of DOX where the comet showed nearly normal nuclei of healthy cells (Figure 2d).

The combination of omega-3 with DOX revealed a significant decrease ($p<0.05$) in all DNA damage parameters in comet assay of liver compared to the DOX-treated group. The decreased percentage of DNA damage upon supplementation with omega-3 indicates the protective action of omega-3 against the genotoxic injury of DNA induced by DOX treatment.

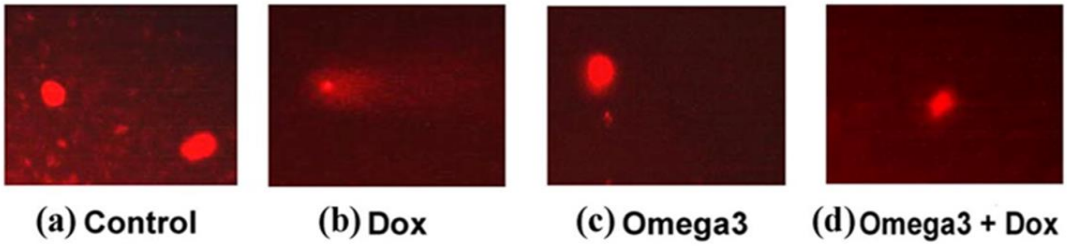

Figure 2. Representative photograph in liver tissue of Albion rat showing (a) typical nuclei of healthy cells of the negative control group; (b) DNA damage appeared as comets in DOX (4 mg/kg) group; (c) Omega-3 group (400 mg/kg); and (d) combined omega-3+ DOX group which is characterized by a marked reduction of DNA damage.

Finally, the results of comets showed similar trends in DNA damage parameters in blood and liver comets (Tables 1,2 and Figures 3,4). In the blood, there are no significant differences between the control and the omega-3-treated group, demonstrating the safety, and absence of genotoxic effects of omega-3. Comparing DOX to the control and omega-3 groups in blood comets, there was a significant ($p<0.001$) increase in tail length, tail moment, tail % DNA damage, and tail % damage, indicating genotoxic effects.

Table1. The effect of omega-3 against doxorubicin on the proportion of DNA damage, tail length, the percentage of DNA in tail and tail moment in blood cells of different groups of Albion rat.

Treatment	% of damage	Tail length(px)	% DNA in Tail	Tail moment
Control	7.15±0.15	7.79±0.09	7.04±0.30	0.44±0.03
DOX	22.65±0.45*	19.56±0.47*	13.65±0.17*	1.06±0.05*
Omega-3	8.25±0.25	8.41±0.09	9.72±0.25	0.59±0.01
DOX+ Omega-3	18.25±0.25*	11.56±0.23*	7.71±0.12*	0.85±0.03*

Data were represented as mean ± SE, (n = 15).
P<0.05 significantly different from control. (one-way ANOVA)

Asignificant increase was showed in the levels of serum AST, and ALT ($p<0.001$) in DOX-treated when compared to the control and omega-3 groups (Table 3 and Figure 5). In comparison to the DOX-treated group, treated with omega-3 along with

DOX caused a significant ($p<0.001$) decrease in the amounts of enzymes close to the normal levels (Table 3).

Table 2. The effect of Omega-3 against doxorubicin on the proportion of DNA damage, tail length, the percentage of DNA in tail and tail moment in liver tissue of different groups.

Treatment	% of damage	Tail length (px)	% DNA in Tail	Tail moment
Control	10.2±0.20	7.808±0.141	8.223±0.255	0.76±0.044
DOX	26.83±0.441*	11.07±0.569*	14.59±0.546*	1.21±0.018*
Omega-3	14.95±0.750	8.1±0.447	8.61±0.234	0.7±0.034
DOX+Omega-3	17.85±0.150*	9.46±0.458*	8.72±0.533*	0.96±0.056*

Data were represented as mean ± SE, (n = 15).
P< 0.05 significantly different from control. (one-way ANOVA)

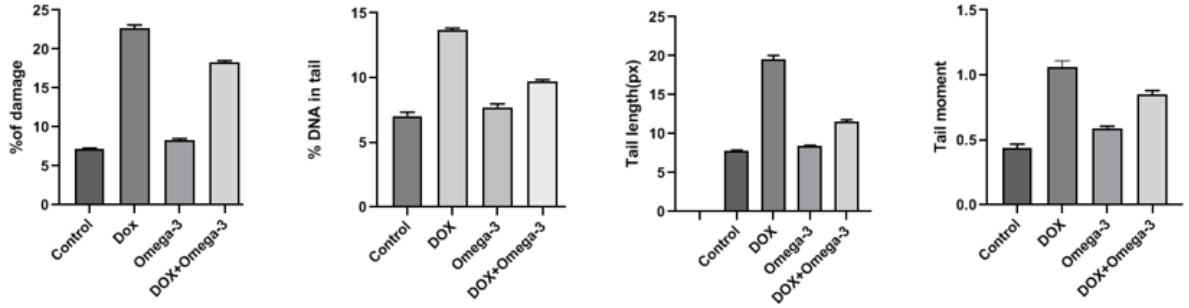

Figure 2. Comparisons between blood demonstrating the effect of Omega-3 (400 mg/kg) on DNA damage after DOX (4 mg/kg) treatment in rats.

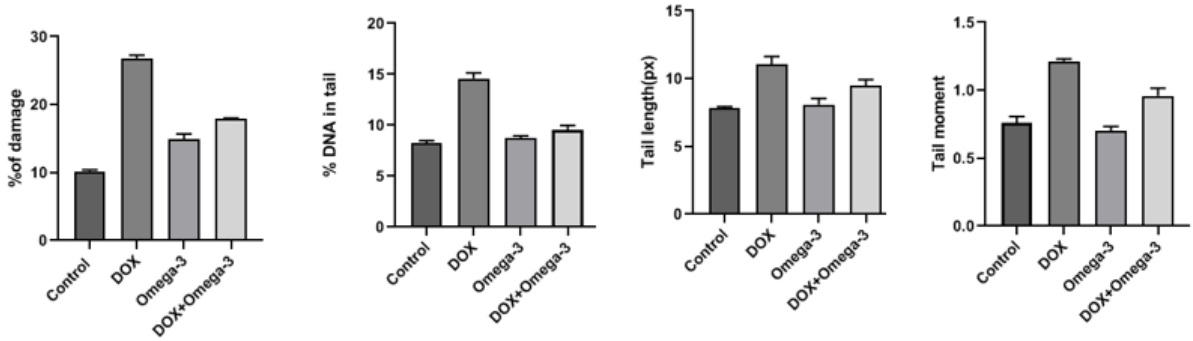

Figure 4. Comparisons between liver tissue of Albion rat demonstrating the effect of Omega-3 (400 mg/kg) on DNA damage after DOX (4 mg/kg) treatment in rats.

Table 3. The effect of omega-3 on some function of liver in different experimental groups of Albion rat.

Parameters (units)	Expermintal groups			
	control	DOX	Omega-3	DOX+Omega
AST (u/L)	178±1.41	282±2.82**	151±1.41	175±1.41**
ALT (u/L)	31±1.41	56±2.82**	26±1.41	29±1.41**

- Values are tabulted reported as means ± SE; (n= 15) for each experimented group.
**Means are statistically significant compared to the control group and (DOX+Omega-3) group. P<0.001

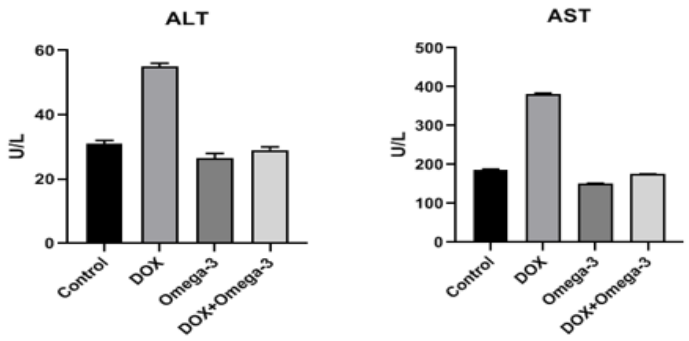

Figure 5. Serum ALT and AST in control, DOX, omega-3, and DOX+omega-3 treated of Albino rat

The biochemical results of liver tissues showed that the mean values of CAT and SOD (Table 4 and Figure 6) were significantly lower in the DOX-treated group than in the control group ($p < 0.05$) and ($p < 0.001$), respectively. The levels of MDA and NO in the liver tissues were significantly raised in the DOX-treated group when compared to the control group ($p < 0.001$). In contrast, omega-3 in the DOX-treated group increased CAT and SOD compared to the DOX-treated group. Furthermore, administration of fish omega-3 fatty acids along with DOX led to significant ($p < 0.05$) decrease in MDA levels and ($p<0.001$) in NO compared to the DOX-treated group.

Table 4. The effects of fish oil omega-3 on MDA, CAT, SOD, and NO in the liver tissue of different experimental groups of albino rat.

Parameters	**Control**	**DOX**	**Omega-3**	**DOX+O**
Catalase(CAT) liver tissue (u/gm)	486±17.7	399.9±15.78*	912.4±31. 3	783.3±24.5**
Superoxide dismutase (SOD)LiverTissue(U/gm)	1080±10.8	702.5±23.3**	1259±23.1	908.3±3.11**
(MDA) (nmol/g)	1.51±0.10	2.39±0.07**	1.44±0.06	1.97±0.02*
Nitric oxide(NO) nmol /mg tissue protein	26.24±1.18	51.25±1.11**	28.2±1.4	35.01±2.03**

Values are reported as the means ±SE; n= 15 for each treatment group
* Means are significantly different between the control group and (DOX+O) group. – (P<0.05)
** Means are significantly different between the control group and (DOX+O) group. – (P<0.001)

Histological examination revealed a normal hepatic architecture in both control and omega-3 administered groups (Figure 7 A, B). The liver parenchyma consisted of hepatic lobules. Hepatocytes arranged in cords around a small central vein. Hepatocytes are large polyhedral cells, with eosinophilic cytoplasm and large, round central nuclei. A few cells were binucleated. Portal triad contains: a portal vein, a hepatic artery and a small bile ductules of cuboidal epithelium. Vascular sinusoids between the anastomosing hepatocytic cords converge on the lobule's central vein. Numerous Kupffer cells (small resident hepatic macrophages) and specialized stellate cells are found in the sinusoid lining.

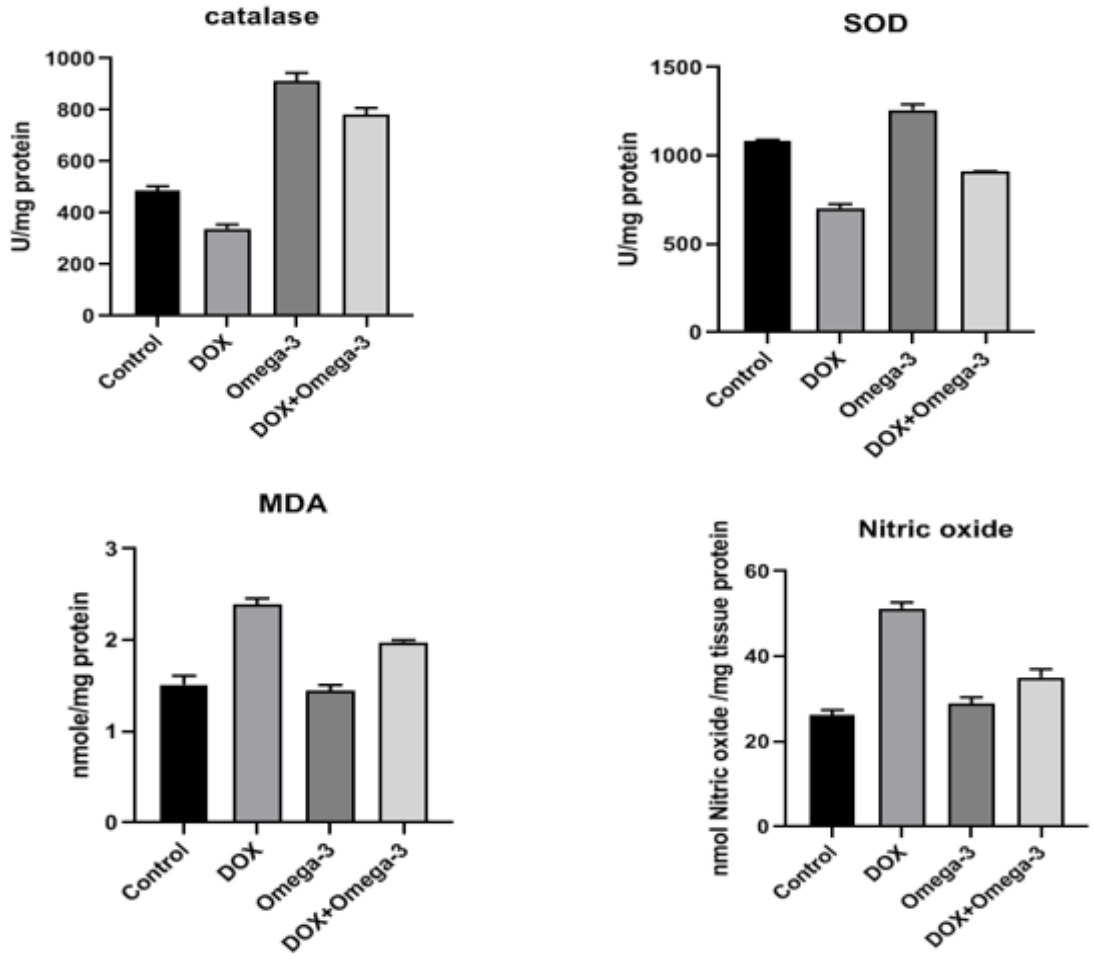

Figure 6 . The effects of DOX, omega-3 and DOX + omega-3 on liver oxidation and antioxidant profile in rats. Values are reported as the means ±SE (n = 15).

Administration of DOX led to focal and multiple changes in the parenchyma, which involved massive areas of the liver. The hepatocytes lost the orderly cord arrangement (i.e., disarray), and the blood sinusoids were hyperaemic, (Figure 7 C). Intermingled foci of coagulative necrosis and hydropic degeneration of hepatocytes (Figure 7 D). These foci contained hepatocytes increase in acidophelia of the cytoplasm and pyknotic nuclei. In the parenchymal tissue and/or the portal tract leucocytic aggregation was observed (Figure 7 E). In addition, pavementation of leucocytes through the blood vessel diffused Kupffer cells and leucocytes in the sinusoid lumen, have been obsreved. In the fourth group, when omega-3 was given along with doxorubicin, the architecture of the liver was obviously improved (Figure 7 F-H). Few hepatocytes with pyknotic nuclei, and less vacuolated cytoplasm were seen. Therefore, the histological results support the obsrevations of the comet assay and biochemical results.

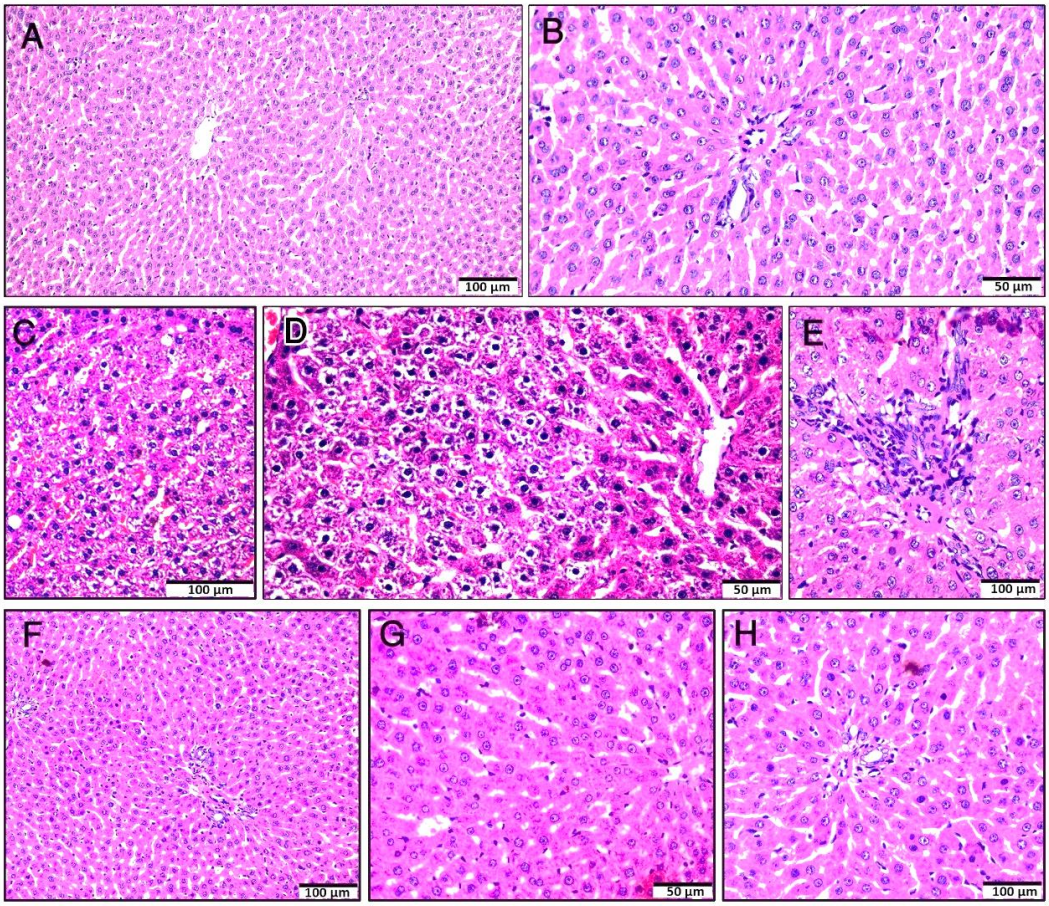

Figure 7. The liver sections doxorubicin and after omega-3 administrated rats (H&E stain). (A) control and (B) Omega-3 liver with normal architecture groups which was normal and comparable. The second row shows the multiple focal lesions in the liver of the DOX-administrated group. (C) They include the disarray of the hepatic cords and congestion of sinusoids, (D) intermingled foci of coagulative necrosis, hydropic degeneration of hepatocytes and (E) leukocytic infiltration. The last raw shows the protective effect of Omega-3 when administrated simultaneously with Dox (F-H).

3. DISCUSSION

In the current study, we utilized the comet test as a rapid tool to detect and quantify of DNA damage at the level of individual cells and, concurrently, to assess the effectiveness of regeneration using omega-3 in DOX-induced liver genotoxicity. This is a promising step in the utility of this test in predicting how cancer patients will respond to treatment. We also used other techniques to study and validate the adverse effects of doxorubicin and the likely protective effects of omega-3. In the present work, the genotoxic impact of doxorubicin on the liver was evidenced by the increase in DNA damage measured by comet assay. The toxic effect of doxorubicin was also recorded as the significant increase in the levels of ALT and AST enzymes and MDA level and NO as well as the decreased levels of CAT and SOD in rat liver tissues compared to the control rat group.

The results revealed that the treatment with DOX has a significant genotoxic action on the liver, as evidenced by the increase in DNA damage measured by comet assay as

demonstrated previously [19,20]. Similar genotoxic effect of DOX treatment observed in blood samples analyzed by comet was consistent with the previously obtained results [21].

The use of comet assay has exploded and is now used in a variety of fields, including genotoxicity testing for the agreement of biologics and chemicals [22,23]. The current work reports the antigenotoxic impact of omega-3 by reducing the genotoxic potential of DOX in the combined DOX and omega-3 groups. It significantly reduced the proportion of DNA damage, tail length, and proportion of DNA in tail and tail moment in comparison to the DOX-treated group in both blood and liver tissues. Our results confirmed many preceding experiments [20,24,30] in male rats, who found that omega-3 administration either before or after treatment with the anticancer DOX quantitatively reduced the fragmentation of DNA.

The aminotransferases ALT and AST serve as clinical diagnostic markers for liver injury. The greater AST and ALT activities observed in the present work may be explained due to severe liver damage brought on by the toxic activity of DOX accumulation, which increased the permeability of hepatic cells and caused cellular degeneration [25]. Recently, similar studies reported a significant increase in levels of ALT and AST in DOX-treated mice than in the negative control group [26]. In the present investigation, the protective effect of omega-3 was observed in the liver function of albino rats administered with doxorubicin, which, significantly minimized the toxic effect of DOX, similar to previous studies [26,27,28]

According to our results, treating albino rats with omega-3 along with DOX led to an improvement in the biochemical levels induced by DOX. DOX treatment significantly decreased CAT, NO and SOD in rat liver tissues compared to the control group. The liver is a sensitive organ to DOX damage as its high metabolic capacity. DOX can creat and initiate reactive molecules, including oxygen radicals. It can also inactivate superoxide dismutase (SOD). Then, the elevated reactive oxygen species (ROS) and the reduced proportion of antioxidants can lead to further tissue destruction [29].

SOD, a metalloenzyme that is an important part of the cellular antioxidant defense system, which catalyzes the superoxide anion dismutation into molecular oxygen and hydrogen peroxide [30]. Relying on the isoform of nitric oxide (NO) synthase, NO has a double effect on liver physiology during ischemia injury (NOS). Because of the reaction between the superoxide anion and peroxynitrite radical, which produces an oxidative toxin that further harms the liver, excessive NO synthesis induced by iNOS may have a detrimental impact on hepatic tissue [31].

Under typical physiological conditions, organisms use a delicate equilibrium between the production of ROS and the complex web of antioxidant defense systems to inactivate and defend themselves from free radical poisoning [32]. A situation known as oxidative stress is caused by a disturbance of the oxidant/antioxidant equilibrium and is recognized to be a factor in cellular and molecular tissue harm in a variety of human disorders [32,33]. Severe oxidative stress can cause necrotic and apoptotic cell death [34].

On the other hand, the levels of CAT and SOD were significantly increased in the DOX + omega 3-treated group compared to the DOX group which is consistent with the results which reported that omega 3 reduced MDA and raise SOD in animals [30,35]. The significant increase in SOD may be illustrate by the potency of omega 3 to stimulate the ROS-sensitive transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2),

which upregulates the antioxidant enzymes [36]. The score of the current study showed that omega-3 had a protective impact against liver toxicity induced by DOX and this might be as its antioxidant activities.

In the current study, MDA, a byproduct of secondary lipid peroxidation, serves as a marker for damage to cell membranes [30,37]. We found that treatment with DOX led to a significant raise MDA levels compared to the control group. However, administration of omega-3 fatty acids in combination with DOX showed a decrease in MDA rates compared to the DOX group. The level of MDA significantly decreased along with an increase in the activity of antioxidant enzymes [2]. The significant decrease in MDA level might be illustrate by the potency of omega-3 to prevent the production of DOX-induced ROS and to the upregulation of antioxidant enzymes [30,38]

Histopatological alteraions were used as biomarkers for diseses or steressors [39]. Our histological findings revealed that the liver tissue underwent several changes due to DOX. These include hepatocyte degeneration, congestion of blood vessels, and necrosis [2,40]. Treating rats with omega-3, along with DOX, improved the histological damage in the liver when compared with DOX treated group. This demonstrated the value of omega-3 in preventing the hepatotoxic effects of DOX. The advantages of administration of Omega-3 in order to lessen the toxicity of DOX on the liver include the restoration of the normal liver architecture and diminishing the congestion and hepatocyte degeneration [2,10,41] This ameliorative effect of Omega-3 present in fish oil could be attributed to its high peroxidizable characteristics, which could raise cancer cells' sensitivity to conventional cancer medication while normal host cells stay unaffected. Consequently, this may lead to an increase in positive responses to cancer treatment with fewer adverse effects [42,43].

CONCLUSION

Taken together, omega-3 fatty acids had protective and antigenotoxic effects by modulating the genotoxic potential of doxorubicin. Furthermore, comet assay is a highly sensitive technique to analyze and quantify the DNA damage induced by doxorubicin. Therefore, the current study recommends: The adminstrating omega-3 along with doxorubicin to get the most effective action

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