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Original article

Doxorubicin-induced hepatic toxicity in rats: Mechanistic protective role of Omega-3 fatty acids through Nrf2/HO-1 activation and PI3K/Akt/GSK-3β axis modulation

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ABSTRACT

Doxorubicin (DOX), a common antibiotic used to treat a variety of tumors, has several substantial adverse effects that limit its clinical use. As a result, finding effective protective agents to combat DOX-induced organ damage is a necessity. The current study was set to delineate the hepatoprotective role of omega-3 fatty acids (ω-3FA) against DOX-mediated acute liver damage in rats and the underlined mechanism of GSK-3β inhibition. Five groups of rats were orally received either saline (groups 1 & 2) or ω-3FA (25, 50 and 100 mg/kg/day; groups 3, 4 & 5, respectively) for 28 consecutive days. Single DOX intraperitoneal injection (20 mg/kg) was used to induce hepatic toxicity in all groups except group 1 (negative control). Blood samples and liver tissues were collected 48-hr after injection. Our results revealed that pre-administration of ω-3FA (25, 50 and 100 mg/kg) to DOX-induced hepatic injured rats showed a significant reduction in serum hepatic injury biomarkers (ALT, AST, total and direct bilirubin) as well as hepatic contents of MDA, GSH, Nrf2 and HO-1. Additionally, hepatic PI3K, pAkt and GSK-3β have been restored significantly in a dose-dependent manner. Furthermore, all the hepatic histopathological features have been retained upon ω-3FA treatment together with the immunostaining intensity of tumor necrosis factor-α and caspase-3. These results suggest that ω-3FA have shown a marked activation of the Nrf2/HO-1 signaling pathway and modulation of the PI3K/pAkt/GSK-3β axis against DOX-induced hepatotoxicity.

1. Introduction

Doxorubicin (DOX) is a common chemotherapeutic antibiotic widely used to treat breast cancer, leukemia, bronchogenic carcinoma, gastric carcinoma, sarcomas and thyroid carcinomas as well as hematological malignancies (Thorn et al., 2011). Although DOX has a prominent antitumor activity, its adverse effects on non-cancerous cells limit its use (Li et al., 2018). It is metabolized by liver microsomal enzymes and cytoplasmic reductases, resulting in the accumulation of toxic and immunogenic intermediates that have been implicated in the induction of liver injury (Licata et al., 2000). According to growing evidence, DOX toxicity is caused by a variety of processes that vary based on the cell type. DOX exerts its activity by causing DNA damage in cancer cells, preventing them from proliferating (Edwardson et al., 2015), while in non-cancerous cells, it triggers oxidative stress and impairs the mitochondrial activity by building up reactive oxygen species (ROS) (Asensio-López et al., 2017). DOX could stimulate oxidative stress by inhibiting the transcription factor; nuclear factor (erythroid-derived 2)-like 2 (Nrf2) that synchronizes cellular redox homeostasis and controls the antioxidant and detoxification responses (Jakobs et al., 2017). The pathway mediating DOX-induced impairment of redox homeostasis has been reported in numerous organs, like heart (Cappetta et al., 2017), kidneys (Elsherbiny and El-Sherbiny, 2014) and liver (AlAsmari et al., 2021; Barakat et al., 2018).

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Glycogen synthase kinase (GSK)-3β, a principal redox-sensitive protein kinase, is abundantly expressed in almost all cells and is participated in numerous extracellular and intracellular activities as well as mediating cell differentiation, proliferation, and apoptosis (Hoffmeister et al., 2020; Martelli et al., 2021). Many signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt could control the GSK-3β activity. Moreover, GSK-3β suppression could modulate oxidative stress in hepatocytes by targeting Nrf2 (Jiang et al., 2015). Evidence has shown that targeting GSK-3β might be a novel approach to counteract the DOX-induced toxicity in many organs (Niringiyumukiza et al., 2019).

Omega-3 fatty acids (ω-3FA) are essential fatty acids found in cell membranes of many body regions (Avramovic et al., 2012; de Batlle et al., 2012). Besides their anti-inflammatory activities (Layé et al., 2018; Talukdar et al., 2010), ω-3FA have been shown to mitigate redox homeostasis imbalance via elevating the levels of the transcription factor; Nrf2 with subsequent heme oxygenase-1 (HO-1) activation via targeting GSK-3β (Beurel et al., 2015), in hepatic tissue and hippocampus (Qi et al., 2017). Several studies have shown that GSK-3β suppression protects many organs against chemotherapy-mediated oxidative and apoptotic damage (Lin et al., 2020; Niringiyumukiza et al., 2019; Wang et al., 2010). However, to the best of our knowledge, targeting GSK-3β on DOX-mediated hepatic damage has not been extensively investigated. Our aim was to delineate the protective role of ω-3FA against DOX-mediated acute hepatotoxicity *in vivo* and to verify the hypothesis that GSK-3β inhibition would have a prominent impact on counteracting the detrimental effect of the chemotherapeutic agent on the liver. Moreover, the involved mechanism in suppressing DOX-induced oxidative damage as a therapeutic target to counteract hepatic toxicity has been investigated.

2. Material and methods

2.1. Drugs and chemicals

Fish oil ω-3FA and DOX were obtained from BIOVEA (Egypt) and Sigma-Aldrich (USA), respectively. Each 100 ml of fish oil contains 95 mg of total ω-3FA (18 mg eicosapentaenoic acid, 12 mg docosahexaenoic acid and other fatty acids). All other used chemicals were of analytical grade.

2.2. Animals

Female Wistar albino rats (180–200 g) were provided by the National Research Centre (NRC, Egypt). Animals were housed in clean plastic cages at a 12-h light/dark cycle with temperatures adjusted at 22 ± 3 °C. They had free access to water ad libitum and food of standard pellets. Rats were acclimated to this setting for 7 days before the start of the experiment. The NRC ethics committee approved all procedures and experiment protocol.

2.3. Experiment design and treatment regimen

Rats were randomly divided into five groups (7–8 rats per group). Group 1 (normal, negative control) and group 2 (DOX, positive control) received saline for 4 consecutive weeks. Groups 3, 4 and 5 received oral ω-3FA (25, 50 and 100 mg/kg daily, p.o.), respectively, for 4 consecutive weeks. Twenty-four hours after receiving the last dose of ω-3FA, all groups except group 1 were injected intraperitoneally (20 mg/kg) DOX as a single dose (AlAsmari et al., 2021).

2.4. Serum collection and tissue preparation

All rats had been anesthetized 48 h after DOX injection with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Blood samples were obtained from retro-orbital plexus venesection and collected on plain tubes for serum preparation. The blood samples were allowed to stand for 10 min then centrifuged using a cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000*g* for 10 min and serum was separated.

Under anesthesia, animals were euthanized by cervical dislocation. Livers were removed and rinsed with ice-cold saline and then weighed. Weighed parts of the liver were frozen at −80 °C. Liver samples were homogenized in ice-cold Tris-HCL buffer (150 mM, PH 7.4) to prepare 10% w/v homogenate. Briefly, tissue samples were ground using a tissue homogenizer (Biospec Products, mini-BeadBeater-8, Bartlesville, USA). The tissue fragments were then dispersed in ice-cold Tris-HCL lysis buffer (150 mM, PH 7.4). The mixture was placed on ice and was sonicated for 20–30 s then centrifuged at 3000 rpm for 15 min. Then, the supernatant was collected. Aliquots of serum and tissue homogenate were stored at −80 °C to be further used for the determination of various biochemical markers. In addition, 10% neutral formalin was used to fix different sections of livers of all groups for histopathological and immunohistochemical examination.

2.5. Serum enzyme markers of liver damage

For evaluating the acute liver injury, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and direct bilirubin serum levels were assessed using enzymatic colorimetric kits (Spectrum diagnostics, Egypt).

2.6. Tissue biochemical analysis

Hepatic levels of malondialdehyde (MDA), a marker of lipid peroxidation, were determined by a thiobarbituric acid method using MDA kit (Biodiagnostic, Egypt). The formed colored thiobarbituric acid reactive product was measured spectrophotometrically at 534 nm (Gavino et al., 1981). Hepatic glutathione (GSH) levels were measured by GSH kit (Biodiagnostic, Egypt) based on the reduction of 5,5'dithiobis (2-nitrobenzoic acid) by GSH. The absorbance of colored product was measured at 405 nm (Beutler, 1963). Aliquots of liver homogenate were used for determination of nuclear Nrf-2, GSK-3β and PI3K levels using rat-specific ELISA kits provided by Wuhan Fine Biological Technology Co., China (catalog no.: ER911, ER0124 and ER1531, respectively). Hepatic levels of HO-1 were estimated by rat-specific ELISA kit supplied by Elabscience Biotechnology Inc., USA (catalog no.: E-EL-R0864) while hepatic rat pAkt concentrations were measured by Cusabio® ELISA kit (China). The Pierce™ bicinchoninic acid (BCA) assay kit supplied by ThermoFisher Scientific, USA (catalog no.: 23227) was used to determine total protein content (Smith et al., 1985). Tissue parameters were expressed per mg of protein.

2.7. Histopathological examination

Formalin-fixed sections of livers were embedded in paraffin blocks and then cut into segments of 4 μm thick. Liver segments were dyed with hematoxylin and eosin (H&E). Hepatic damage was semi-quantitively analyzed in a low-power field based on the severity and tissue damage percentage as described previously (Orabi et al., 2020). A grading scale (0–4) was used, where 0; normal tissue, 1; <25% of tissue damaged, 2; 26–50% of tissue damaged, 3; tissue damage in 51–75%, and 4; tissue damage >75%. The pathological parameters used for assessment were acute cellu-

lar swelling, vacuolar degeneration of hepatocytes, hepatocellular necrosis and apoptosis.

2.8. Immunohistochemical analysis

Cleaved caspase-3 and tumor necrosis factor (TNF)-α in liver sections were detected according to the method of Abd Eldaim et al., (2020). The liver sections were deparaffinized, hydrated in alcohol and incubated in 3% H_2O_2. After that, tissues were incubated with primary antibodies; rabbit monoclonal anti-caspase-3 (EPR18297, Abcam) or rabbit polyclonal anti-TNF-α (ab6671, Abcam) at a concentration of 1 μg/ml in 5% bovine serum albumin overnight. The immune reactivity was visualized by diaminobenzidine (DAB; Sigma, USA) and semi-quantitatively analyzed in high-power fields (HPF, 40X) based on the percentage of positively immune stained cells. The grading scale (0–3) was used, where 0; negative staining, 1; positively stained cells per field <25%, 2; positively stained cells 25–60%, 3; positively stained cells >60% (Abd Eldaim et al., 2020).

2.9. Statistical analysis

Data were represented as mean ± S.E.M. and statistically performed using GraphPad Prism 5.0 Software (USA). Comparison between groups was conducted by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pretreatment with ω-3FA reduces serum hepatotoxicity biomarkers in DOX-treated groups

As shown in Table 1, the intraperitoneal injection of DOX induced acute liver damage that was observed by significant elevation of enzyme activity of serum ALT and AST by 3.5 and 3.7 folds, respectively, compared to negative control rats. Oral pretreatment with 25, 50 and 100 mg/kg ω-3FA alleviated the ALT serum activity to 42.28, 27.45 and 23.15 U/L by about 42%, 63% and 68% decrease, respectively, relative to DOX-treated rats (73.40 U/L). It is noticeable that serum ALT levels after oral administration of ω-3FA (100 mg/kg) decreased to almost the levels of the negative control group (21.18 U/L). Additionally, serum AST activities showed a significant reduction upon pretreatment with ω-3FA (25, 50 and 100 mg/kg) to 86.35, 69.99 and 58.07 U/L by about 23%, 38% and 49% decrease, sequentially, in comparison to the positive control group (112.77 U/L).

Furthermore, DOX-induced hepatotoxicity is manifested by increased serum total and direct bilirubin (1.71 and 0.325 mg/dL, respectively) compared to negative control rats (0.61 and 0.128 mg/dL, respectively). Pretreatment with oral doses of ω-3FA (25, 50 and 100 mg/kg) revealed suppression of serum total bilirubin levels to 1.28, 0.87 and 0.78 mg/dL, respectively by about 25%, 49% and 54% decrease, respectively, in comparison to Dox-injected rats. In addition, oral doses of ω-3FA (25, 50 and 100 mg/kg) decreased the levels of direct bilirubin to 0.27, 0.19 and 0.16 mg/dL, respectively, when compared to DOX-injected rats (Table 1).

3.2. Oral administration of ω-3FA alleviates hepatic oxidative stress biomarkers in DOX-treated groups

Imbalanced redox homeostasis has been noticed evidenced by elevated hepatic MDA levels in DOX-treated rats compared to negative control rats (0.256 ± 0.011 versus 0.109 ± 0.04 nmol/mg protein). Also, hepatic GSH levels were significantly lower in DOX-injected rats (7.70 ± 0.15 mg/mg protein) than in normal rats (19.83 ± 0.57 mg/mg protein). Pretreatment with ω-3FA (25, 50 and 100 mg/kg) lowered the hepatic MDA levels to 0.191, 0.163 and 0.123 nmol/mg protein by 25%, 36% and 51% decrease, respectively, and increased the hepatic GSH levels to 12.36 ± 0.20, 14.72 ± 0.47 and 16.62 ± 0.49 mg/mg protein by about 61%, 91% and 116%, sequentially, when compared to DOX-treated rats, as demonstrated in Table 2. It is important to mention that high-dose of ω-3FA could restore the normal levels of both hepatic MDA and GSH levels in DOX-injected animals.

3.3. ω-3FA modulates hepatic Nrf2/HO-1 pathway in DOX-injected rats

Fig. 1 demonstrates the hepatic Nrf2/HO-1 pathway in DOX-injected rats whereas, Fig. 1a presents that DOX injection was accompanied by a remarkable reduction in hepatic nuclear Nrf2 levels (141.45 ± 5.17 pg/mg protein versus 236.45 ± 8.50 pg/mg protein), in comparison to the healthy control group. However, oral ω-3FA administration (25, 50 and 100 mg/kg) caused a marked elevation in tissue nuclear Nrf2 levels to 157.68 ± 3.28, 214.49 ± 3.03 and 211.08 ± 7.75 pg/mg protein, respectively. This represents about 11%, 52% and 49% increase, sequentially, when compared to the positive control group. Moreover, Fig. 1b shows a prominent suppression in hepatic levels of HO-1 that was reported in DOX-treated rats (1.17 ± 0.05 ng/mg protein) versus (2.45 ± 0.04 ng/m g protein) in the negative control group. Pre-administration of ω-3FA (50 and 100 mg/kg) elevated the hepatic HO-1 levels to 1.56 ± 0.15 and 2.21 ± 0.06 ng/mg protein, respectively, in comparison to DOX-treated rats. It is noticeable that normal hepatic Nrf2 levels were maintained in DOX-injected rats by medium and high doses of ω-3FA while HO-1 levels were restored only by high doses of ω-3FA.

Table 1
Effect of ω-3FA on serum hepatic function parameters in DOX-injected rats.

Groups	Parameters			
	Serum ALT (U/L)	Serum AST (U/L)	Total Bilirubin (mg/dL)	Direct Bilirubin (mg/dL)
Normal	21.18 ± 0.33	30.2 ± 0.69	0.61 ± 0.016	0.128 ± 0.007
DOX	73.40 ± 2.73*	112.77 ± 3.57*	1.71 ± 0.036*	0.325 ± 0.008*
DOX + ω-3FA (25 mg/kg)	42.28 ± 1.63*@	86.35 ± 1.98*@	1.28 ± 0.017*@	0.27 ± 0.007*@
DOX + ω-3FA (50 mg/kg)	27.45 ± 0.46*@	69.99 ± 0.97*@	0.87 ± 0.008*@	0.19 ± 0.002*@
DOX + ω-3FA (100 mg/kg)	23.15 ± 0.99@	58.07 ± 1.07*@	0.78 ± 0.047*@	0.16 ± 0.002*@

Oral administration of omega 3 fatty acids (ω-3FA; 25, 50 and 100 mg/kg) for 28 consecutive days before single intraperitoneal injection of doxorubicin (20 mg/kg) reduced serum hepatotoxicity biomarkers in DOX-treated groups manifested as decreased serum levels of ALT, AST, total and direct bilirubin.
Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test.
*P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; DOX, doxorubicin; ω-3FA, omega-3 fatty acids.

Table 2
Effect of ω-3FA on hepatic oxidative stress biomarkers in DOX-injected rats.

Groups	Parameters	
	Hepatic MDA (nmol/mg protein)	Hepatic GSH (mg/mg protein)
Normal	0.109 ± 0.004	19.83 ± 0.57
DOX	0.256 ± 0.011*	7.70 ± 0.15*
DOX + ω-3FA (25 mg/kg)	0.191 ± 0.005*@	12.36 ± 0.20*@
DOX + ω-3FA (50 mg/kg)	0.163 ± 0.005*@	14.72 ± 0.47*@
DOX + ω-3FA (100 mg/kg)	0.123 ± 0.008@	16.62 ± 0.49@

Oral administration of omega 3 fatty acids (ω-3FA; 25, 50 and 100 mg/kg) for 28 consecutive days before single intraperitoneal injection of doxorubicin (20 mg/kg) caused a significant dose-dependent reduction of hepatic MDA levels and a marked elevation in hepatic GSH levels when compared to their levels in DOX-treated groups.
Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test.
*P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats.
DOX, doxorubicin; GSH, glutathione; MDA, malondialdehyde; ω-3FA, omega-3 fatty acids.

3.4. Pretreatment with ω-3FA regulates hepatic PI3K/pAkt/GSK-3β pathway in DOX-treated groups

As shown in Fig. 2, intraperitoneal injection of DOX caused a notable increment in hepatic GSK-3β levels to 1.64 ± 0.02 ng/mg protein versus 0.86 ± 0.02 ng/mg protein in the normal group. Oral pretreatment with ω-3FA (25, 50 and 100 mg/kg) showed dose-dependent inhibition of hepatic GSK-3β levels to 1.26 ± 0.04, 1.05 ± 0.05 and 0.91 ± 0.06 ng/mg protein, respectively, by about 23%, 36% and 45% decrease, respectively when compared to DOX-treated rats. It is noticeable that oral administration of high-dose of ω-3FA restored the GSK-3β levels in DOX-injected rats.

As illustrated in Fig. 3, our results reported a 2.4-fold decrease in hepatic PI3K levels in DOX-treated rats as compared to negative control rats (266.21 ± 18.66 versus 620.15 ± 2.17 pg/mg protein, respectively). This decline has been improved by pretreatment with ω-3FA (25, 50 and 100 mg/kg) as their levels reached 420.8 0 ± 15.52, 450.70 ± 29.88 and 504.15 ± 15.07 pg/mg protein (Fig. 3a). Similarly, induction of hepatotoxicity by DOX injection showed a 1.7-fold reduction in hepatic pAkt concentration as compared to the healthy control group (21.99 ± 0.94 versus 38.08 ± 0. 33 pg/mg protein, respectively). In comparison to the DOX-treated group, rats pretreated with ω-3FA (25, 50 and 100 mg/kg) exhibited a dose-dependent increase in hepatic levels of pAkt to 23.62 ± 0.19, 29.51 ± 0.31 and 34.95 ± 0.48 pg/mg protein, respectively (Fig. 3b). It is noticeable that hepatic pAKT levels increased to almost the normal levels of the negative control group after oral administration of 100 mg/kg ω-3FA.

3.5. ω-3FA restores hepatic histopathological alterations associated with DOX injection

Table 3 and Fig. 4 show the total pathologic score recorded in the hepatic tissues of the studied groups. Liver of the normal group demonstrated normal histological structure with no evidence of acute cellular swelling (Fig. 4a and b). Meanwhile, the liver of DOX-treated rats revealed extensive vacuolar degeneration of hepatocytes, with remarkable acute cellular swelling and apoptotic figures (Fig. 4c and d). Focal hepatocellular necrosis infiltrated with

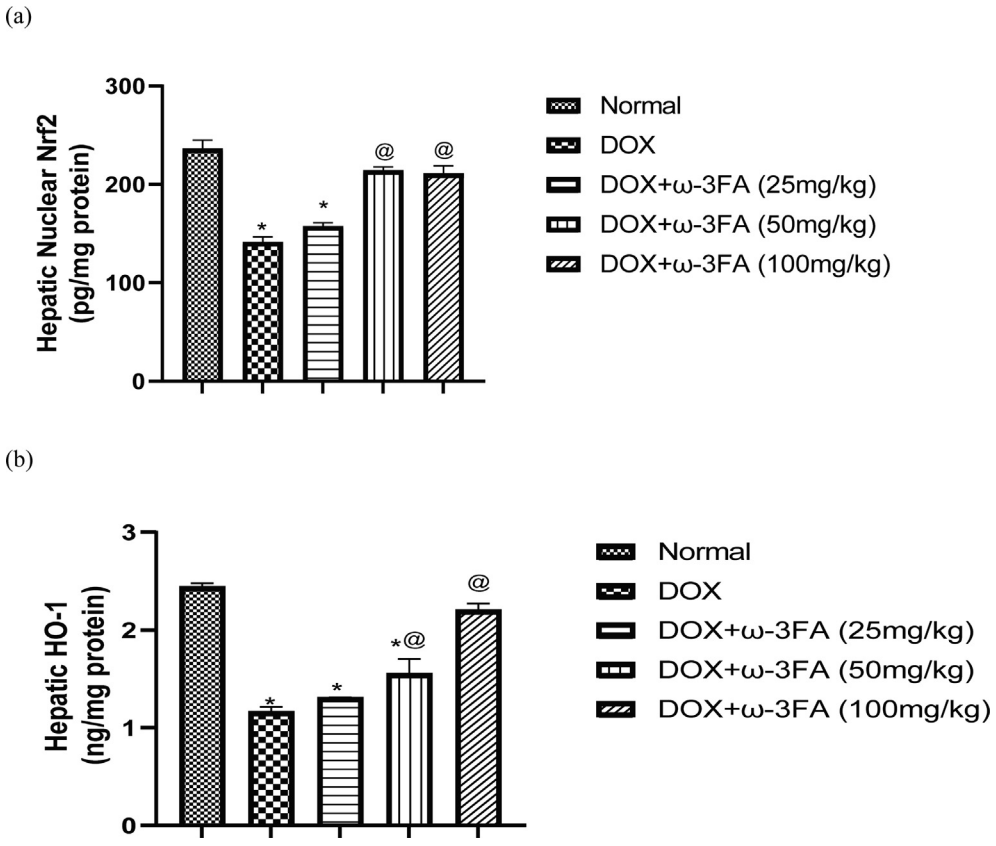


Fig. 1. Pretreatment with ω-3FA modulates hepatic Nrf2/HO-1 pathway in DOX-injected rats. Oral administration of omega 3 fatty acids (ω-3FA; 25, 50 and 100 mg/kg) for 28 consecutive days before a single intraperitoneal injection of doxorubicin (20 mg/kg) caused a significant dose-dependent elevation of hepatic Nrf2 and HO-1 levels compared to their levels in DOX-treated rats. Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test *P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats. DOX, doxorubicin; HO-1, hemeoxygenase-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ω-3FA, omega-3 fatty acids.

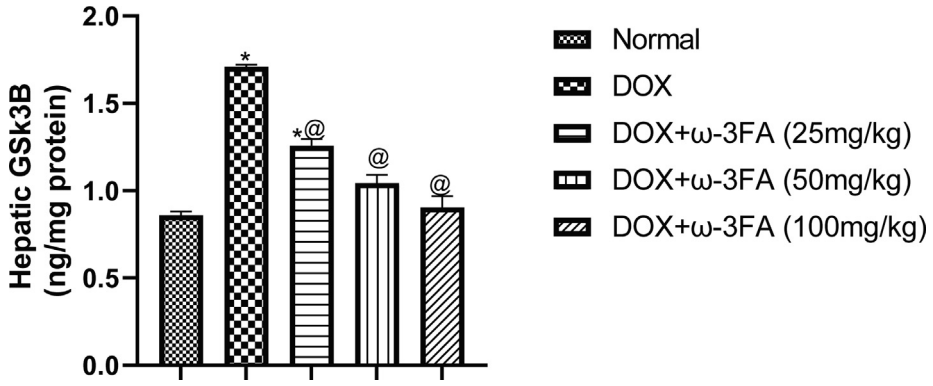
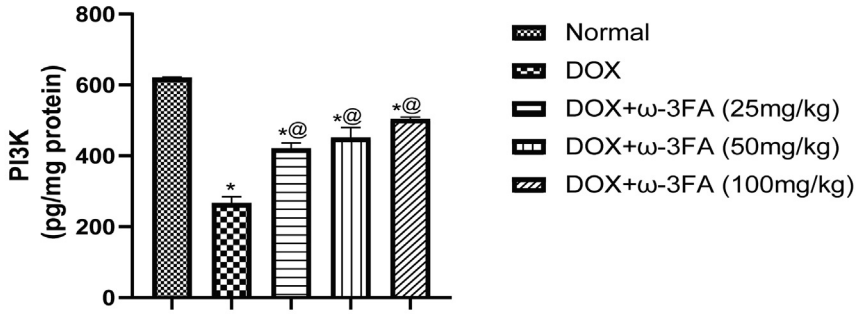


Fig. 2. Pretreatment with ω-3FA down-regulates hepatic GSK-3β in DOX-treated groups. Oral administration of omega 3 fatty acids (ω-3FA; 25, 50 and 100 mg/kg) for 28 consecutive days before single intraperitoneal injection of doxorubicin (20 mg/kg) caused a significant dose-dependent inhibition of hepatic GSK-3β levels compared to DOX-treated rats. Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test. *P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats. DOX, doxorubicin; GSK-3β; Glycogen synthase kinase-3β−1; ω-3FA, omega-3 fatty acids.

(a)



(b)

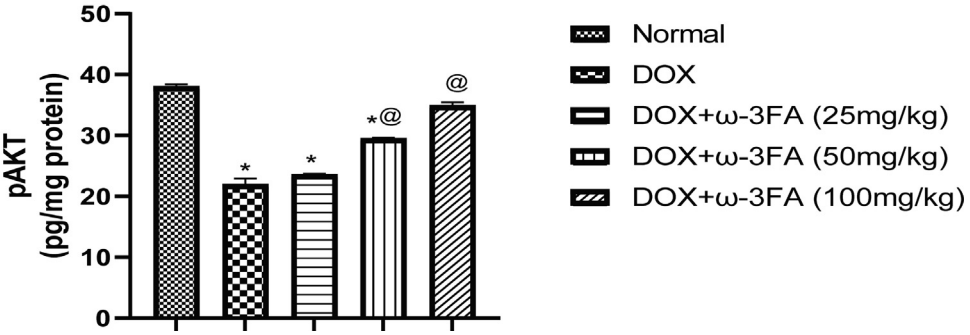


Fig. 3. Oral administration of ω-3FA regulates hepatic PI3K/pAkt pathway in DOX-injected rats. Pretreatment with oral omega 3 fatty acids (ω-3FA; 25, 50 and 100 mg/kg) for 28 consecutive days before a single intraperitoneal injection of doxorubicin (20 mg/kg) caused a marked dose-dependent elevation of hepatic PI3K and pAKT levels compared to DOX-treated rats. Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test. *P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats. DOX, doxorubicin; ω-3FA, omega-3 fatty acids; PI3K; phosphatidylinositol-3-kinase.

macrophages and a significant increase in the pathologic score were recorded in this group. Treatment with ω-3FA revealed a significant improvement in a dose-dependent manner, with a significant decrease in the pathologic score. Hepatocytes of the ω-3FA (25 mg/kg) group appeared less swollen, with cytoplasmic vacuolation, compared to the DOX-treated group (Fig. 4e and f). Much better improvement, with a significant decrease in the pathologic score, was recorded in groups pretreated with ω-3FA (50 and 100 mg/kg). Mild vacuolar degeneration, with a pronounced decrease in hepatocellular swelling, was demonstrated in ω-3FA (50 mg/kg) group (Fig. 4g and h). On the other side, normal hepatocytes with no evidence of acute hepatocellular swelling were demonstrated in ω-3FA (100 mg/kg) group (Fig. 4i and j).

3.6. Effect of ω-3FA on hepatic TNF-α immunohistochemical expression

The hepatic TNF-α expression pattern in different studied groups is shown in Fig. 5. No TNF-α expression was reported in hepatocytes of the normal group, but a weak expression was

Table 3
Effect of ω-3FA on the total pathologic score recorded in the liver of normal and different treated groups.

Groups	Parameters Pathologic score
Normal	0.10 ± 0.10
DOX	3.50 ± 0.22*
DOX + ω-3FA (25 mg/kg)	2.85 ± 0.17*@
DOX + ω-3FA (50 mg/kg)	2.50[b] ± 0.31*@
DOX + ω-3FA (100 mg/kg)	1.80[c] ± 0.25*@

Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test.
*P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats.
DOX, doxorubicin; ω-3FA, omega-3 fatty acids.

observed in the hepatic sinusoids of this group (Fig. 5a). In contrast, remarked elevation of TNF-α expression, with an increased % of positively stained hepatocytes and sinusoidal cells, was reported in the liver of DOX-treated rats (Fig. 5b). Decreased TNF-α expression, with a remarked decrease of % of positively stained hepatocytes and sinusoidal cells, was recorded in low and medium-dose treated groups, with no significant difference between them (Fig. 5c and d, respectively). On the other hand, sparse positively stained hepatocytes and sinusoids were demonstrated in the high dose-treated group (Fig. 5e).

3.7. Effect of ω-3FA on hepatic caspase-3 immunohistochemical expression

Fig. 6 shows the liver caspase-3 expression of normal and different treated groups. There is no hepatic caspase-3 expression observed in hepatic tissues of negative control rats (Fig. 6a). Conversely, increased cleaved caspase-3 expression, with profound elevation in positively stained cells percentage, was demonstrated in the liver of DOX-treated rats (Fig. 6b). Pretreatment with ω-3FA revealed dose-dependent inhibition of cleaved caspase-3 hepatic expression. When compared to DOX-treated rats, there is a remarked decrease in the percentage of caspase-3 positively stained cells reported in low and medium ω-3FA dose-treated groups, with no significant difference between them (Fig. 6c and d). But a significant difference was recorded in the high dose-treated group, which revealed scanty positively stained cells around the central vein (Fig. 6e).

4. Discussion

Doxorubicin-induced hepatic toxicity was evidenced in the current investigation by increment in serum ALT and AST activities as well as direct and total bilirubin in DOX-treated rats compared to the negative control rats, indicating serious liver injury, which was validated by histological investigations. These findings come in accordance with previous observations on DOX-induced hepatotoxicity and apoptosis in subjects possessing some sort of liver damage upon receiving DOX (AlAsmari et al., 2021; Barakat et al., 2018; Song et al., 2019). The histological abnormalities, as well as the higher hepatic enzyme activity observed in DOX-treated rats, are dramatically restored in the ω-3FA-pretreated groups, implying protection against DOX-induced liver injury.

The mechanisms underlying DOX-prompted hepatotoxicity are complex and caused by a variety of processes. Recent research has identified oxidative stress as a significant mechanism of oxidation-induced hepatotoxicity (Tan et al., 2018). DOX has been shown to cause redox homeostasis imbalance, which is defined by an elevation in ROS and a reduction in antioxidant defenses, resulting in oxidization of DNA and other macromolecules including lipids leading to liver damage (AlAsmari et al., 2021; Song et al., 2019). Allied with previous results, our results revealed that DOX intake showed a marked elevation in hepatic MDA, a lipid peroxidation product, and a significant reduction in hepatic GSH.

The current study focused attention on the crucial modulator of the antioxidant response, Nrf2, in response to chemical insults and oxidative/electrophilic stresses. Nrf2 is a redox-dependent transcription factor that regulates the expression of major antioxidant and detoxifying enzymes. Of these downstream enzymes, HO-1 is a phase II enzyme that controls the rate-limiting step in heme metabolism and production of various antioxidant intermediates. Accumulating shreds of evidence highlight the role of HO-1 in protecting the liver and kidney against oxidative damage and inflammation. Many speculations regarding the process of DOX-

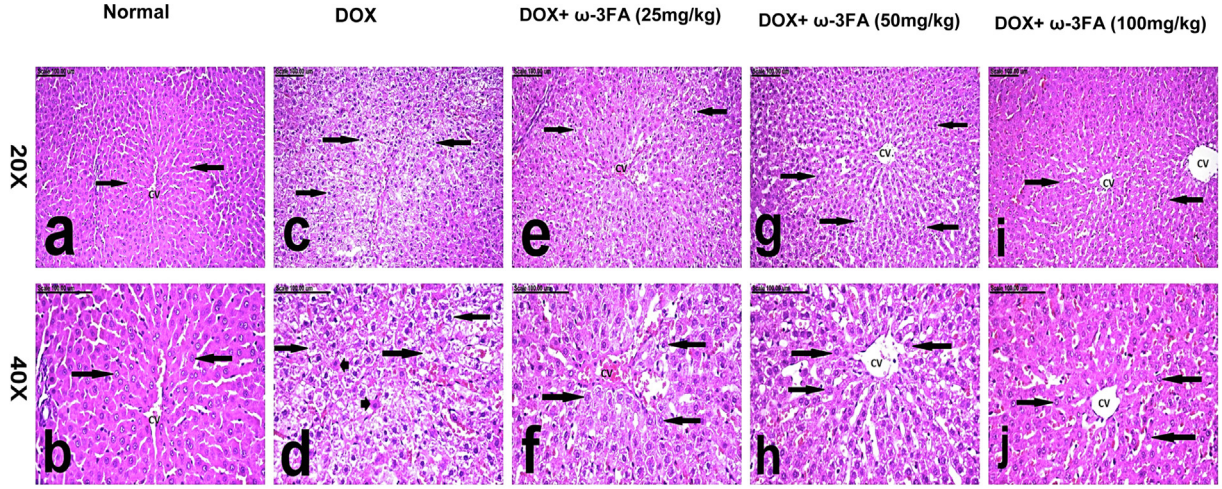


Fig. 4. Pretreatment with oral ω-3FA restores hepatic histopathological alterations in DOX-injected rats. Photomicrograph of liver tissue stained with H&E, of (a, b) normal rats showing normal histological structure (arrows) with normal central vein (CV) (a) and no evidence of acute cellular swelling (arrows) (b), (c, d) DOX-treated rats (single intraperitoneal injection of 20 mg/kg) showing extensive vacuolar degeneration of hepatocytes (arrows) (c) with remarkable acute cellular swelling (arrows) and apoptotic figures (arrow heads) (d), (e, f) low dose-treated rats (oral pretreatment with 25 mg/kg ω-3FA for 28 consecutive days) showing appeared less swollen hepatocytes (arrows) (e) with cytoplasmic vacuolation (arrows) (f), (g, h) medium-dose treated rats (50 mg/kg ω-3FA) showing mild vacuolar degeneration (arrows) (g) with pronounced decrease of hepatocellular swelling (arrows) (h), (i, j) high-dose treated group (100 mg/kg ω-3FA) showing normal hepatocytes (arrows) (i) with no evidence of acute hepatocellular swelling (arrows) (j). (scale bar = 100 μm). DOX, doxorubicin; ω-3FA, omega-3 fatty acids.

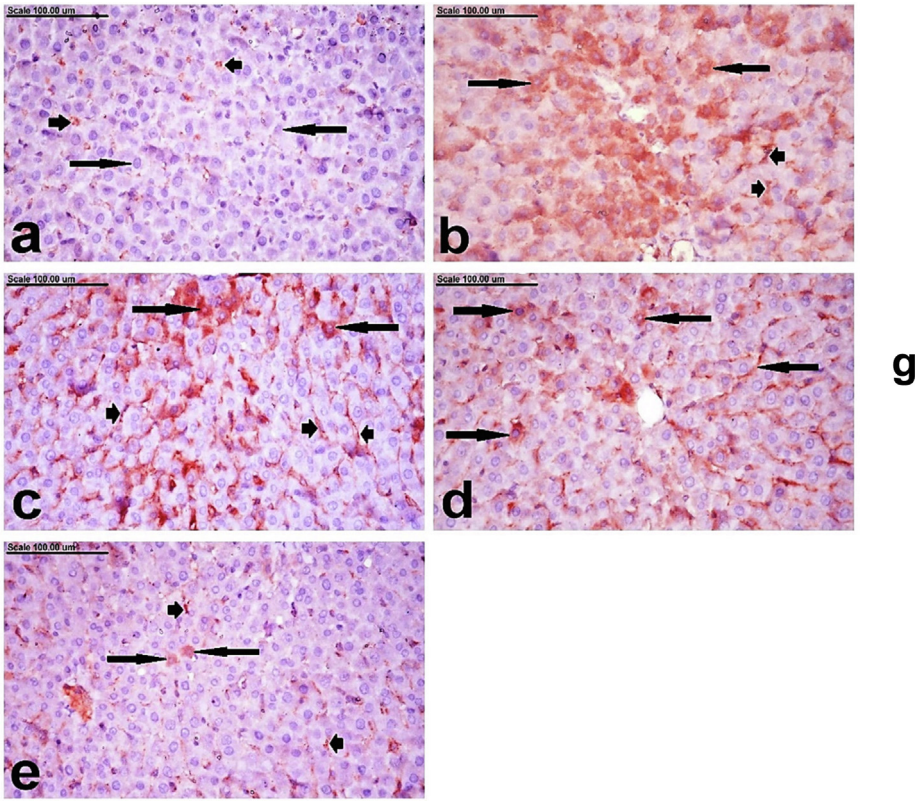


Fig. 5. Effect of ω-3FA on hepatic immunohistochemically assessed TNF-α in DOX-injected rats. Photomicrograph of liver tissue immunohistochemically stained with anti-TNF-α of (a) normal rats showing no TNF-α expression in hepatocytes (arrows), but weak expression in hepatic sinusoids (arrowheads), (b) DOX-treated rats (single intraperitoneal DOX injection of 20 mg/kg) showing increased TNF-α expression, with increased % of positively stained hepatocytes (arrows) and sinusoidal cells (arrowheads), (c) low dose-treated rats (oral pretreatment with 25 mg/kg ω-3FA for 28 consecutive days) showing decreased TNF-α expression, with a significant decrease of % of positively stained hepatocytes (arrows) and sinusoidal cells (arrowheads), (d) medium-dose treated rats (oral pretreatment with 50 mg/kg ω-3FA) showing decreased % of TNF-α positively stained cells (arrows), and (e) high dose group (oral pretreatment with 100 mg/kg ω-3FA) showing sparse positively stained hepatocytes (arrows) and sinusoids (arrowheads). (scale bar = 100 μm). The quantification graph of immunohistochemical analysis of TNF- α. (g). Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test. *P < 0.05 compared with the normal rats; @P < 0.05 compared with the DOX-injected rats. DOX, doxorubicin; HPF, high power field; ω-3FA, omega-3 fatty acids; TNF-α, tumor necrosis factor-alpha.

induced hepatic damage might be ascribed to the decreased hepatic expression of HO-1 and Nrf2 protein as well as elevated hepatic MDA triggering oxidative stress along with hepatocyte apoptosis evidenced by caspase-3 expression (Barakat et al., 2018). In our work, we observed that rats treated with DOX revealed lower hepatic Nrf2 and HO-1 levels than those in negative control rats. This reduction in hepatic content of Nrf2 and HO-1 is attenuated by pretreatment with ω-3FA, suggesting the possible therapeutic effect of these nutrients to alleviate DOX-induced oxidative stress.

Based on the cellular redox state, the Keap1-Nrf2-antioxidant response element (ARE) signaling system is controlled by many layers of regulation (Lu et al., 2016). Among these various layers of regulation, GSK-3β-mediated Nrf2 degradation and nuclear export are of particular importance. It was found that GSK-3β could phosphorylate serine residues of Nrf2 promoting Keap1-independent degradation (Xing et al., 2015). Concomitant administration of ω-3FA with DOX reduced hepatic MDA and GSK-3β levels and restored hepatic GSH levels, suggesting the GSK-3β inhibitor's protective properties against DOX-induced oxidative stress.

Notably, it has been also shown in the present study that GSK-3β inhibition provides guarding against DOX-prompted oxidative stress in the hepatic tissues by elevating the protein expression of Nrf2, a crucial transcription factor controlling redox homeostasis, while reducing the increment lipid peroxidation that is reported in the liver from rats cotreated with ω-3FA DOX. Herein, attenuation of oxidative stress via GSK-3β inhibition and Nrf2/HO-1 activation might be one of the critical strategies of ω-3FA to combat DOX-induced hepatotoxicity.

In addition to oxidative stress, inflammation is evidenced to be one of the major mechanisms involved in hepatotoxicity. DOX treatment may trigger the pro-inflammatory response by increasing pro-inflammatory cytokines like TNF-α and inhibiting anti-inflammatory supporting components (Supriya et al., 2016). Immunohistochemistry results demonstrated elevated hepatic expression of TNF-α with an increased percentage of hepatic and sinusoidal cells expressing TNF-α in DOX-treated rats than in normal rats. Supplementing with ω-3FA, on the other hand, can modify the inflammatory response by suppressing hepatic TNF-α levels. This comes in accordance with earlier studies that reported the effect of fish oil to attenuate inflammation in experimental models (Azuma et al., 2018; Lobo et al., 2016).

Our results revealed that DOX induces apoptosis evidenced by increased hepatic expression of caspase-3, the promoter and the terminal effector in the apoptotic cascade. On the other hand, pretreatment with ω-3FA decreased hepatic caspase-3 expression and the percentage of caspase-3 positive cells in dose-related behavior. One of the mechanisms to explain reduced apoptosis is the modulation of the PI3K/Akt signaling pathway, which also boosts cell development, and promotes cell proliferation (Liu et al., 2020). PI3K/Akt activation is closely regulated in normal settings and is controlled by different types of sensors that stimulate downstream kinases by suitable binding in the PI3K family. Phosphorylated Akt activates several downstream proteins. Akt mediates signals that

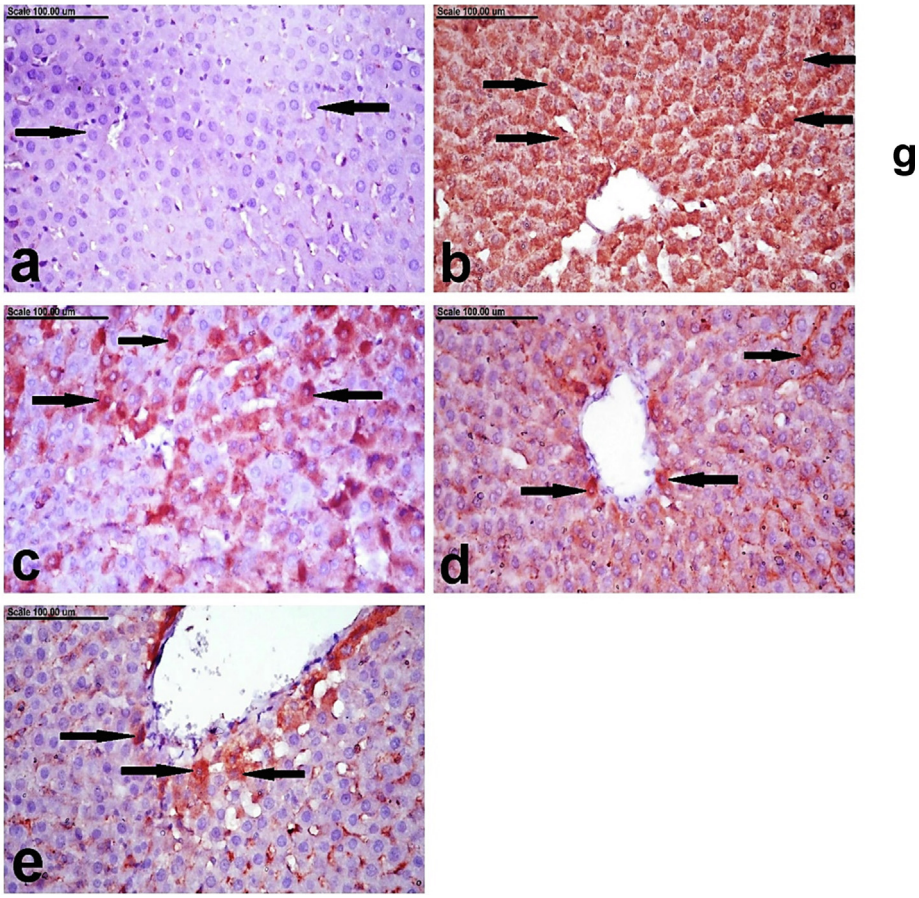


Fig. 6. Effect of ω-3FA on hepatic immunohistochemically assessed caspase-3 in DOX-injected rats. Photomicrograph of liver tissue immunohistochemically stained with anti-caspase-3 of (a) normal rats showing no cleaved caspase-3 expression (arrows), (b) DOX-treated rats showing a significant increase of % of positively stained cells with intense brown cytoplasmic staining (arrows), (c) low dose-treated rats (oral pretreatment with 25 mg/kg ω-3FA for 28 consecutive days) showing decreased % of caspase-3 positively stained cells (arrows), (d) medium-dose treated rats (oral pretreatment with 50 mg/kg ω-3FA for 28 consecutive days) showing decreased % of caspase-3 positively stained cells with faint brown cytoplasmic staining (arrows), and (e) high dose group (oral pretreatment with 25 mg/kg ω-3FA for 28 consecutive days) showing scanty caspase-3 positively stained cells around the central vein (arrows). (Scale bar = 100 μm). The quantification graph of immunohistochemical analysis of caspase-3 (g). Results are expressed as mean ± SEM and analyzed by ANOVA followed by Tukey's post-hoc test. $^{*}P < 0.05$ compared with the normal rats; $^{@}P < 0.05$ compared with the DOX-injected rats. DOX, doxorubicin; HPF, high power field; ω-3FA, omega-3 fatty acids.

promote cell proliferation, differentiation, and angiogenesis, as well as preventing apoptosis, via its various substrates (Long et al., 2021; Somanath et al., 2006). Activated Akt could phosphorylate GSK-3β and inhibit its activity. In addition, DOX treatment was found to attenuate pAkt activity with subsequent activation of GSK-3β resulting in apoptosis induction via caspase-3 activation. In the present study, oral treatment of ω-3FA for successive 28 days revealed dose-dependent suppression of hepatic GSK-3β levels as compared to the DOX-treated group as well as restoration in hepatic levels of PI3K and pAkt. In addition, pretreatment with ω-3FA decreased caspase-3 positively stained cells in dose-related behavior. These findings highlight that the anti-apoptotic effect of ω-3FA; evidenced by attenuation of caspase-3, is contributed to the modulation of PI3K/Akt/GSK-3β axis as illustrated in the Graphical Abstract.

5. Conclusion

The present study provides further evidence that DOX-induced hepatic toxicity is due to oxidative damage, evidenced by elevated hepatic MDA levels in accordance with reduced hepatic GSH, levels which have a great impact on HO-1, and Nrf2 protein downregulation as well as the inflammatory biomarker; TNF-α. Moreover, the role PI3K/Akt/GSK-3β signaling pathway has been reinforced in regulating apoptosis. The current study also supports the hypothesis that modulating PI3K/Akt/GSK-3β pathway via ω-3FA would have a significant influence on mitigating the chemotherapeutic agent's negative effects on the liver. Finally, from all the previous findings, one can conclude that the beneficial effects of ω-3FA against DOX-induced acute hepatotoxicity in rats through the modulation of PI3K/Akt/GSK-3β axis and activation of the HO-1/Nrf2 defense pathway with subsequent downstream antioxidant proteins.

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Dalia O. Saleh: Conceptualization, Methodology, Investigation, Resources, Writing – original draft. **Sawsan S. Mahmoud:** Concep-

tualization, Methodology. **Azza Hassan:** Investigation, Formal analysis. **Eman F. Sanad:** Conceptualization, Investigation, Formal analysis, Resources, Writing – original draft and revising.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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